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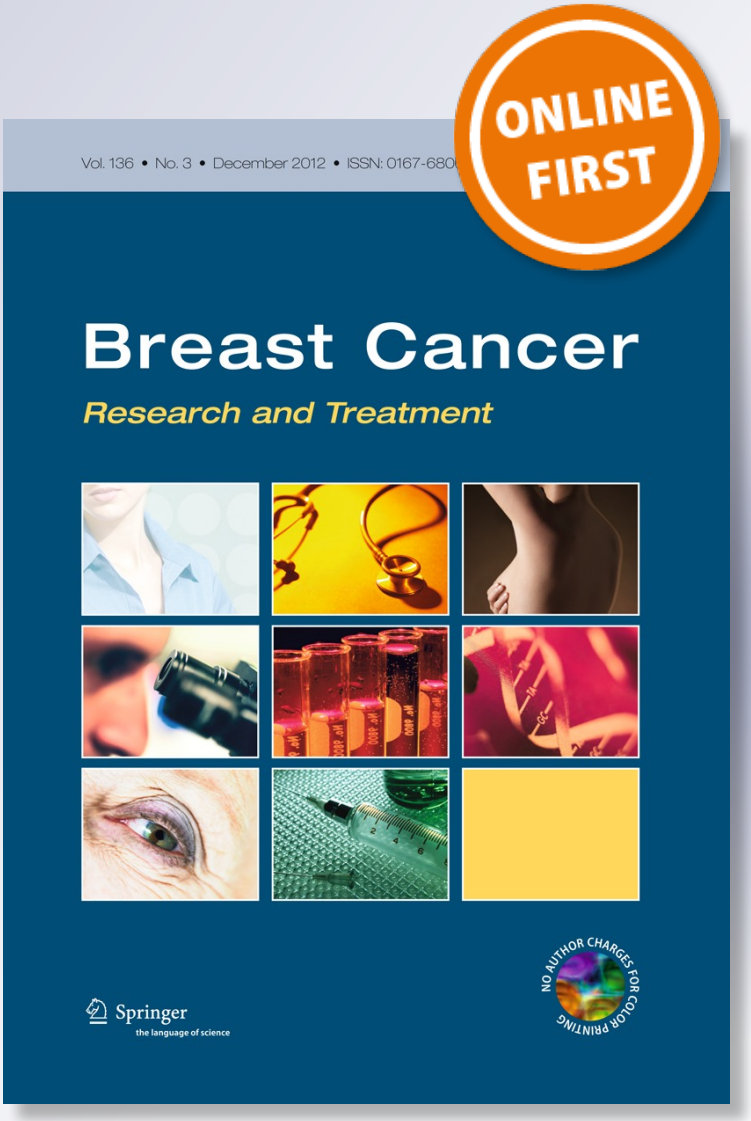
Breast Cancer Research and Treatment

ISSN 0167-6806

Breast Cancer Res Treat
DOI 10.1007/s10549-012-2353-5



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PRECLINICAL STUDY

Vimentin DNA methylation predicts survival in breast cancer

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Received: 13 June 2012 / Accepted: 21 November 2012

Abstract The Vimentin gene plays a pivotal role in epithelial-to-mesenchymal transition and is known to be overexpressed in the prognostically poor basal-like breast cancer subtype. Recent studies have reported Vimentin DNA methylation in association with poor clinical outcomes in other solid tumors, but not in breast cancer. We therefore quantified Vimentin DNA methylation using MALDI-TOF mass spectrometry in breast tumors and matched normal pairs in association with gene expression and survival in a hospital-based study of breast cancer patients. Gene expression data via qRT-PCR in cell lines and oligomicroarray data from breast tissues were correlated with percent methylation in the Vimentin promoter. A threshold of 20 percent average methylation compared with matched normal pairs was set for bivariate and multivariate tests of association between methylation and tumor subtype, tumor histopathology, and survival. Vimentin was differentially methylated in luminal breast cancer cell lines, and in luminal A, luminal B, and HER2-enriched breast tumor subtypes, but was rare in basal-like cell lines and tumors. Increased methylation was strongly correlated with decreased mRNA expression in cell lines, and had a moderate inverse correlation in breast tumors. Vimentin methylation predicted poor overall survival independent of race, subtype, stage, nodal status, or metastatic disease and holds promise as a new prognostic biomarker for breast cancer patients.

Keywords DNA methylation · Breast cancer · Vimentin · Epigenetic · Survival

Abbreviations

EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
HR	Hormone receptor
HER2	Epidermal growth factor receptor
OS	Overall survival
RFS	Recurrence free survival
mRNA	messenger RNA
IHC	Immunohistochemical stain
UNC	University of North Carolina

Electronic supplementary material The online version of this article (doi:10.1007/s10549-012-2353-5) contains supplementary material, which is available to authorized users.

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Published online: 13 December 2012

Introduction/background

The Vimentin gene encodes an intermediate filament protein reported to have varied roles in cytoskeletal architecture [13, 17, 20], immune response [21], and in stabilization of collagen mRNAs [1]. Given these multiple functions, Vimentin is considered to have a pivotal role in epithelial-to-mesenchymal transition (EMT), including upregulation of other EMT associated genes, adaptive responses to wound healing, and pathological responses during cell invasion and metastasis [11]. In breast cancer, Vimentin expression is upregulated during EMT, and is highly expressed in the prognostically poor basal-like subtype of breast tumors [17, 35, 36, 39].

Although upregulation of Vimentin expression during EMT has been well characterized in breast cancer, only one study to our knowledge has reported epigenetic changes in Vimentin derived from breast tumors [8]. Recent studies have explored epigenetic changes in Vimentin in general, with data showing differential Vimentin DNA methylation in other solid tumors such as colorectal, gastric, cervical, bladder, and pancreatic cancer [2, 4, 10, 12, 14, 15, 23, 37, 38].

We therefore sought to quantify Vimentin methylation in $N = 154$ samples using the SEQUENOM® mass spectrometry platform in breast cell lines, breast tumors, and matched normal tissue, and to test for associations of methylation with gene expression, molecular subtypes, and clinical outcomes. Given the poor prognosis of breast cancers having elevated Vimentin expression, our central hypothesis was that if Vimentin methylation was correlated with decreased gene expression, then increased methylation would be associated with better survival. We found high levels of Vimentin methylation negatively correlated with gene expression only in the HER2+ and ER+ "luminal"-like cell lines. This pattern persisted in breast tumors, with elevated methylation in the HER2-enriched luminal A, and the HER2-enriched luminal B subtypes, while extremely low-to-nil methylation was found in 91 % of basal-like tumors. Importantly, Vimentin methylation strongly predicts poor overall survival (OS) independent of race, subtype, stage, nodal status, or metastatic disease.

Methods

Cell culture and molecular subtype assignments

All 14 cell lines were grown at 37 °C and 5 % carbon dioxide. MCF-7, ZR-75-1, HCC1937, T47D, SUM149PT, SUM159PT, SUM102, MDA-MB-231, SKBR3, and MDA-MB-468 were obtained from Asterand or the American Tissue Culture Collection (Manassas, Virginia). MCF-7, ZR-75-1, HCC1937, and T47D were cultured in PRMI medium with 10 % fetal bovine serum and penicillin and streptomycin solution (500ug/ml). SUM149PT and SUM159PT were cultured in HAM's F-12 medium with 5 % fetal bovine serum, insulin (5 ug/ml), Hydrocortisone (1 ug/ml), Amphotericin B (0.5 ug/ml), and Gentamycin (5 ug/ml). SUM102 and ME16C were cultured in HuMeC with 0.5 % BPE, 1 % HuMeC supplement, and Gentamycin (5 ug/ml). MDA-MB-231, SKBR3, and MDA-MB-468 were cultured in DMEM, McCoy's 5A, and L-15, respectively, each with 10 % fetal bovine serum and penicillin and streptomycin solution (500 ug/ml). MCFDCIS, MCF10AT1, and MCF10DCIS were cultured in DMEM/F-12, with 20 ng/ml epidermal growth factor, 0.5 ug/ml hydrocortisone, 0.1 ug/mL cholera toxin, 10 ug/ml insulin and penicillin and streptomycin solution (500 ug/ml).

Cell lines were authenticated by gene expression, and all cell lines tested negative for *Mycoplasma* contamination using the MycoProbe *Mycoplasma* detection assay cat. no. UL001B (R&D Systems, Minneapolis, MN).

Cell line categories by hormone/growth receptor status and molecular subtype

The seven estrogen receptor (ER) and progesterone receptor (PR) negative cell lines; HCC1937, SUM102, SUM149PT, MDA-MB-468, ME16C, SUM159PT, and MDA-MB-231 were collectively grouped as "hormone receptor negative" (HR−), and the HER2 + SKBR3 and three ER+ cell lines (T47D, MCF-7, and ZR-75) were grouped as "hormone receptor positive" (HR+). The basal-like and luminal molecular subtype classifications of the cell lines used in this study were based on previous studies using hierarchical clustering of oligoarray data from approximately 50 breast cancer cell lines [24, 29]. The classification used by Neve et al., (2006) distinguished basal A from basal B cell lines. For simplicity, however, we used the broader designation of "basal-like" to encompass both basal A and basal B cell lines.

RNA and DNA isolation from cell lines and human tissues

Human breast tumor and paired normal tissues were collected from fresh frozen samples following Biomedical Institutional Review Board approval through the UNC Office of Human Research Ethics. All breast tumors used in this study were selected to have greater than 50 % tumor cells by pathology/histology analysis, and on average had 70 % tumor epithelium. Depending on sample type, DNA extraction was carried out with either the Qiagen Puregene® Core Kit A or the Qiagen DNAeasy® Blood and Tissue Kit (Qiagen, Germantown, MD, USA). The Qiagen RNeasy® Mini Kit RNA was used to extract RNA before

DNA isolation and served as a template for both oligo array and qRT-PCR experiments. For qRT-PCR, RNA was reverse transcribed using miScript Reverse Transcription Kit (Qiagen, cat. no. 218061), and the product was treated with RNAse-free DNase I.

Previously obtained DNA oligoarray expression data in breast tumor and matched normal tissues

Methylation studies were carried out on DNAs extracted from previously analyzed breast tumors and matched normal breast tissues that had already been profiled for gene expression using DNA oligonucleotide arrays (Agilent Technologies, Santa Clara, CA, USA) [9]. Pending publication, all microarray data are temporarily available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dvqrtwaeewcyuns&acc=GSE35629, which are deposited in the Gene Expression Omnibus (GEO) under the accession number GSE35629. Array data for individual breast tumors are found in hypertext at the bottom of the above URL. Molecular subtypes of breast tumors were assigned using the PAM50 algorithm as previously described [26]. In total, five classifications were assigned that included: Luminal A, Luminal B, Basal-like, HER2-enriched, and Normal-Breast-Like. The normalized $\log_2$ ratios (Cy5 sample/Cy3 control) of Vimentin probe P161190 were median-centered before generating relative gene expression values. These values were used to correlate gene expression with percent methylation values for the CpG units interrogated in the Vimentin amplicon by means of mass spectrometry (Table 1).

Patient clinical and demographic data, molecular subtypes for each tumor and matched normal pair, and GEO accession numbers are listed in Supplemental File 1. There were $n = 83$ tumors and $n = 57$ matched tumor normal pairs ($N = 140$ total breast samples). In brief, this cohort was 63 % white, 29 % black, and 8 % other with an average breast cancer onset age of 56 years. Three of $N = 83$ breast cancer cases were male. Distribution of molecular subtypes were as follows: 29 % Luminal A, 28 % Luminal B, 27 % Basal-like, 12 % HER2 enriched, and 2 % Normal-like. The two Normal-like tumors identified with the PAM50 predictor were subsequently excluded from methylation scatter plots, gene expression correlation, and survival analyses. The mean and median years of follow-up were 5.5 and 5.2 years, respectively; over a third of the breast cancer cases had between 7 and 15 years of follow-up.

Table 1 Gene expression versus Vimentin methylation in tumors

CpG Units	Pearson R	*p* value
1.2	−0.264	0.019
3	−0.228	0.045
4	−0.234	0.039
5.6	−0.381	0.001
7.8	−0.345	0.002
16	−0.236	0.037
17	−0.304	0.007

Pearson correlation coefficients were calculated based on $\log_2$ expression values from oligo DNA microarray (Agilent) Vimentin probe P161190 with percent methylation values for CpG units 1–17. Methylation was significantly inversely correlated with Vimentin expression, with coefficients ranging from −0.228 to −0.381 ($p = 0.019$–0.001). Results are based on $n = 76$ tumors for which there were both complete methylation and oligoarray data

qRT-PCR gene expression analysis in breast cell lines

Gene expression in breast cell lines was quantified using qRT-PCR on a 7500 Real-Time PCR Platform (Applied Biosystems, Foster City, CA). Relative cDNA quantity was measured using pre-designed ABI TaqMAN probes and primers for Vimentin (VIM-Hs00185584_m1) and GAPDH (GAPDH-Hs02758991_g1) as the endogenous expression control (Applied Biosystems, Foster City, CA.) Cell line cDNAs were examined in triplicate, with qRT-PCR cycling as follows: 50 °C for 2 min, denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C annealing for 1 min.

Positive and negative DNA methylation controls

Artificially synthesized methylation controls were made as previously described [40]. In brief, 2 ug of human sperm DNA (unmethylated control) or 2 ug MDA-MB-231 DNA were treated in separate 20-µl reaction volumes of SSsI CpG methyltransferase (New England Biolabs, Beverly, MA) for 1.5 h at 37 °C before sodium bisulfite (NaBi) conversion. Percent mixtures were made of unmethylated and artificially methylated (positive controls) ranging from 0 to 100 % and quantified for both Vimentin and GAPDH amplicons using MassARRAY (See Supplementary Table 3). Non-NaBi-converted DNAs were used as negative template controls.

Sodium bisulfite conversion of DNA

The EZ DNA Methylation-Direct Kit (Zymo Research, Irvine,CA, USA) was used to sodium bisulfite convert genomic DNA extracted from cell lines, methylated controls, and breast tissue. As per protocol, the 5.5 h C → U conversion reaction (all U's are converted to T during PCR) was performed on a thermocycler at 95 °C for 30 s and 50 °C for 15 min for 20 cycles.

Quantifying Vimentin methylation using mass spectrometry

Percent Vimentin methylation was quantified using mass spectrometry with the EpiTYPER® T complete reagent kit. The kit included reagents for PCR, transcription and t-cleavage reactions, Clean Resin, and 10 silicon matrix SpectroCHIPs® (with a capacity of 384 samples per chip) to perform the mass spectrometry. The SEQUENOM EpiTYPER® methylation assay has been validated in numerous studies and previously described in detail [3, 6, 7, 31, 41]. We first custom designed the primers for an amplicon that included the core Vimentin promoter (Fig. 1), as predicted by http://linux1.softberry.com/berry.phtml?topic=fprom&group=programs&subgroup=promoter and http://www.cbs.dtu.dk/services/Promoter/ PCR was then carried out on 5–10 ng of sodium bisulfite-converted sample DNA using NaBi conversion-specific primers with lower case letters representing the T7 tag sequence (Forward–aggaagagag GAGAGTGGTAGAGGATTGGATTT and Reverse–cagtaatacgactcactatagggagaaggctCTTTTTCAACACCCCAA AATAAAC) in a 5-µl reaction volume under the following conditions: 95 °C for 2 min, with a series of touch down reactions for two cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; two cycles each with 59, 58, and 57 °C annealing, respectively; followed by 40 cycles at 56 °C annealing. Final extension was at 72 °C for 5 min. Unincorporated dNTPs were dephosphorylated with shrimp alkaline phosphatase before adding 2 µl of the reaction as a template for the in vitro transcription reaction as per the EpiTYPER protocol. RNase-A was added in the final T cleavage reaction that rendered unmethylated and methylated CG-containing fragments.

Methylated versus unmethylated CGs peaks are easily identified because of a 16 Dalton mass shift between the two peaks. The EpiTYPER® software then calculates the relative ratio of methylated to unmethylated CGs as percent methylation within a 5 % methylation confidence margin [3, 6, 32]. After the cleavage reaction, resulting fragments may contain more than one CG dinucleotide, and are therefore referred to as "CpG units." In such cases, percent methylation based on the ratio of methylated to unmethylated fragments is calculated as previously described [3]. Because the MassARRAY platform allows accurate percent methylation calculations between 1,000 and 8,000 Dalton windows, values for CG-containing fragments falling near or outside this mass window cannot be reliably quantified and are assigned an "N/A." For example, there are no data for CpG # 9, 11, 12, and 13 of the Vimentin amplicons because their cleavage fragments fell outside the EpiTYPER mass detection window. In addition , CpG #10 and 15 were excluded from the statistical analysis because they trended with similar methylation values across all sample types, including matched normal tissues. All analyses in this study were therefore performed on CpG sequence numbers 1, 2, 3, 4, 5, 6, 7, 8, 16, and 17 as shown in Fig. 2a. Percent methylation data for both GAPDH and Vimentin in positive and negative methylation controls, and in breast cancer cell lines, are provided in Supplemental File 3. Methylation data for each tumor and matched normal pair are provided in Supplemental File 2. Because the magnitude of methylation differs between sample types, the terms "hypermethylation" and "hypomethylation" throughout this article broadly refer to any sample with >20 and <20 % Vimentin methylation, respectively.

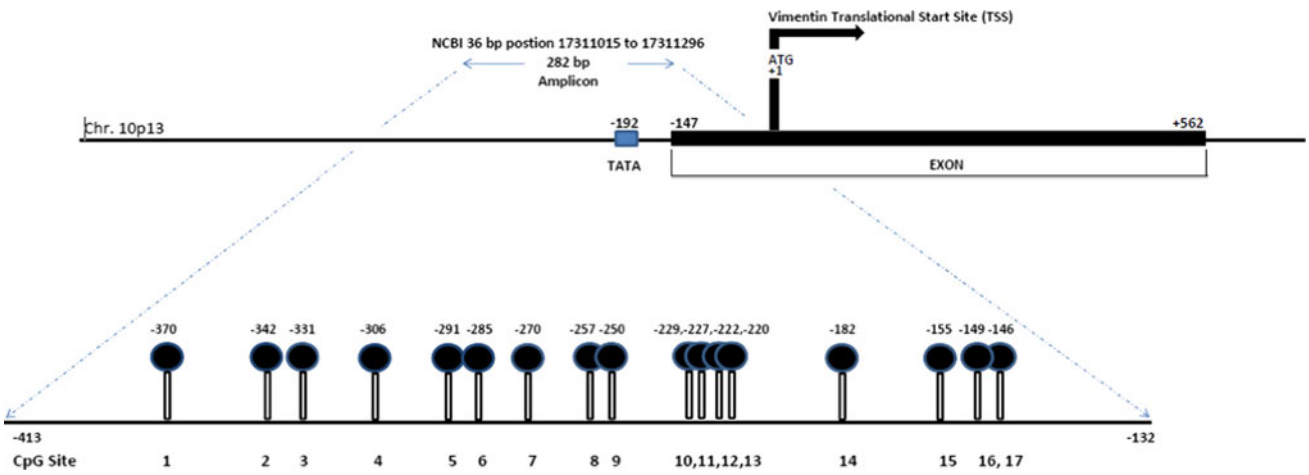


Fig. 1 Position of CpG dinucleotides interrogated for methylation. *Top Row* Base pair positions for the Vimentin amplicon and CG dinucleotides are listed relative to the ATG translational start site (TSS). The TATA box in the Vimentin promoter is at −192 bp. *Bottom row* Expanded view of the 282-bp amplicon shows each of the 1–17 CG sequences (represented by a *circle* on top of a *vertical line*), interrogated for methylation relative to the TSS. The entire sequence was based on human build NCBI 36/hg19, with the Vimentin amplicon spanning bp 17311015–17311296

Statistical analyses

Average linkage hierarchical clustering of Vimentin methylation in cell lines (Fig. 2a) was performed and displayed using MeV (version 4.8.1) of the TM4 software suite [33]. The Student's t test was used to determine the difference between log-relative gene expressions in cell lines (Fig. 2c), as measured using an ABI real-time platform. In addition, gene expression differences were verified using the more conservative Wilcoxon Rank test (Mann–Whitney U). Relative gene expression in tumors was measured by normalized $\log_2$ ratios (Cy5 sample/Cy3 control) of the Agilent Vimentin oligoarray probe P161190. The Pearson r statistic was used to correlate relative gene expression and percent methylation in both tumors (Table 1) and cell lines. Differences in mean percent methylation between molecular breast cancer subtypes (Fig. 3c) was assessed using analysis of variance (ANOVA). Cox proportional hazards models (Table 2) and Kaplan–Meier survival curves (Fig. 4a–f) were generated in SAS (SAS institute, Inc., Cary, NC), and the survival curves were compared by the log-rank statistical test. Reported p values are two-sided. A complete description of the hazard modeling and model selection is provided in Supplemental File 4. All other statistical analyses were performed in R (http://www.R-project.org), unless otherwise noted.

Results

Vimentin methylation in breast cell lines

We designed bisulfite conversion-specific primers to the Vimentin gene promoter located 5' of the ATG start site. CpG sequences in the 282-bp amplicon spanning from −413 to −132 bp from the translational start site (TSS) were interrogated based on NCBI build 36/hg 19 (Fig. 1). Percent Vimentin methylation for CpG sequences interrogated in 14 breast cancer cell lines are shown in Fig. 2a and in Supplemental File 3.

Previous studies have characterized breast tumor cell lines and hTERT immortalized cells from breast tissues as having intrinsic molecular subtypes by means of DNA microarray analysis [22, 24, 29]. The basal-like and luminal subtype characterizations previously described mirrored hormone receptor (HR) status and were applied to the cell lines used in this study [24]. The luminal, HR+ breast tumor cell lines (ZR75, T47D, and MCF7) were hypermethylated for CpGs 1–17, as was the luminal, HER2+ cell line, SKBR3. In contrast, the remaining basal-like HR− cell lines (SUM159PT, ME16C, SUM102, SUM149PT, HCC1937, MDA-MB-468, MDA-MB-231, MCF10A, MCF10AT1, and MCF10DCIS) were hypomethylated across the Vimentin amplicon (Fig. 2a).

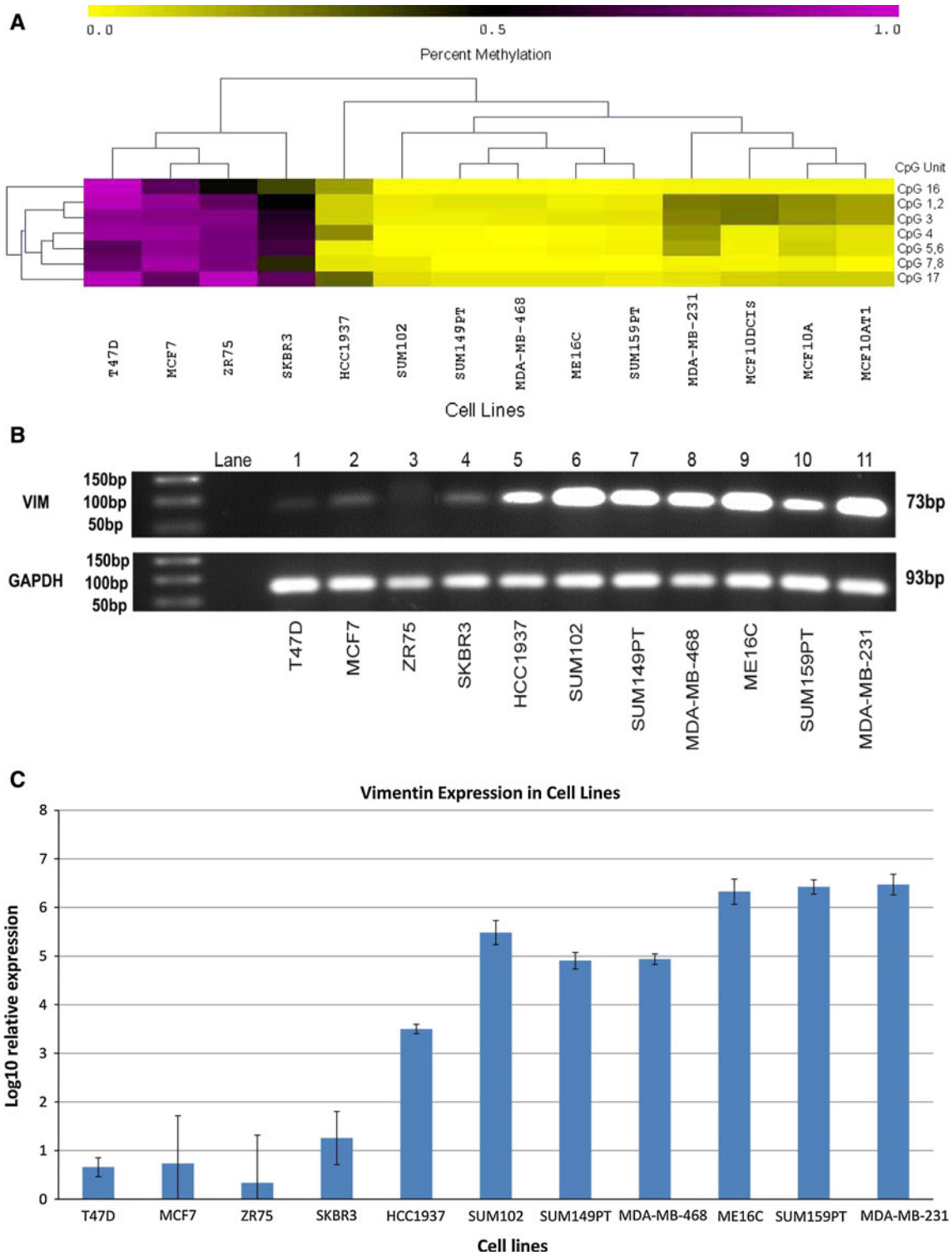
Correlations with vimentin methylation and gene expression in cell lines

Percent methylation per CpG sequence was compared to relative expression of Vimentin mRNA in cell lines using qRT-PCR. As seen in Fig. 2b, the Vimentin mRNA product band is boldly visible in the basal, HR− cell lines and weak or absent in the luminal, HR+ cell lines (lanes 1–4). Both linear and exponential regressions were applied to individual CpG sites as well as to weighted and un-weighted averages across the Vimentin amplicon. Exponential regression fit the un-weighted data with a Pearson correlation value of -0.92 ($p < 0.0001$). CpG site 17 was the most inversely correlated with gene expression at -0.96 ($p < 0.00001$), while the remaining CpG sites had correlations ranging from -0.78 to -0.96 ($p < 0.01$).

A student's t test was used to compare mean relative quantity (RQ), the differences of Vimentin expression between the HR+ and HR− groups (Fig. 2c). Hypomethylated HR− cell lines had significantly higher Vimentin mRNA than hypermethylated HR+ cell lines ($t = 10.12$; $p < 0.00001$). In addition , a more statistically conservative non-parametric Mann–Whitney test was applied because of the potential violation of normality assumptions for percent methylation values (Fig. 2c). Again, differences in mean gene expression between the HR− and HR+ cell lines remained significant ($U = 32.0$; $p < 0.01$).

Vimentin methylation in breast tumors and matched normal pairs

Molecular subtypes for breast tumors were assigned based on Agilent oligoarray $\log_2$ gene expression values using the PAM50 predictor as previously described [29]. The magnitude of Vimentin methylation varied according to sample type. Overall, Vimentin methylation values were higher in luminal cell lines (>50 % average methylation for SKBR3 and >75 % average methylation for the three ER+ cell lines) than those found in breast tumors (>20 % average methylation) and matched normal tissue (~5 % average methylation). The scatter plot in Fig. 3a shows percent Vimentin methylation distributions per CpG unit for breast tumors grouped by molecular subtype. Luminal and HER2-enriched (HER2 over-expressing) subtypes are differentially methylated in comparison with paired normal tissues, whereas basal-like tumors are not significantly more methylated than their paired normal tissues, with the exception of 9830-030619 and UNC01-0318 (Fig. 3b). We measured methylation in paired tumor normal breast tissues as an indicator of possible field effects and as a base line reference.



As per Fig. 3b, the dynamic range of methylation varied greatly in tumors (particularly luminal and HER2 enriched tumors) but was very low in matched normal pairs taken from the ipsilateral breasts of the tumor samples. Only one matched pathologically normal breast tissue sample had average methylation of 11 % (Luminal B normal sample BC00017, Fig. 3b). With the exception of four samples with average methylation ranging between 6 and 8 %, the remaining normal samples had <5 % average methylation (Supplemental File 2).

Analysis of variance (ANOVA) results in Fig. 3c show statistically different methylation values at the CpG#17 site

◀**Fig. 2** **a** Cluster analysis of differential Vimentin methylation in breast cancer cell lines. Vimentin DNA methylation was quantified on a mass array platform per CpG unit across the amplicon. Percent methylation is represented on a color continuum from zero percent methylation (*gold*) to 50 % methylation (*black*) to 100 % methylation (*purple*). ER+ and HER2+ luminal cell lines (T47D, MCF7, ZR75 and SKBR3) were hypermethylated while the remaining hormone receptor (HR−) cell lines were hypomethylated. **b** Vimentin qRT-PCR electrophoresis in breast cancer cell lines. qRT-PCR was carried out in a subset of breast cell lines on an ABI 7500 real-time platform in 20 µl reaction volumes. GAPDH was amplified in tandem and used as the reference control. Vimentin product bands (73 bp) are faint or absent for the luminal, hypermethylated cell lines (*lanes 1–4*), while robust product bands are seen in the HR− cell lines (*lanes 5–11*). **c** Vimentin expression in breast cancer cell lines. qRT-PCR was used to quantify Vimentin expression relative to GAPDH using commercially available ABI TaqMan probes and primers. All breast cell lines were examined in triplicate. *Error bars* represent the standard error of the mean. HCC1937 was used as the referent cell line, and $\log_{10}$ (RQ) was set at 3.5. Differences between luminal and HR− cell line mRNA expression were highly significant by the student's *t* test ($t = 10.12$; $p < 0.00001$) and by the Mann–Whitney test ($U = 32.0$; $p < .01$)

between tumor subtypes ($p < 0.0001$). Only results for CpG 17 are shown; however, equally significant differential methylation values were observed for the other CpG units. For each CpG unit, the median percent methylation of HER2-enriched, luminal B, or luminal A subtypes is higher than that of basal-like tumors and matched normal tissues (Fig. 3c). Given the published sensitivity of the Sequenom Epityper MassARRAY to quantify methylation in biological samples, including from FFPE or samples with limited cellularity [32, 41], the baseline methylation results we obtained from matched normal breast tissues enabled us to set relevant methylation thresholds in our survival analyses.

Correlating vimentin methylation in breast tumors with gene expression

In contrast to cell lines, correlation coefficients in breast tumors were lower, but remained significant. Table 1 shows inverse correlations ranging from −0.228 to −0.381 ($p < 0.03$), which were derived from $\log_2$ expression (R/G) of the Vimentin Agilent microarray probe P161190 versus percent methylation per Vimentin CpG unit. Lower negative correlations are likely due in part to the increased cellular heterogeneity found within tumors, relative to much higher cellular homogeneity observed in cell lines. Although correlations in breast tumors were lower overall, they nevertheless remained statistically significant (Table 1).

Survival analysis: Vimentin methylation in association with clinical factors

Distribution plots of average Vimentin CpG methylation across the amplicon revealed a threshold of methylation between 15 and 20 % relative to matched normal controls (Fig. 3a, b). In the light of the significant negative correlation findings with gene expression, we conservatively chose 20 % Vimentin methylation thresholds to model survival in Cox proportional hazards bivariate analyses. Figure 4a–d shows Vimentin methylation significantly predicted OS and recurrence-free survival (RFS) for all tumor subtypes, as well as in the subset where the basal-like subtypes were removed from the analysis (Fig. 4c, d). After further parsing the tumor samples by ER status, Vimentin methylation predicted OS in ER+ but not in ER− tumors (Fig. 4e, f).

In order to test if other confounding predictors of survival explained the Vimentin methylation finding, we considered other available predictors one at a time in Cox regression bivariate analyses (Table 2). For predictors with more than two values, e.g., molecular subtype, tumor size; and tumor stage, reference values, were assigned to the least clinically severe category. The *p* values reported for the other categories within a multicategory predictor address if the unadjusted hazard rate for the time to death was different for that category as compared with the reference category.

Predictors with no distinct effects for the survival in the bivariate analysis were not considered in subsequent multivariate analyses (Supplemental File 4). Multivariate Cox regression models of the time to death were then tested in combinations of marginally significant ($p < 0.10$) predictors from the bivariate analyses: methylation >20 %; female; luminal B subtype; tumor size 2–5 cm; tumor size >5 cm; nodal status at diagnosis; metastatic disease at diagnosis; and stage II, stage III, and stage IV breast cancer. Using these ten predictors of time to death, we generated composite models through forward selection, stepwise selection, and backward elimination, with $p = 0.05$ as the basis for removing and retaining variables. All three alternatives generated the same model with the following predictors as shown in Multivariate model 1A (Table 2). We also repeated the process for Multivariate Model 1B, except that the three male breast cancer cases were removed from the analysis. The hazard ratios in Table 2 were based on multivariate model 1B, which show that after controlling for the effects of lymph node status and metastatic disease on survival, the estimated hazard for females dying from breast cancer is 2.6 times greater for those with average Vimentin methylation >20 %, as compared to those with <20 % methylation (95 % CI, 1.2–5.3).

Discussion

Vimentin methylation was observed in both luminal cell lines and in luminal and HER2-enriched molecular tumor

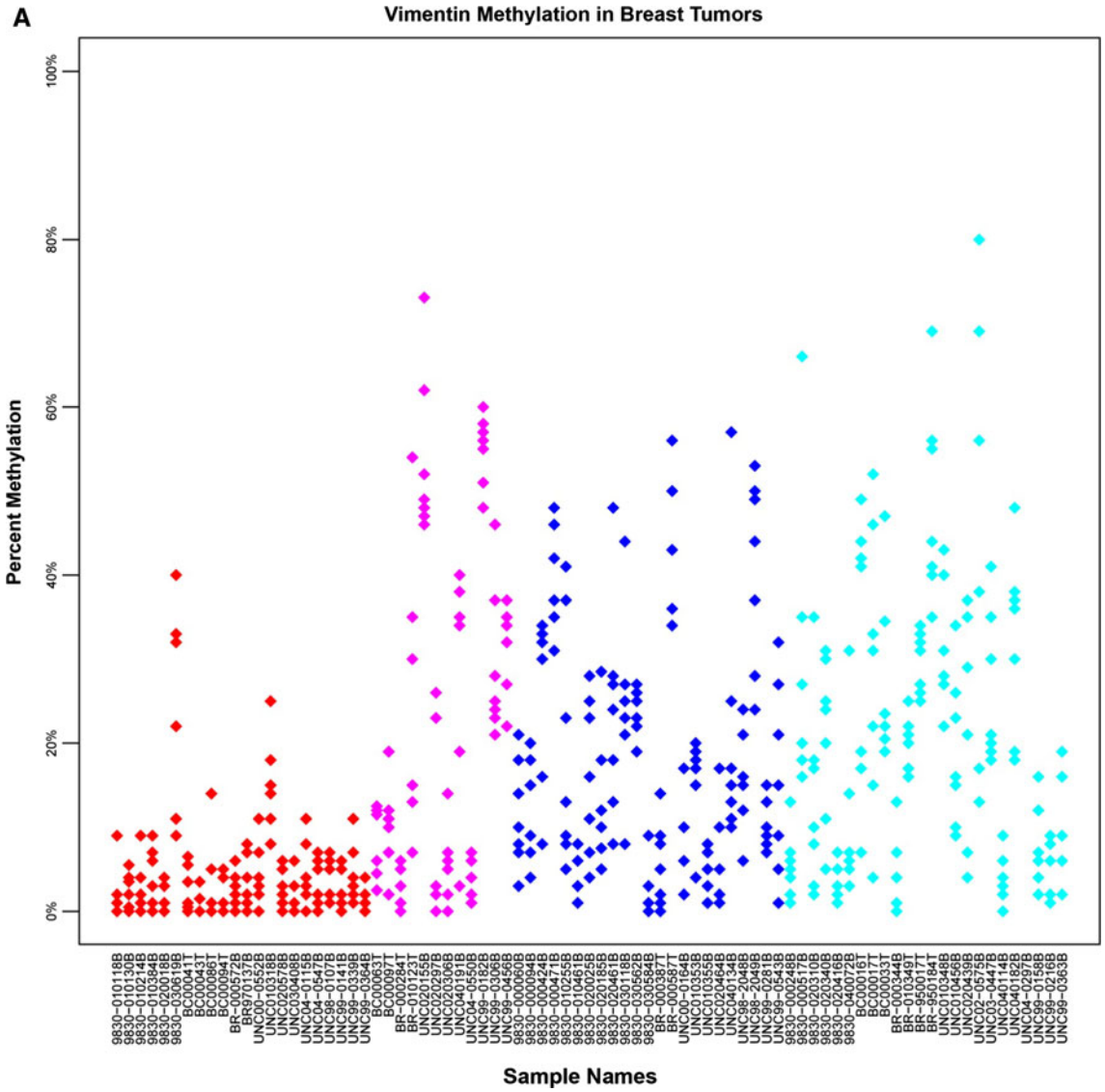


Fig. 3 **a, b** Vimentin methylation in breast tumors and matched tumor normal pairs by molecular subtype**. a** Breast tumors ($n = 81$) are listed on the horizontal axis and percent methylation on the vertical axis. Diamonds represent percent methylation for each CpG unit within the Vimentin amplicon. *Note* Similar percent methylation values for either CpGs 1, 2, 3, 4, 5, 6, 7, 8, 16, and 17 can overlap and may appear as "one diamond." Tumors are grouped by molecular subtypes assigned from previous oligoarray analysis. (Basal = *red*, HER2-enriched = *pink*, Luminal A = *Dark Blue*, and Luminal B = *Light blue*). **b** *Top row* Methylation of $n = 57$ tumors and $n = 57$ matched adjacent normal breast tissue pairs. Differential methylation is seen in tumors, while paired normal tissues have average baseline methylation values of 11 % or less. **c** ANOVA of Vimentin CpG 17 methylation by molecular subtype. ANOVA was performed in $n = 83$ tumors + $N = 57$ matched normal pairs ($n = 140$ total samples) and grouped by molecular subtype (*x axis*). The *boxplot* shows percent methylation distributions where the upper and lower whiskers represent 1.5 times the interquartile range (IQR). Significant differences ($p < 0.00001$) were observed in percent methylation between tumor subtypes

subtypes, but infrequent in the basal-like tumors. Overall, increased methylation was negatively correlated with Vimentin mRNA levels quantified via qRT-PCR in cell lines and by oligomicroarray in breast tumors. Our results mirror other tumor studies where Vimentin methylation was also shown to be inversely correlated with gene expression in cervical cancer [12] and in bladder cancer cell lines [4]. In general, previous studies showed that Vimentin methylation was differentially methylated in gastric, cervical, hepatocellular, and colorectal tumor versus normal tissues [12, 14–16, 37] and was associated with a higher tumor stage and grade in liver cancer [15].

Because previous studies have shown up-regulated Vimentin expression is associated with EMT, poor prognosis, and the basal-like subtype [18, 26, 29, 39], our strong association finding of increased Vimentin methylation with

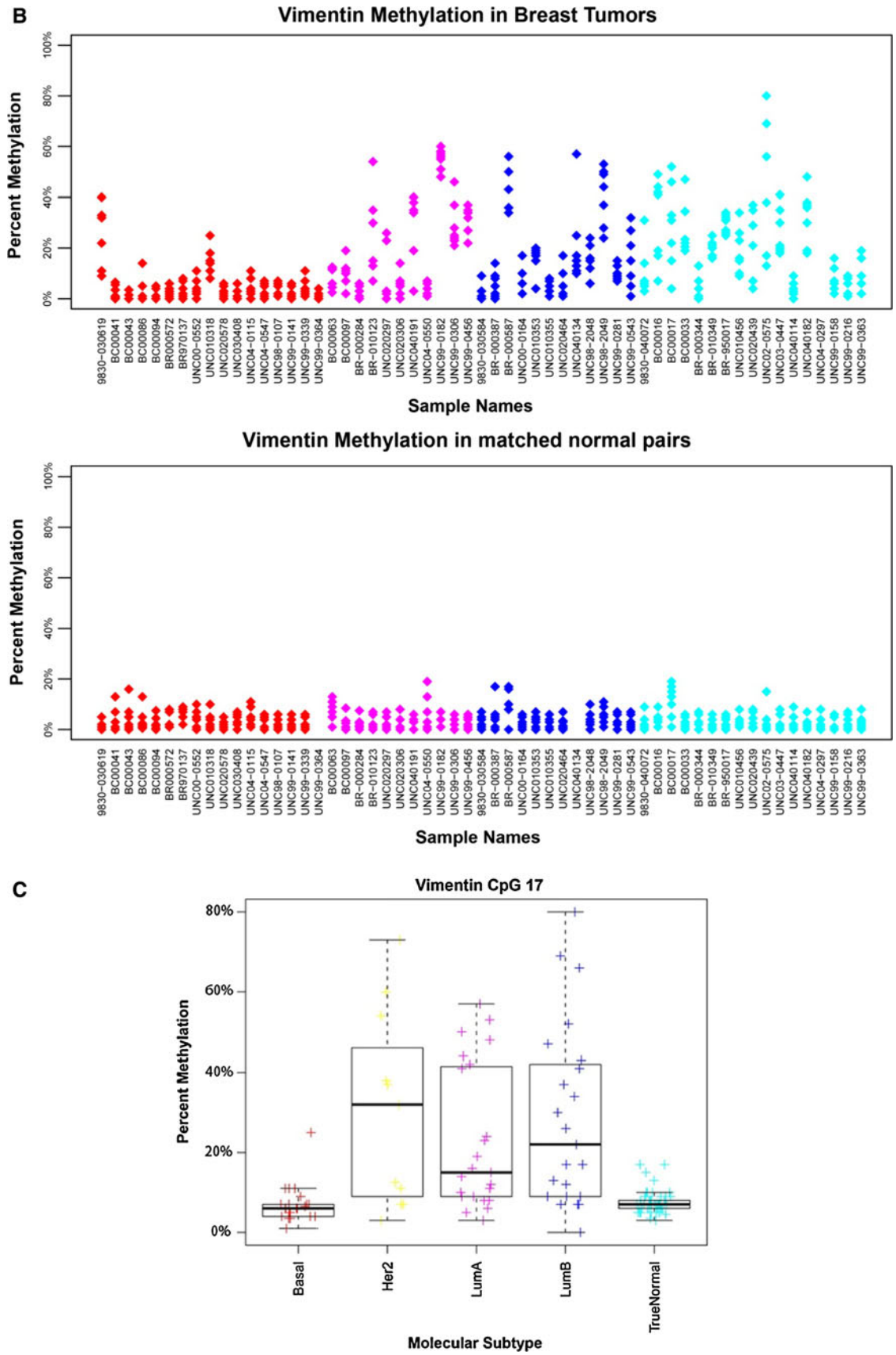


Fig. 3 continued

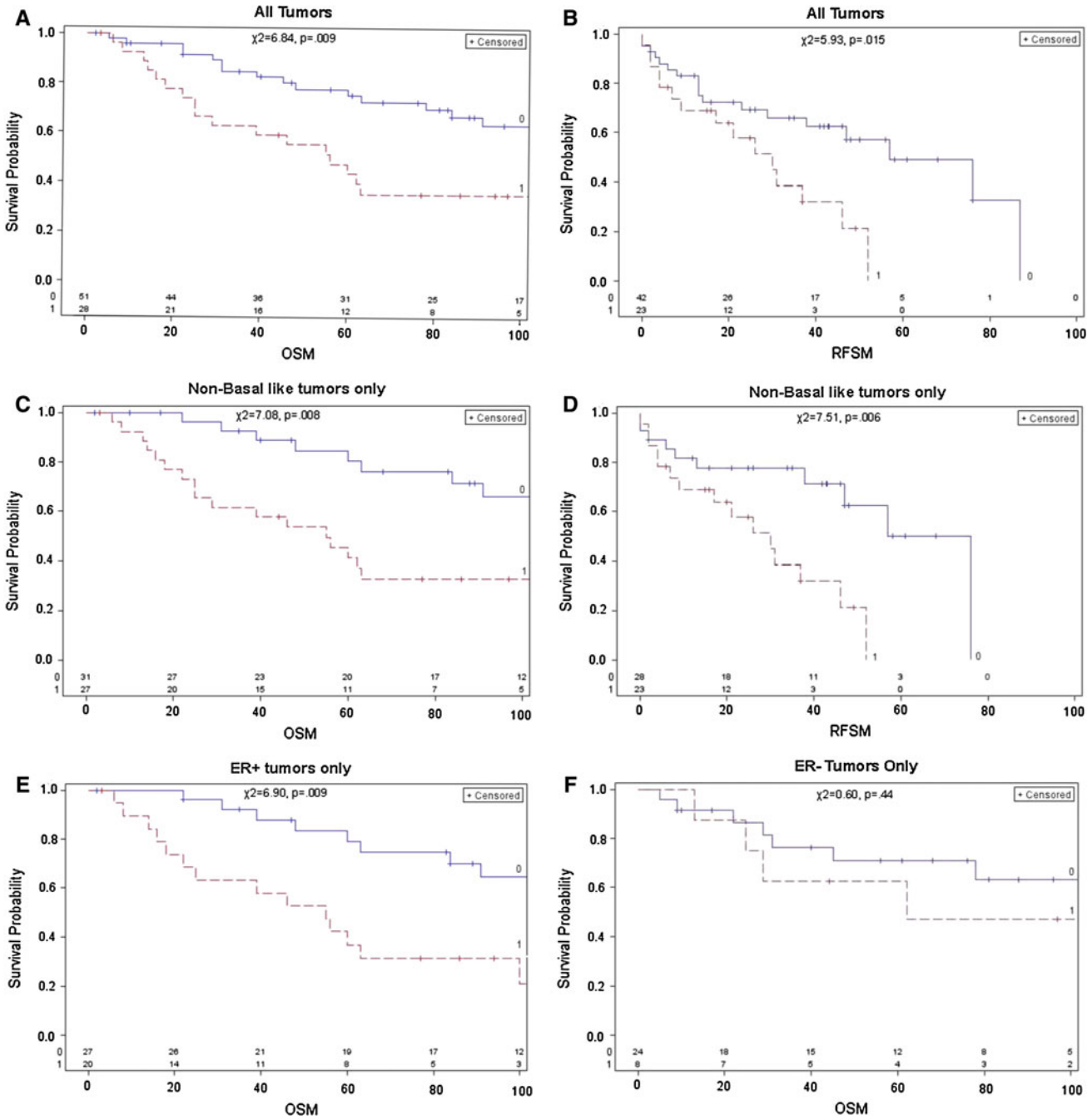


Fig. 4 a–f Overall and recurrence free survival by Vimentin methylation status. Kaplan–Meier overall (OS) and recurrence free (RFS) survival analyses were carried out by stratifying breast cancer patients with average Vimentin methylation thresholds above 20 percent (1 = *red line*) and below 20 percent (0 = *blue line*) based on relative methylation in matched normal tissues. OS and RFS were recorded in months (*horizontal axis*). The number of tumors in each analysis varied according to complete data available for the variables under study: **a** OS in $N = 79$ breast tumors, **b** RFS in $N = 65$ breast tumors, **c** OS in $N = 58$ tumors after removing the basal-like tumors from the analysis, **d** RFS in $N = 51$ tumors after removing 22 basal-like tumors from the analysis, **e** OS in $N = 47$ ER+ tumors only, **f** OS in $N = 32$ ER− tumors only. Results for Fig. 4a–e were highly significant and showed breast cancer patients with <20 % average Vimentin methylation survive much longer than those with >20 % methylation (range log rank $\chi^2 = 5.93$–7.51, $p = 0.015$–0.006). Survival differences were not significant in ER− breast tumors (**f**)

markedly decreased survival, particularly within ER + disease, was counter to our hypothesis and merits further exploration. Breast cancer is inherently heterogeneous and represents at least two different diseases [27, 28]. At minimum, therefore, the methylation marks we quantified in the Vimentin promoter are likely to have a complex etiology that

Table 2 Cox proportional hazards modeling of vimentin methylation

Predictor	Bivariate model *p* value	*N*	Multivariate 1A* *p* value	Multivariate 1B* *p* value
Methylation >20 %	0.012	79	0.019	0.011
Lymph Node Status	<0.001	79	0.005	0.004
Metastatic Disease	<0.001	80	0.001	<0.001
Female	0.015	80	0.043	
Black	0.122	77		
Age	0.212	80		
ER Positive	0.588	80		
HER2 Positive	0.593	73		
Molecular Subtype		78		
Luminal A	–			
Basal	0.973			
Luminal B	0.054			
HER2 Over-expressing	0.990			
Tumor Size		80		
<=2 cm	–			
2–5 cm	0.024			
>5 cm	0.004			
Tumor Stage		80		
Stage 1	–			
Stage II	0 058			
Stage III	0.005			
Stage IV	0.001			

Parameter	Hazard ratio	95 % Confidence intervals***	
		Lower limit	Upper limit
Methylation >20 %	2.6	1.2	5.3
Lymph node positivity	3.1	1.4	6.6
Metastatic disease	4.3	1.9	9.7

Bivariate analysis identified marginally significant ($p < 0.1$) predictors of survival later tested in multivariate Cox regression models. Reference categories were set for those predictors with more than two categories (Supplemental file 3)

Possible predictors in the multivariate model were used to generate composite models through forward, backward, and stepwise selection, with $p = 0.05$ as the basis for removing and retaining variables. All three approaches generated the same model with multivariate model

* 1A based on $N = 75$ female and $N = 3$ male (total $N = 78$ cases), and multivariate model

** 1B based on $N = 75$ female-only breast cancer cases

*** Hazard ratios were based on multivariate model 1B and show women with >20 % average Vimentin methylation were 2.6 times more likely to die compared to those with lower Vimentin methylation levels

differs between basal and non-basal like tumors. Nevertheless, there may be several possible contributors to this finding.

First, even in homogenous cell culture, high levels of methylation did not completely silence expression in the luminal and SKBR3 cell lines (Fig. 2a, b), suggesting the dose–response of Vimentin methylation may differ between mRNA and protein. We were not able to assess Vimentin protein because only DNA remained from the previously analyzed tumor and tumor normal samples for which oligoarray gene expression data were already available. However, past studies have found Vimentin protein being consistently high in most basal tumors, but being infrequently expressed in most luminal breast tumors [18, 25, 39]. Therefore, it is likely that associations of Vimentin methylation with homogeneously low Vimentin protein previously reported in luminal tumors would not have attained significance in this dataset, given the heterogeneous methylation seen in our luminal A, luminal B, and HER2-enriched tumors (Fig. 3a–c). Specifically, we suggest that the frequent Vimentin methylation seen in luminal, but not in basal-like tumors, is part of a larger phenotype.

Indeed, future studies may show that Vimentin methylation is predictive of survival only in luminal and/or HER2-enriched subtypes: breast tumors which contrast sharply with basal-like tumors possessing their own distinct molecular signatures and biological origins [26, 27, 29, 34, 35]. For example, the majority of basal-like tumors are ER− by IHC, and we found that Vimentin methylation predicted OS in ER+ but not ER− tumors (Fig. 4e, f). However, differences in survival observed in Vimentin hypermethylated versus hypomethylated (greater vs <20 % average methylation, respectively) ER− tumors may not have attained statistical significance because of the smaller number of ER− tumors in our sample set. Therefore, future studies will interrogate a larger sample of ER− tumors to determine if Vimentin methylation is a useful biomarker in predicting survival in these breast cancers.

Perhaps the most compelling explanation for Vimentin methylation predicting poor survival may be ascribed to the previously described role of "drivers" in mediating deterministic and predictable genetic and epigenetic changes in multiple downstream "passengers" [5, 40, 42]. In such a scenario, Vimentin methylation may be a passenger event resulting from non-random epigenetic changes, and therefore may function as a reliable surrogate of a pathway that is deterministic for poor survival. Previous studies support such a possibility in that numerous gene promoters can be highly methylated without apparent effects on gene expression at either the RNA or protein level [30, 40, 43].

The potential for selection bias is a concern for any tumor marker study [19]. However, apart from the higher survival rates in the subset of basal-like tumors (Table 2, bivariate analysis), OS characteristics of our sample were similar to previous breast cancer studies. In order to address potential bias, we therefore excluded basal-like tumors in a subset of survival analyses (Fig. 4c, d) and still obtained significant findings. As expected, other variables previously known to confer poor survival, such as tumor size, stage, luminal B subtype, nodal status, and metastatic disease, were significant in the bivariate analyses (Table 2). Several strengths of our study are that (1) we quantified methylation for individual CpG sequences spanning approximately 300 bp, (2) we assayed methylation in luminal versus basal-like breast cell lines and then validated our findings in breast tumors, (3) we tested Vimentin methylation in a bivariate model and also in a multivariate model side by side with other strong predictors of survival, and (4) we carefully assayed matched tumor-normal pairs (e.g., histologically normal ipsilateral breast tissues from cases), which in turn allowed us to set relevant Vimentin methylation thresholds for our statistical analyses.

In summary, regardless of whether it is a surrogate, or has a more direct biological role in survival, the clinical implications of Vimentin methylation as a prognostic marker are great. It is especially encouraging that the strength of the methylation effect on survival remained robust in a multivariate model, even after including such strong predictors as nodal status and metastatic disease at diagnosis. Future studies will determine the sensitivity and specificity of Vimentin methylation to predict recurrence free and OS in both ER+ and ER− tumors in a larger population-based study of breast cancer.

Additional material

Supplemental File 1 contains the geo names, and clinical and histopathological data for the breast cancer cases. Supplemental File 2 provides methylation data for all the breast tumor/matched normal pairs. Supplemental File 3 has Vimentin and GAPDH methylation in positive and negative controls and breast cell lines. Supplemental File 4 details the bivariate and multivariate analyses and Cox proportional hazards modeling.

Acknowledgments The authors thank Dr. Tim Wiltshire and the Lineberger Cancer Center for permitting the use of the Mass Array Platform to carry out the methylation studies; Dr. Lisa Carey and Amy Drobish for clinical updates and medical records abstraction; and Dr. Scott Bultman for his thoughtful review of the paper. The authors gratefully acknowledge the support of the NIH/NCI Breast SPORE CA058823 to C.M. Perou, and the KL2RR025746 from the National Center for Research Resources and the Susan G. Komen Foundation KG090180 to T. Swift-Scanlan.

Conflicts of interest The experiments described in this study comply with the current laws of the country in which they were performed. CMP is a major stockholder of Bioclassifier LLC and co-founder and managing partner of University Genomics. The other authors declare no competing interest.

References

1. Challa AA, Stefanovic B (2011) A novel role of vimentin filaments: binding and stabilization of collagen mRNAs. Mol Cell Biol 31:3773–3789. doi:10.1128/MCB.05263-11
2. Chen WD, Han ZJ, Skoletsky J, Olson J, Sah J, Myeroff L, Platzer P, Lu S, Dawson D, Willis J, Pretlow TP, Lutterbaugh J, Kasturi L, Willson JK, Rao JS, Shuber A, Markowitz SD (2005) Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst 97:1124–1132. doi:10.1093/jnci/dji204
3. Coolen MW, Statham AL, Gardiner-Garden M, Clark SJ (2007) Genomic profiling of CpG methylation and allelic specificity using quantitative high-throughput mass spectrometry: critical evaluation and improvements. Nucleic Acids Res 35:e119. doi:10.1093/nar/gkm662
4. Costa VL, Henrique R, Danielsen SA, Duarte-Pereira S, Eknaes M, Skotheim RI, Rodrigues A, Magalhaes JS, Oliveira J, Lothe RA, Teixeira MR, Jeronimo C, Lind GE (2010) Three epigenetic

biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. Clin Cancer Res 16:5842–5851. doi:10.1158/1078-0432.CCR-10-1312

5. Dumont N, Wilson MB, Crawford YG, Reynolds PA, Sigaroudinia M, Tlsty TD (2008) Sustained induction of epithelial to mesenchymal transition activates DNA methylation of genes silenced in basal-like breast cancers. Proc Natl Acad Sci USA 105:14867–14872. doi:10.1073/pnas.0807146105
6. Ehrich M, Bocker S, van den Boom D (2005) Multiplexed discovery of sequence polymorphisms using base-specific cleavage and MALDI-TOF MS. Nucleic Acids Res 33:e38. doi:10.1093/nar/gni038
7. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102:15785–15790. doi:10.1073/pnas.0507816102
8. Fackler MJ, Umbricht CB, Williams D, Argani P, Cruz LA, Merino VF, Teo WW, Zhang Z, Huang P, Visvananthan K, Marks J, Ethier S, Gray JW, Wolff AC, Cope LM, Sukumar S (2011) Genome-wide methylation analysis identifies genes specific to breast cancer hormone receptor status and risk of recurrence. Cancer Res 71:6195–6207. doi:10.1158/0008-5472.CAN-11-1630
9. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, Nobel A, Parker J, Ewend MG, Sawyer LR, Wu J, Liu Y, Nanda R, Tretiakova M, Ruiz Orrico A, Dreher D, Palazzo JP, Perreard L, Nelson E, Mone M, Hansen H, Mullins M, Quackenbush JF, Ellis MJ, Olopade OI, Bernard PS, Perou CM (2006) The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 7:96. doi:10.1186/1471-2164-7-96
10. Itzkowitz SH, Jandorf L, Brand R, Rabeneck L, Schroy PC 3rd, Sontag S, Johnson D, Skoletsky J, Durkee K, Markowitz S, Shuber A (2007) Improved fecal DNA test for colorectal cancer screening. Clin Gastroenterol Hepatol 5:111–117. doi:10.1016/j.cgh.2006.10.006
11. Ivaska J (2011) Vimentin: central hub in EMT induction? Small Gtpases 2:51–53. doi:10.4161/sgtp.2.1.15114
12. Jung S, Yi L, Kim J, Jeong D, Oh T, Kim CH, Kim CJ, Shin J, An S, Lee MS (2011) The role of vimentin as a methylation biomarker for early diagnosis of cervical cancer. Mol Cells 31:405–411. doi:10.1007/s10059-011-0229-x
13. Katz E, Dubois-Marshall S, Sims AH, Gautier P, Caldwell H, Meehan RR, Harrison DJ (2011) An in vitro model that recapitulates the epithelial to mesenchymal transition (EMT) in human breast cancer. PLoS ONE 6:e17083. doi:10.1371/journal.pone.0017083
14. Kisiel JB, Yab TC, Taylor WR, Chari ST, Petersen GM, Mahoney DW, Ahlquist DA (2011) Stool DNA testing for the detection of pancreatic cancer: assessment of methylation marker candidates. Cancer. doi:10.1002/cncr.26558
15. Kitamura Y, Shirahata A, Sakuraba K, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K (2011) Aberrant methylation of the Vimentin gene in hepatocellular carcinoma. Anticancer Res 31:1289–1291
16. Kitamura YH, Shirahata A, Sakata M, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K (2009) Frequent methylation of Vimentin in well-differentiated gastric carcinoma. Anticancer Res 29:2227–2229
17. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M (2007) Vimentin and epithelial-mesenchymal transition in human breast cancer—observations in vitro and in vivo. Cells Tissues Organs 185:191–203. doi:10.1159/000101320
18. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, Perou CM (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 19:264–271. doi:10.1038/modpathol.3800528
19. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM (2005) Reporting recommendations for tumor marker prognostic studies. J Clin Oncol 23:9067–9072. doi:10.1200/JCO.2004.01.0454
20. Mendez MG, Kojima S, Goldman RD (2010) Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. The FASEB J 24:1838–1851. doi:10.1096/fj.09-151639
21. Mor-Vaknin N, Punturieri A, Sitwala K, Markovitz DM (2003) Vimentin is secreted by activated macrophages. Nat Cell Biol 5:59–63. doi:10.1038/ncb898
22. Nagaraja GM, Othman M, Fox BP, Alsaber R, Pellegrino CM, Zeng Y, Khanna R, Tamburini P, Swaroop A, Kandpal RP (2006) Gene expression signatures and biomarkers of noninvasive and invasive breast cancer cells: comprehensive profiles by representational difference analysis, microarrays and proteomics. Oncogene 25:2328–2338. doi:10.1038/sj.onc.1209265
23. Ned RM, Melillo S, Marrone M (2011) Fecal DNA testing for colorectal cancer screening: the ColoSure test. PLoS Currents 3:RRN1220. doi:10.1371/currents.RRN1220
24. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10:515–527. doi:10.1016/j.ccr.2006.10.008
25. Park SY, Lee HE, Li H, Shipitsin M, Gelman R, Polyak K (2010) Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. Clin Cancer Res 16:876–887. doi:10.1158/1078-0432.CCR-09-1532
26. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauron C, He X, Hu Z, Quackenbush JF, Stijleman IJ, Palazzo J, Marron JS, Nobel AB, Mardis E, Nielsen TO, Ellis MJ, Perou CM, Bernard PS (2009) Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 27:1160–1167. doi:10.1200/JCO.2008.18.1370
27. Perou CM (2011) Molecular stratification of triple-negative breast cancers. Oncologist 16(Suppl 1):61–70. doi:10.1634/theoncologist.2011-S1-61
28. Prat A, Ellis MJ, Perou CM (2012) Practical implications of gene-expression-based assays for breast oncologists. Nat Rev. Clin Oncol 9:48–57. doi:10.1038/nrclinonc.2011.178
29. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X, Perou CM (2010) Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 12:R68. doi:10.1186/bcr2635
30. Pruitt K, Zinn RL, Ohm JE, McGarvey KM, Kang SH, Watkins DN, Herman JG, Baylin SB (2006) Inhibition of SIRT1 reactivates silenced cancer genes without loss of promoter DNA hypermethylation. PLoS Genet 2:e40. doi:10.1371/journal.pgen.0020040
31. Radpour R, Haghighi MM, Fan AX, Torbati PM, Hahn S, Holzgreve W, Zhong XY (2008) High-throughput hacking of the methylation patterns in breast cancer by in vitro transcription and thymidine-specific cleavage mass array on MALDI-TOF silicochip. Mol Cancer Res 6:1702–1709. doi:10.1158/1541-7786.MCR-08-0262
32. Radpour R, Kohler C, Haghighi MM, Fan AX, Holzgreve W, Zhong XY (2009) Methylation profiles of 22 candidate genes in breast cancer using high-throughput MALDI-TOF mass array. Oncogene 28:2969–2978. doi:10.1038/onc.2009.149

33. Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. Methods Enzymol 411:134–193. doi:10.1016/S0076-6879(06)11009-5
34. Sarrio D, Franklin CK, Mackay A, Reis-Filho JS, Isacke CM (2011) Epithelial and mesenchymal subpopulations within normal basal breast cell lines exhibit distinct stem cell/progenitor properties. Stem Cells. doi:10.1002/stem.791
35. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J (2008) Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 68:989–997. doi:10.1158/0008-5472.CAN-07-2017
36. Satelli A, Li S (2011) Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci. doi:10.1007/s00018-011-0735-1
37. Shirahata A, Sakata M, Sakuraba K, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K (2009) Vimentin methylation as a marker for advanced colorectal carcinoma. Anticancer Res 29:279–281
38. Shirahata A, Sakuraba K, Goto T, Saito M, Ishibashi K, Kigawa G, Nemoto H, Hibi K (2010) Detection of vimentin (VIM) methylation in the serum of colorectal cancer patients. Anticancer Res 30:5015–5018
39. Sousa B, Paredes J, Milanezi F, Lopes N, Martins D, Dufloth R, Vieira D, Albergaria A, Veronese L, Carneiro V, Carvalho S, Costa JL, Zeferino L, Schmitt F (2010) P-cadherin, vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. Histol Histopathol 25:963–974
40. Swift-Scanlan T, Vang R, Blackford A, Fackler MJ, Sukumar S (2011) Methylated genes in breast cancer: associations with clinical and histopathological features in a familial breast cancer cohort. Cancer Biol Ther 11:853–865. doi:10.4161/cbt.11.10.15177
41. Vanaja DK, Ehrich M, Van den Boom D, Cheville JC, Karnes RJ, Tindall DJ, Cantor CR, Young CY (2009) Hypermethylation of genes for diagnosis and risk stratification of prostate cancer. Cancer Invest 27:549–560. doi:10.1080/07357900802620794
42. Veeck J, Esteller M (2010) Breast cancer epigenetics: from DNA methylation to microRNAs. J Mammary Gland Biol Neoplasia 15:5–17. doi:10.1007/s10911-010-9165-1
43. Zinn RL, Pruitt K, Eguchi S, Baylin SB, Herman JG (2007) hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. Cancer Res 67:194–201. doi:10.1158/0008-5472.Can-06-3396