# Histone-modifier Gene Expression Profiles Are Associated with Pathological and Clinical Outcomes in Human Breast Cancer

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**Abstract.** *Background: Epigenetic regulation of gene expression is under normal circumstances tightly controlled by the specific methylation of cytosine residues in CpG dinucleotides and coordinated by adjustments in the histone-dependent configuration of chromatin. Following our original report, providing the first description of potential tumor suppressor function associated with the histone methyltransferase SET domain containing 2 (SETD2) in breast cancer, the objective of this study was to determine the expression profiles of 16 further histone-modifier genes in a well annotated cohort of patients with primary operable breast cancer. Materials and Methods: Breast cancer tissues (n=127) and normal tissues (n=33) underwent RNA extraction and reverse transcription, and histone-modifier gene transcript levels were determined using real-time quantitative PCR. The histone-modifier genes included: histone acetyltransferases (cAMP response element-binding protein-binding protein (CREBBP)); class I (histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2)), II (histone deacetylase 5 (HDAC5)) and III (sirtuin 1 (SIRT1)) histone deacetylases; and histone methyltransferases (SET domain containing suppressor of variegation 3-9 homolog 1 (SUV39H1) and suppressor of variegation 3-9 homolog 2 (SUV39H2)) amongst others. Expression levels were analysed against tumor size, grade, nodal involvement, histological subtype, receptor status, TNM stage, Nottingham Prognostic Index, and disease-free and overall survival over a 10-year follow-up period. Results: Expression of histone-modifier genes in breast cancer differed significantly from those in normal tissue (HDAC5, HDAC1, lysine (K)-specific demethylase 4A (KDM4A) and lysine (K)-specific demethylase 6A (KDM6A)). Differences in expression profiles were also found to exist between individual breast tumors and, in some cases, were significantly associated with conventional pathological parameters and prognostic indices: tumor grade (K (lysine) acetyltransferase 5 (KAT5), HDAC1, KDM4A, SUV39H1 and KDM6A)); TNM stage (SUV39H1, K (lysine) acetyltransferase 2B (KAT2B), lysine (K)-specific demethylase 1A (KDM1A), KDM4A, lysine (K)-specific demethylase 5C (KDM5C), K (lysine) acetyltransferase 8 (KAT8), HDAC5 and KAT5)); Nottingham Prognostic Index (KDM5C, myeloid/lymphoid or mixed-lineage leukemia (MLL), KAT8 and SET and MYND domain containing 3 (SMYD3)); receptor status (KAT5, SMYD3 and KDM1A); histological type (KAT5, KDM5C, KAT8, KDM4A and MLL); disease-free survival (SUV39H1, SMYD3, HDAC5, KDM6A, HDAC1, KDM1A, KDM4A, KAT8, KDM5C, KAT5 and MLL) and overall survival (KAT8). Significant correlations were identified between the differential expression profiles of particular histone-modifying genes. Conclusion: Expression levels of histone-modifier genes in breast cancer differ significantly from normal tissue. Differences in expression profiles exist between breast tumors and are significantly associated with conventional pathological parameters and clinical outcomes. Further study is warranted to determine the consequences of altered expression for each specific histone-modifier gene and the biological and clinical implications of combinatorial variations in expression profiles. Histone-modifier enzymes offer utility as biomarkers and potential for targeted therapeutic strategies.*

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Epigenetic regulation of gene expression is tightly controlled and inherently stabilised under normal circumstances by the specific methylation of cytosine residues in CpG dinucleotides and coordinated adjustments in the configuration of chromatin

(1). The organisation of DNA within the chromatin template depends upon highly conserved histone proteins, the properties of which continue to exceed the simplistic packaging role originally assigned to them (2). Chromatin remodelling, orchestrated by the reversible enzymatic post-translational modification of histones, represents a key regulatory mechanism for gene expression (3-5). Histone-modifier genes (HMGs) are classified by the manner in which they transform the amino-terminal tails of constituent amino acids and include: acetyltransferases (HAT), deacetylases (HDAC) and methyltransferases (HMT), in addition to those mediating phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (6). The combination of these events imparts a dynamic 'histone code', and specific permutations have significant implications for chromatin topology and the functional configuration of promoters (7-9). The genes associated with these covalent modifications have characteristic tissue type-dependent expression profiles (3). In this way, specific nucleosome remodelling complexes can demarcate genomic areas and actively mediate interactions between transcription factors and their targets. Hence, disturbance of the 'epigenetic equilibrium' may result from aberrant methylation and/or altered histone modification, both of which have been implicated in tumorigenesis and cancer progression (2, 10). Aberrant transcriptional regulation, resulting from epigenetic alteration of the accessibility of specific genomic regions and the affinity of activators and repressors within the transcription machinery, is increasingly understood to cooperate with genetic alterations in the molecular pathogenesis of human cancer (1, 2).

The assignment of breast cancer (BC) subtypes to distinct epigenetic categories requires consideration of both methylation status and histone modifications. Genomic methylation studies in BC have revealed significant differences in the methylome between subtypes and association with clinicopathological outcomes (11-13). Similarly, global histone modifications in BC have also been associated with tumor phenotypes, prognostic factors and patient outcomes (14-17). Hence, there is considerable interest in mechanisms which underlie the transition between transcriptionally active and inactive chromatin states and the extent to which these are influenced by the multiplicity of histone modification (7, 18).

Following our original report, providing the first description of potential tumor suppressor function associated with the HMT SET domain containing 2 (SETD2) in BC (19), the objective of the present study was to determine the expression profiles of 16 other HMGs in a well described cohort of patients with primary operable BC (Table I). The HMGs evaluated in this study were selected to include HATs (cAMP response element-binding protein-binding protein (CREBBP)); class I (histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2)), II (histone deacetylase 5 (HDAC5)) and III (sirtuin 1 (SIRT1)) HDACs (20); and HMTs (SET domain containing suppressor of variegation 3-9 homolog 1 (SUV39H1) and suppressor of variegation 3-9 homolog 2 (SUV39H2)) amongst others (Table II). Expression levels were compared with those of normal background tissues and evaluated against established pathological parameters and clinical outcome over a 10-year follow-up period.

Table I. *Clinical and pathological data.*

| Parameter | Category | Number of cases |
|---|---|---|
| Node status | Positive | 54 |
| | Negative | 73 |
| Tumor grade | 1 | 24 |
| | 2 | 43 |
| | 3 | 58 |
| Tumor type | Ductal | 98 |
| | Lobular | 14 |
| | Medullary | 2 |
| | Tubular | 2 |
| | Mucinous | 4 |
| | Non specific | 7 |
| TNM Stage | 1 | 70 |
| | 2 | 40 |
| | 3 | 7 |
| | 4 | 4 |
| NPI | NPI1 | 68 |
| | NPI2 | 38 |
| | NPI3 | 16 |
| Clinical outcome | Disease free | 90 |
| | Alive with metastasis | 7 |
| | With local recurrence | 5 |
| | Died from breast cancer | 16 |
| | Died of unrelated disease | 9 |
| ER Status | ERα | |
| | Negative | 75 |
| | Positive | 38 |
| | ERβ | |
| | Negative | 91 |
| | Positive | 24 |

Note: missing values reflect discarded/un-interpretable values. Abbreviations: Tumor Node Metastasis (TNM), Nottingham Prognostic Index (NPI), oestrogen receptor-alpha (ERα), oestrogen receptor-beta (ERβ).

## Materials and Methods

*Materials*. BC tissues (n=127) and normal background tissues (n=31) were collected from the University Hospital of Wales and St. George's Hospital and Medical School in accordance with institutional guidelines, including ethical approval and informed consent. Specimens were obtained immediately after excision during surgery and stored at −80˚C until use. A consultant pathologist examined haematoxylin and eosin-stained frozen sections to verify the presence of tumor cells in the collected samples. Normal tissue

Table II. *Forward and reverse primers used in this study.*

| Gene symbol | Gene name | Primer | Sense | Sequence |
|---|---|---|---|---|
| *CREBBP* | cAMP response element-binding protein-binding protein | CREBBPF1 | Forward | gctttgtctacacctgcaac |
| | | CREBBPZr1 | Reverse | actgaacctgaccgtacacgtgttatagcagttgatgc |
| | | CREBBPF2 | Forward | gtttccccgcaaatgact |
| | | CREBBPZr2 | Reverse | actgaacctgaccgtacagtccaaatggacttgtgttc |
| *HDAC5* | Histone deacetylase 5 | HDA5F1 | Forward | ttctctgcccaacatctc |
| | | HDA5Zr1 | Reverse | actgaacctgaccgtacactgctgtgtcgacagctt |
| | | HDA5F2 | Forward | agttcctggagaagcagaa |
| | | HDA5Zr2 | Reverse | actgaacctgaccgtacagtcagctcctcctctgtct |
| *HDAC1* | Histone deacetylase 1 | HDAC1F1 | Forward | ggtggttacaccattcgtaa |
| | | HDAC1Zr1 | Reverse | actgaacctgaccgtacatgtatggaagctcattaggg |
| | | HDAC1F2 | Forward | acacgaatgagtacctggag |
| | | HDAC1Zr2 | Reverse | actgaacctgaccgtacacttgtcagggtcgtcttc |
| *HDAC2* | Histone deacetylase 2 | HDAC2F1 | Forward | tgctactactacgacggtga |
| | | HDAC2Zr1 | Reverse | actgaacctgaccgtacaaacagcaagttatgggtcat |
| | | HDAC2F2 | Forward | ccagtgtttgatggactctt |
| | | HDAC2Zr2 | Reverse | actgaacctgaccgtacatcctgatgcttctgatttct |
| *KAT2B* | K (lysine) acetyltransferase 2B | KAT2BF1 | Forward | cctcttgaaaaacgaactct |
| | | KAT2BZr1 | Reverse | actgaacctgaccgtacaagaaaatcctgatcccagat |
| | | KAT2BF2 | Forward | tcatcattaaaaagcagaagg |
| | | KAT2BZr2 | Reverse | actgaacctgaccgtacaaacatgaaagtccagggtaa |
| *KAT5* | K (lysine) acetyltransferase 5 | KAT5F1 | Forward | gaattgtttgggcactga |
| | | KAT5Zr1 | Reverse | actgaacctgaccgtacagcttcgatcagacaccag |
| | | KAT5F2 | Forward | ccttctttgagattgatgga |
| | | KAT5Zr2 | Reverse | actgaacctgaccgtacacgtagaagaggaaagggtct |
| *KDM1A* | Lysine (K)-specific demethylase 1A | KDM1AF1 | Forward | agtggaaaacatctgcagtc |
| | | KDM1AZr1 | Reverse | actgaacctgaccgtacaccgaacaaattgacacttg |
| | | KDM1AF2 | Forward | actgccgagttcttagtgaa |
| | | KDM1AZr2 | Reverse | actgaacctgaccgtacatccaactcctgaagtttttc |
| *KDM4A* | Lysine (K)-specific demethylase 4A | KDM4AF1 | Forward | agcgacaatctttatcctga |
| | | KDM4AZr1 | Reverse | actgaacctgaccgtacaaacttggctccatagacttg |
| | | KDM4AF2 | Forward | gatggcaccagcatactc |
| | | KDM4AZr2 | Reverse | actgaacctgaccgtacacataaacagcagtcctcctc |
| *KDM5C* | Lysine (K)-specific demethylase 5C | KDM5CF1 | Forward | aggatatgcctaaggtccag |
| | | KDM5CZr1 | Reverse | actgaacctgaccgtacagcagctccagatctctctta |
| | | KDM5CF2 | Forward | gactctgcggaagaaagata |
| | | KDM5CZr2 | Reverse | actgaacctgaccgtacagtcttaggcgatgttgactc |
| *KDM6A* | Lysine (K)-specific demethylase 6A | KDM6AF1 | Forward | aatgttggtccacttacagc |
| | | KDM6AZr1 | Reverse | actgaacctgaccgtacatgaaccatgggtactattgac |
| | | KDM6AF2 | Forward | ccaatggacccttttctg |
| | | KDM6AZr2 | Reverse | actgaacctgaccgtacattcctgttatatgattattgccta |
| *MLL* | Myeloid/lymphoid or mixed-lineage leukemia | MLL2F1 | Forward | cgatcagagcctaaaatcc |
| | | MLL2Zr1 | Reverse | actgaacctgaccgtacatgtgaaggtgctctgatatg |
| | | MLL2F2 | Forward | cttttgctcagggagtgat |
| | | MLL2Zr2 | Reverse | actgaacctgaccgtacatcagctgcatctgtaggag |
| *KAT8* | K (lysine) acetyltransferase 8 | MYST1F1 | Forward | gccgagaggaattctatgta |
| | | MYST1Zr1 | Reverse | actgaacctgaccgtacaacagcatccttcactgtctt |
| | | MYST1F2 | Forward | cgagtactgcctcaagtaca |
| | | MTST1Zr2 | Reverse | actgaacctgaccgtacatcttgcggtagatctctttc |
| *SIRT1* | Sirtuin 1 | SIRT1F1 | Forward | gatccaagaccattcttcaa |
| | | SIRT1Zr1 | Reverse | actgaacctgaccgtacagcgaagtagttttccttcct |
| | | SIRT1F2 | Forward | tcattgttattgggtcttcc |
| | | SIRT1Zr2 | Reverse | actgaacctgaccgtacagatgaggcaaaggttctcta |
| *SMYD3* | SET and MYND domain containing 3 | SMYD3F1 | Forward | gtgtgcaaggggagtcgt |
| | | SMYD3Zr1 | Reverse | actgaacctgaccgtacatttttctgacacttagcactaca |
| | | SMYD3F2 | Forward | ggaaggaagttcaagaatcc |
| | | SMYD3Zr2 | Reverse | actgaacctgaccgtacacaccttcagctggtagatgt |
| *SUV39H1* | suppressor of variegation 3-9 homolog 1 | SUV39H1F1 | Forward | gagaagattcgcaagaacag |
| | | SUV39H1Zr1 | Reverse | actgaacctgaccgtacaacgtagtccaggtcaaagag |
| | | SUV39H1F2 | Forward | gtgcgattacaagaagatcc |
| | | SUV39H1Zr2 | Reverse | actgaacctgaccgtacatacgcacacacttgagattc |
| *SUV39H2* | suppressor of variegation 3-9 homolog 2 | SUV39H2F1 | Forward | ggtgtaaagacccttgtgaa |
| | | SUV39H2Zr1 | Reverse | actgaacctgaccgtacagaactgtcctcgtctttcag |
| | | SUV39H2F2 | Forward | gcaatgtgtctcattttgtg |
| | | SUV39H2Zr2 | Reverse | actgaacctgaccgtacatggttcttgtggaaaacaat |

was derived from the background breast parenchyma of BC patients within the study group. Medical notes and histology reports were used to extract clinicopathological data (Table I). A customized database was established to record the data.

*Tissue processing, RNA extraction and cDNA synthesis*. Frozen sections of tissue were cut at a thickness of 5-10 μm and kept for routine histological analysis. An additional 15-20 sections were mixed and homogenized using a hand-held homogenizer in ice-cold RNA extraction solution. RNA from cells was extracted using an RNA extraction kit (AbGene Ltd, Surrey, UK). RNA concentration was quantified using a UV spectrophotometer (Wolf Laboratories, York, UK). Reverse transcription (RT) was carried out using a standard kit; cDNA was synthesised using first-strand synthesis with an anchored oligo$^{dt}$ primer (AbGene Ltd). The polymerase chain reaction (PCR) was performed using sets of primers (Table II) with the following conditions: 5 min at 95˚C, 20 s at 94˚C, 25 s at 56˚C, 50 s at 72˚C for 36 cycles and finally 72˚C for 7 min. PCR products were separated on a 0.8% agarose gel (AbGene Ltd), visualised under UV light, photographed using a Unisave™ camera (Wolf Laboratories, York, UK) and recorded with Photoshop™ software (Adobe Systems Incorporated, Middlesex, UK).

*Quantitative analysis of histone-modifier genes*. HMG transcript levels of the prepared cDNA were determined using real-time quantitative PCR, based on Amplifluor™ technology, as modified from previous reports (21). Pairs of PCR primers were designed using the Beacon Designer™ software (Version 2; Palo Alto, CA, USA) and synthesized by Sigma-Aldrich (Dorset, UK). Added to the reverse primer was an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca-'3) which is complementary to the universal Z probe (Intergen Inc., Oxford, UK). The product expands one intron (Table II). The reaction was carried out using the following: custom-made hot-start Q-master mix (AbGene Ltd), 10 pmol of specific forward primer, 1 pmol reverse primer with the Z sequence (Table II), 10 pmol of fluorogenic reporter dye, carboxyfluorescein (FAM)-tagged probe (Intergen Inc.), and cDNA generated from 50 ng RNA. The reaction was carried out using IcyclerIQ™ (Bio-Rad, Hemel Hempstead, UK) which is equipped with an optical unit that allows real-time detection of 96 reactions, under the following conditions: 94˚C for 12 min, 50 cycles at 94˚C for 15 s, 55˚C for 40 s and 72˚C for 20 s. The transcript levels were generated from an internal standard of reference cDNA that was simultaneously amplified with the samples. With every PCR run, a negative control without template and a known cDNA reference sample as a positive control, were included.

*Statistical analysis*. The Mann-Whitney *U*-test (comparison of median transcript copy numbers) and two-sample *t*-test (comparison of mean transcript copy numbers) were used for statistical analysis of transcript copy numbers. For normality, the Anderson-Darling test was used. The transcript levels within BC specimens were compared to those of normal tissues and analysed against conventional pathological parameters and clinical outcome over a 10-year follow-up period. In each case, the true copy number was used for statistical analysis. The statistical analysis was carried out using Minitab version 14.1 (Minitab Ltd., Coventry, UK) using a custom-written macro (Stat 2005.mtw). Correlation coefficients between HMGs were determined with SigmaStat (Systat Software, Inc., Hounslow, UK) using ranked Spearman results. For purposes of the Kaplan-Meier survival analysis, the samples were divided into two groups, 'positive' and 'negative', defined by the ability of the assay to detect the transcript of interest. Survival analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Nottingham Prognostic Index (NPI)=tumor size (cm)×0.2 + lymph node stage (1, no nodes affected; 2, up to 3 nodes affected; 3, more than 3 nodes affected) + grade (1-3, Scarff-Bloom-Richardson grade). NPI scores were classified into three groups: <3.4=NPI-1, 3.4-5.4=NPI-2, >5.4=NPI-3. Within tumor samples, oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) status were classified according to transcript copy number per 50 ng of RNA: <1=negative, ≥1=positive.

## Results

*Tissue expression*. HMGs were expressed in both normal/benign breast tissue and BC specimens. Significantly higher transcript levels in BC specimens, compared to normal/benign tissue, were found for *HDAC5* (mean, $p$=0.055), *HDAC1* (mean, $p$=0.0033), lysine (K)-specific demethylase 4A (*KDM4A*) (mean, $p$=0.025) and lysine (K)-specific demethylase 6A (*KDM6A*) (mean, $p$=0.025). In subgroup analysis, statistical significance was maintained comparing invasive ductal carcinomas (IDC) to normal/benign tissue for *HDAC1* (mean, $p$=0.0085), *KDM4A* (mean, $p$=0.045) and *KDM6A* (mean, $p$=0.036).

*Tumor grade*. Higher HMG transcript levels were associated with increasing tumor grade: grade 1 *vs*. 2 for K (lysine) acetyltransferase 5 (*KAT5*) (mean, $p$=0.014), grade 1 *vs*. 3 for *HDAC1* (mean, $p$=0.031), grade 2 *vs*. 3 for lysine (K)-specific demethyldase 4A (*KDM4A)* (mean, $p$=0.035) and *SUV39H1* (median, $p$=0.0200), grade 1 *vs*. 2 and 3 for *KAT5* (mean, $p$=0.016) and *HDAC1* (mean, $p$=0.024). Similarly, in subgroup analysis of IDCs: grade 1 *vs*. 3 for lysine (K)-specific demethylase 6A (*KDM6A)* (mean, $p$=0.044), *SUV39H1* (mean, $p$=0.034 and median, $p$=0.0387) and *HDAC1* (mean, $p$=0.018), grade 1 *vs*. 2 and 3 for *SUV39H1* (mean, $p$=0.0095), *HDAC1* (mean, $p$=0.0074), *KAT5* (mean, $p$=0.044) and *KDM6A* (mean, $p$=0.029). Higher HMG transcript levels were also associated with lower tumor grade: grade 1 *vs*. 2 for *KDM4A* (median, $p$=0.0001), grade 1 *vs*. grade 3 for *KDM4A* (median, $p$=0.0407), grade 1 *vs*. 2 and 3 for *KDM4A* (median, $p$=0.0022). Statistical significance was maintained in subgroup analysis of IDCs: grade 1 *vs*. 2 for *KDM4A* (median, $p$=0.0020), grade 1 *vs*. 2 and 3 for *KDM4A* (median, $p$=0.0309).

*TNM stage*. Higher HMG transcript levels were associated with increasing TNM stage: TNM 1 *vs*. 2 for *SUV39H1* (median, $p$=0.0054 and mean, $p$=0.035) and K (lysine) acetyltransferase 2B (*KAT2B*) (median, $p$=0.0414), TNM 1 *vs*. TNM 2,3 and 4 combined for *SUV39H1* (mean, $p$=0.032 and median, $p$=0.0195), TNM 1 *vs*. 4 for lysine (K)-specific demethylase 1A (*KDM1A*) (median, $p$=0.0329), TNM 2 *vs*. 4

for *KDM1A* (median, $p$=0.0350). Similarly, in subgroup analyses of IDCs: TNM 1 *vs*. 2 for *SUV39H1* (median, $p$=0.0344) and *KDM4A* (median, $p$=0.0318), TNM 1 *vs*. 4 for lysine (K)-specific demethylase 5C (*KDM5C*) (median, $p$=0.0438). In contrast, lower transcript levels were also associated with increasing TNM stage: TNM 1 *vs*. 3 for *SUV39H1* (mean, $p$=0.027), *KDM4A* (mean, $p$=0.031) and K (lysine) acetyltransferase 8 (*KAT8*) (mean, $p$=0.049), TNM 1 *vs*. 4 for *HDAC5* (mean, $p$=0.0061), *KAT5* (mean, $p$=0.0088) and *KAT8* (mean, $p$=0.031), TNM 2 *vs*. 3 for *SUV39H1* (mean, $p$=0.011) and *KDM4A* (mean, $p$=0.032). Similarly, in subgroup analyses of IDCs: TNM 1 *vs*. 3 for *SUV39H1* (mean, $p$=0.051), TNM 1 *vs*. 4 for *KAT5* (mean, $p$=0.049).

*Nottingham prognostic index (NPI)*. Higher HMG transcript levels were associated with increasing NPI: NPI 1 *vs*. 3 for *KDM5C* (median, $p$=0.0187), NPI 2 *vs*. 3 for *KDM5C* (median, $p$=0.0115) and myeloid/lymphoid or mixed-lineage leukemia (*MLL*) (median, $p$=0.0196). In contrast, lower transcript levels were also associated with increasing NPI: NPI 1 *vs*. 2 for *MLL* (median, $p$=0.0378 and mean, $p$=0.041), NPI 1 *vs*. 3 for *KAT8* (mean, $p$=0.024) and SET and MYND domain containing 3 (*SMYD3*) (mean, $p$=0.0087). Significance was maintained in subgroup analysis of IDCs: NPI 1 *vs*. 3 for *KAT8* (mean, $p$=0.037) and *SMYD3* (mean, $p$=0.038).

*Receptor status*. Higher HMG transcript levels were associated with ER negativity for *KAT5* (mean, $p$=0.030). This relationship remained significant in subgroup analysis of IDCs for *KAT5* (mean, $p$=0.047) and approached significance for *KDM1A* (mean, $p$=0.062). Similarly, higher HMG transcript levels were associated with HER2 negativity for *SMYD3* (mean, $p$=0.027) and within subgroup analysis for *KDM1A* (mean, $p$=0.047).

*Histological type*. Higher HMG transcript levels were associated with ductal type for *KAT5* (mean, $p$=0.044), *KDM5C* (mean, $p$=0.028) and KAT8 (median, $p$=0.0541). Conversely, lower transcript levels of some HMGs were associated with ductal type, including *KDM4A* (median, $p$=0.0448) and *MLL* (median, $p$=0.0504).

*Disease-free survival (DFS)*. Higher HMG transcript levels were associated with patients who remained disease free compared to those diagnosed with local recurrence for: *HDAC1* (mean, $p$=0.011), *KDM6A* (mean, $p$=0.028), *KDM1A* (mean, $p$=0.014). Similarly, in subgroup analysis of IDCs, significance was noted for: *HDAC1* (mean, $p$=0.02), *KDM6A* (mean, $p$=0.047), *KDM1A* (mean, $p$=0.014).

Higher HMG transcript levels were associated with patients who remained disease free compared to those diagnosed with distant recurrence for: *SUV39H1* (mean, $p$=0.014), *SMYD3* (mean, $p$=0.0024), *HDAC5* (mean, $p$=0.014) and *KDM6A* (mean, $p$=0.026). Similarly, in subgroup analysis of IDCs, significance was noted for: *SUV39H1* (mean, $p$=0.030), *SMYD3* (mean, $p$=0.021), *HDAC5* (mean, $p$=0.047) and *KDM6A* (mean, $p$=0.044).

Higher HMG transcript levels were associated with patients who remained disease free compared to those with bone metastasis for: *KAT5* (mean, $p$=0.035), *KDM6A* (mean, $p$=0.027), *KDM1A* (mean, $p$=0.014), *KAT8* (median, $p$=0.0450). Similarly, in subgroup analysis of IDCs, significance was noted for: *HDAC1* (mean, $p$=0.02), *KDM6A* (mean, $p$=0.047), MLL (mean, $p$=0.0052) and *KDM1A* (mean, $p$=0.014). Furthermore, within subgroup analysis of IDCs, in comparison to patients with bony recurrence or other metastasis, significantly higher HMG transcript levels were associated with patients who remained disease free (DF): *HDAC1* (mean, $p$=0.02), *KDM6A* (mean, $p$=0.047), *KAT8* (median, $p$=0.0467) and *KDM1A* (mean, $p$=0.014).

Higher HMG transcript levels were associated with patients who remained disease free compared to those who died of BC for: *KDM4A* (mean, $p$=0.018), *KDM1A* (mean, $p$=0.014) and KAT8 (median, $p$=0.0263). Similarly, in subgroup analysis of IDCs, significance was noted for: *HDAC1* (mean, $p$=0.021), *KDM4A* (mean, $p$=0.028), *KDM1A* (mean, $p$=0.014), *KAT8* (median, $p$=0.0203). Conversely, lower transcript levels of *KDM5C* were associated with patients remaining DF compared to those who died of BC (median, $p$=0.0038).

Higher HMG transcript levels were associated with patients who remained DF compared to those diagnosed with metastasis or who died of BC for *KDM1A* (mean, $p$=0.014) and *KAT8* (median, $p$=0.0268). Higher transcript levels were also associated with patients who remained DF compared to those who had recurrence (local or distant) or died of BC for: *KDM1A* (mean, $p$=0.014), *KAT8* (median, $p$=0.0138), both of which retained significance in subgroup analysis of IDCs: *KDM1A* (mean, $p$=0.014) and *KAT8* (median, $p$=0.0048). In addition, within subgroup analysis of IDCs, lower transcript levels of *SUV39H1* were noted in patients with distant recurrence compared to those who died of BC (mean, $p$=0.059).

Kaplan-Meier analysis for *KAT8* (Figure 1A) illustrates that overall, 20 events (n=92) occurred during follow-up, 18 of which occurred in patients classified as 'negative' (n=61) who had a mean DFS of 121.6 months (95% confidence interval, CI=107.8-135.3), compared to 2 events in patients classified as 'positive' (n=31), who had a mean DFS of 135.3 months (95% CI=123.9-146.8), $p$=0.037. Equivalent analysis for *KDM1A* was comparable (Figure 2A), with 15 events in total (n=63), all of which occurred in patients classified as 'negative' (n=59); however, this did not reach statistical significance ($p$=0.253).

In contrast, Kaplan-Meier analysis for *SUV39H1* (Figure 3A) illustrates that patients classified as 'negative' had a

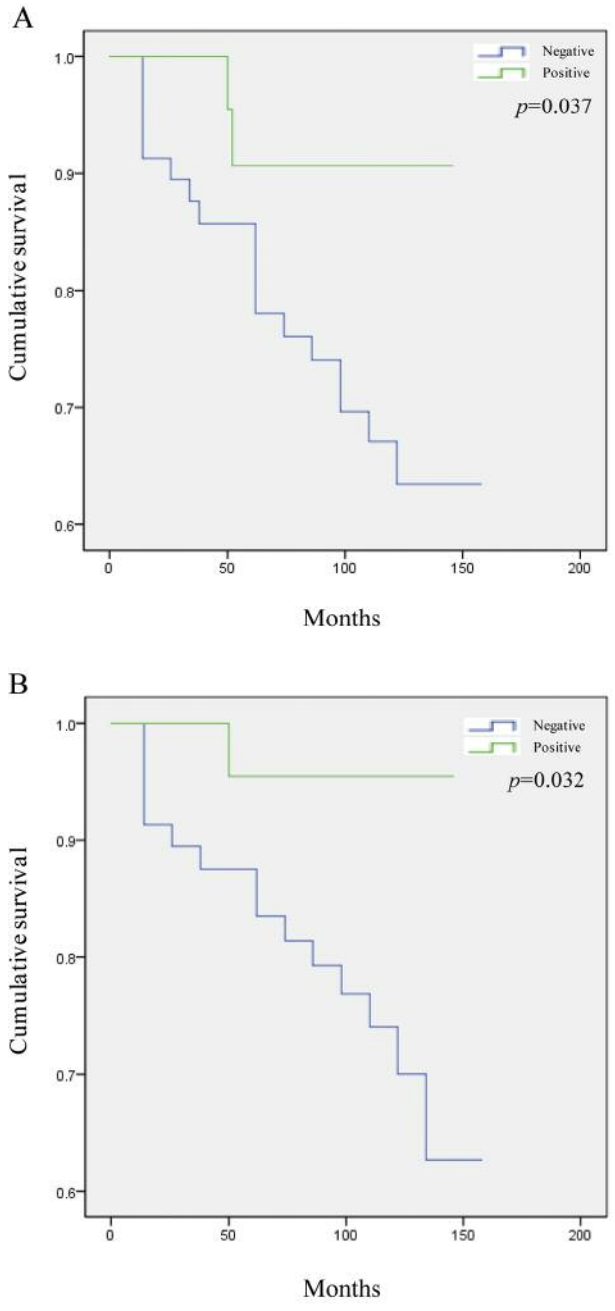


Figure 1. *KAT8 expression and Kaplan-Meier analysis for disease free survival (DFS) (A) and overall survival (OS) (B).*

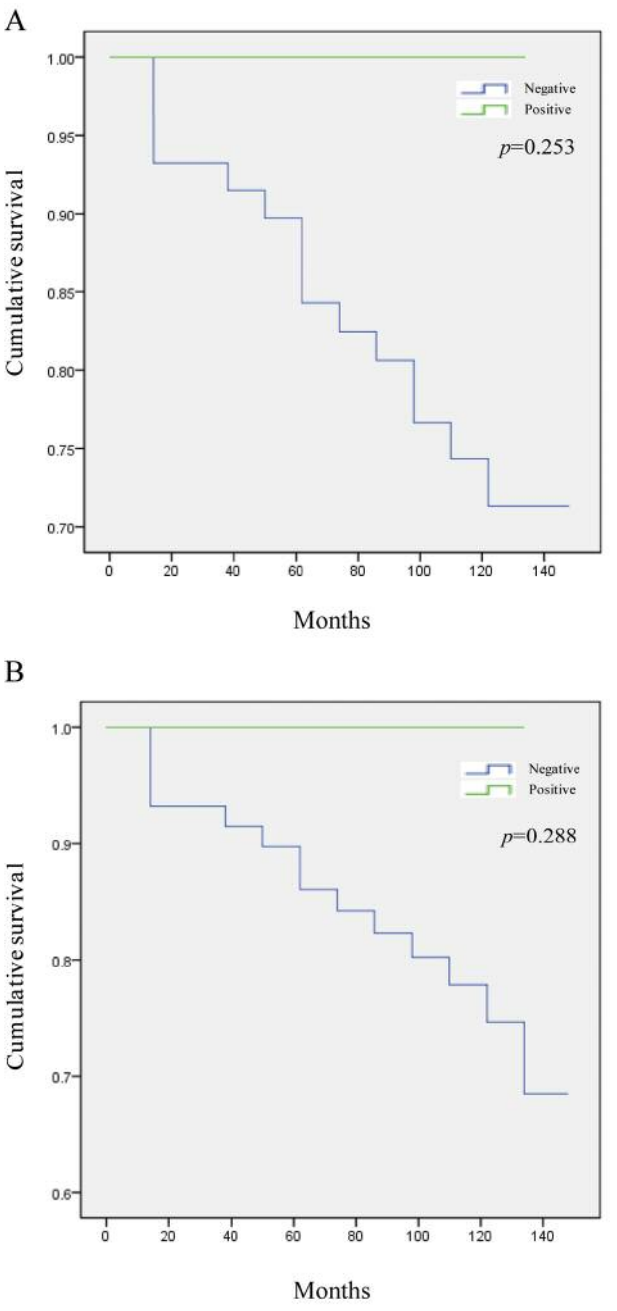


Figure 2. *KDM1A expression and Kaplan-Meier analysis for disease free survival (DFS) (A) and overall survival (OS) B).*

higher mean DFS of 133.9 months (95% CI=121.9-145.9) compared to their 'positive' counterparts, who had a mean DFS of 108.2 (95% CI=91.1-125.2), however, this did not reach statistical significance ($p$=0.211). The equivalent analysis for *KDM6A* (Figure 4A) failed to demonstrate any appreciable or statistically significant difference ($p$=0.529) between patients classified as 'positive' or 'negative'.

*Overall survival (OS).* Kaplan-Meier analysis for *KAT8* (Figure 1B) illustrates that of the 16 deaths (n=92), 15 occurred in patients classified as 'negative' (n=61), who had a mean OS of 126.5 months (95% CI=113.3-139.8) compared to only 1 death amongst patients classified as 'positive' (n=31), who had a mean OS of 139.8 months (95% CI=131.8-147.8), $p$=0.032. Equivalent analysis for *KDM1A* was comparable

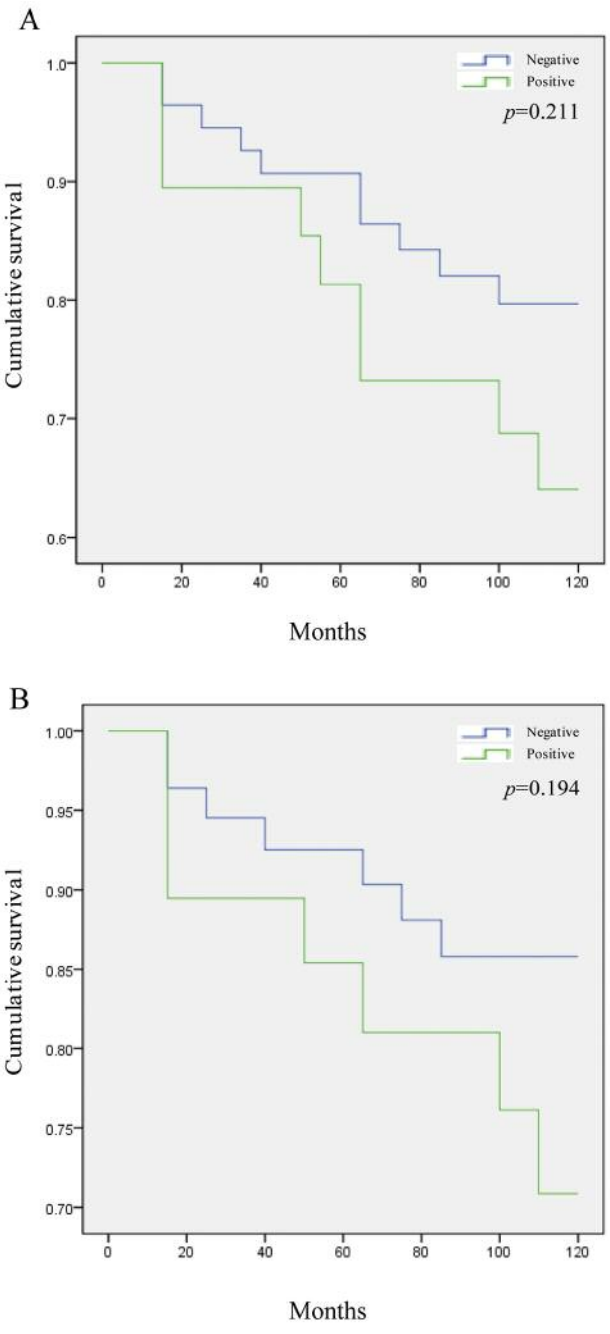


Figure 3. *SUV39H1 expression and Kaplan-Meier analysis for disease free survival (DFS) (A) and overall survival (OS) (B).*

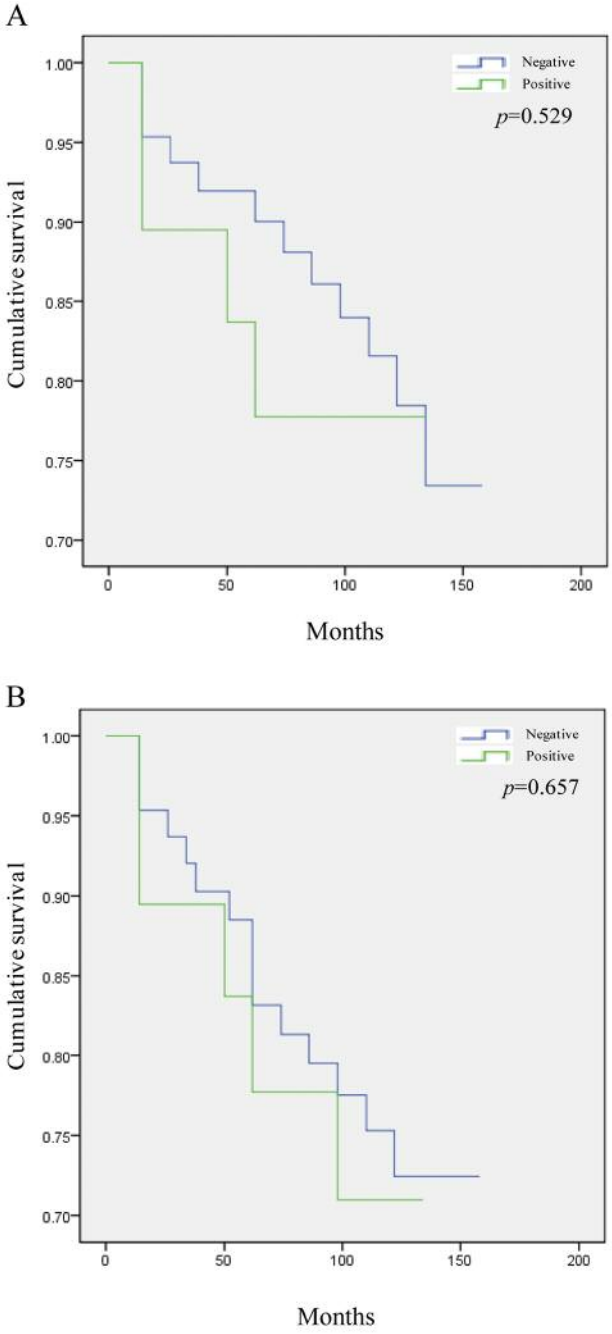


Figure 4. *KDM6A expression and Kaplan-Meier analysis for disease free survival (DFS) (A) and overall survival (OS) B).*

(Figure 2B), with 14 deaths in total (n=63), all of which occurred in patients classified as 'negative' (n=59); however, this did not reach statistical significance ($p$=0.288).

In contrast, Kaplan-Meier analysis for *SUV39H1* (Figure 3B) illustrates that patients classified as 'negative' had a higher mean OS of 139.3 months (95% CI=128.3-150.3) compared to their 'positive' counterparts, who had a mean OS of 113.8 months (95% CI=97.8-129.8); however, this did not reach statistical significance. The equivalent analysis for *KDM6A* (Figure 4B) failed to demonstrate any appreciable or statistically significant difference ($p$=0.657) between patients classified as 'positive' or 'negative'.

Table III. *Summary of correlation coefficients between expressions of histone-modifier genes ($p<0.05$).*

| Gene | *HDA5* | *HDAC1* | *HDAC2* | *KAT2B* | *KAT5* | *KDM1A* | *KDM4A* | *KDM5C* | *KDM6A* | *MLL* | *KAT8* | *SIRT1* | *SMYD3* | *SUV39H1* | *SUV39H2* | *SETD2* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *CREBBP* | 0.44 | | 0.29 | | | 0.43 | 0.23 | | 0.28 | | | 0.30 | 0.30 | 0.19 | | |
| *HDA5* | | 0.35 | | 0.28 | 0.31 | 0.29 | 0.30 | 0.30 | 0.50 | | | 0.19 | 0.26 | 0.38 | | |
| *HDAC1* | | | | 0.48 | 0.25 | 0.35 | | 0.31 | 0.38 | 0.22 | 0.23 | 0.35 | 0.24 | 0.41 | | 0.19 |
| *HDAC2* | | | | | | 0.23 | | | 0.19 | | | | | | 0.24 | |
| *KAT2B* | | | | | 0.30 | 0.19 | | 0.34 | 0.23 | | 0.28 | | | | | |
| *KAT5* | | | | | | | | 0.23 | | –0.24 | | | | | | |
| *KDM1A* | | | | | | | | 0.22 | 0.34 | | | 0.68 | 0.30 | 0.34 | | |
| *KDM4A* | | | | | | | | | 0.25 | 0.31 | | | | 0.18 | 0.41 | |
| *KDM5C* | | | | | | | | | 0.28 | | 0.19 | | 0.23 | 0.27 | | |
| *KDM6A* | | | | | | | | | | | | 0.23 | 0.23 | 0.28 | | |
| *MLL* | | | | | | | | | | | | | 0.38 | 0.31 | 0.43 | |
| *KAT8* | | | | | | | | | | | | | | | | |
| *SIRT1* | | | | | | | | | | | | | 0.29 | 0.19 | | |
| *SMYD3* | | | | | | | | | | | | | | 0.39 | | |
| *SUV39H1* | | | | | | | | | | | | | | | 0.35 | |
| *SUV39H2* | | | | | | | | | | | | | | | | |

*Correlation of histone-modifier gene expression*. Given the extent to which HMG activity is coordinated and co-localised by the formation of distinct complexes, correlations were sought and identified between the differential expression profiles of particular HMGs. The correlation coefficients which reached statistical significance ($p<0.05$) are detailed in Table III.

## Discussion

The comprehensive epigenomic portrayal of BC requires integration of our understanding of the dynamic relationship between the tumor methylome and the determinants of chromatin structure. Indeed, considerable overlap and concordance has been reported in BC, consistent with a degree of interdependency between DNA methyltransferases and histone-modifying enzymes (1, 11, 14, 22). Immunohistochemical evaluation has identified high levels of global histone acetylation (hypermodified cluster) to be associated with favourable prognosis and luminal-like BC, whereas lower levels (hypomodified cluster) correlate with poor prognostic groups, such as basal carcinomas and HER2 positivity (14). Similarly, global hypoacetylation has also been associated with progression from normal to *in situ* and invasive BC (23). However, comprehensively deciphering the 'histone code' will necessitate understanding the network consequences of each specific mark and the complexity of combinatorial variations for the maintenance of hetero- and euchromatin and the governance of gene expression. These alterations are likely to be substantially influenced by the relative expression levels of HMGs within particular tumors, some of which may act in concert with one another, whilst others antagonise their counterparts (24).

In the present study, the expression profiles of 16 HMGs were evaluated in a well characterized cohort of BC patients, extending our original report of potential tumor suppressor function associated with the HMT *SETD2* (19). The genes evaluated were selected to be representative of the major classes of HMGs. In keeping with previous reports, our results demonstrate that HMG expression in BC differs significantly from that in normal tissue, specifically with regard to *HDAC5, HDAC1, KDM4A* and *KDM6A*. In their study of chromatin-modifier enzymes in 26 primary cases of BC (of which *HDAC1, HDAC2, HDAC5, SIRT1, SUV39H1, SUV39H2* and *CREBBP* were evaluated in the present study), Ozdag *et al*. (3) found BC to be distinguished from normal breast tissues by the expression of *EZH2, CREBBP* and *HDAC4*. In the present study, significant differences in HMG expression profiles were also found to exist between BC cases. The resulting epigenetic differences appear to have biological relevance, reflected by the associations with pathological parameters identified for a number of HMGs and prognostic indices associated with *KAT8, KDM1A* and *SUV39H1*.

In the present study, lower *KAT8* transcript levels were associated with increasing TNM stage and NPI, whereas higher levels were found in patients who remained disease free compared to those who developed recurrence, metastasis or died of BC. *KAT8* positivity was associated with a significantly improved DFS and OS (Figure 1). KAT8 specifically acetylates histone H4 lysine 16 (H4K16) (25), which is frequently reduced in human cancer (26). In keeping with our findings, Pfister *et al*. (27) reported down-regulation of *KAT8* in BC, in 41% of cases at the mRNA level and 18% at the protein level, the latter correlating closely with *H4K16* acetylation. Furthermore, CpG island-

associated alterations in histone H4 modifications, in particular loss of histone H4K16 acetylation, contribute to the aberrant methylation and epigenetic silencing of tumor suppressor genes in BC, such as target of methylation-induced silencing (*TMS1*), whose activity is maintained by KAT8 (28-30). KAT8-dependent acetylation of H4K16 has recently been implicated in the control of DNA transcription by RNA polymerase II (28). KAT8 also interacts with male-specific lethal 1 homolog (MSL1v1) which is specifically required for optimal transcriptional activation of p53 target genes *in vitro* and *in vivo* (31).

The expression of *KDM1A* has been reported to be significantly lower in BC compared to adjacent normal tissue (5). In our cohort, *KDM1A* expression was found to be significantly higher in patients who remained disease free compared to those diagnosed with recurrence, or metastasis, or who died of BC, and exhibited a trend towards being associated with improved DFS and OS which did not reach statistical significance. Despite these associations, our data are also consistent with the observation that *KDM1A* expression is considerably higher in ER- and progesterone receptor (PR)-negative tumors and clinically advanced lesions (32). KDM1A regulates gene expression by enabling transcription factors or co-repressor complexes to initiate or repress transcription via de-methylation of lysine 4 and 9 of histone H3 (H3K4/9) (32). KDM1A is an integral component of the Mi-2/nucleosome remodelling and deacetylase complex, regulating transforming growth factor beta 1 (TGFβ1) signalling pathways involved in proliferation, survival, and epithelial-to-mesenchymal transition. KDM1A has been found to be down-regulated in BC and negatively correlated with TGFβ1 (24). The latter study also found KDM1A to inhibit BC cell invasion *in vitro* and suppress metastasis *in vivo*. However, induction and alteration of the sub-cellular localisation of KDM1A appears to represent an early response to carcinogen exposure, influencing the expression of multiple genes in mammary carcinogenesis (33). KDM1A has been shown to repress p53-mediated transcription and inhibit p53-promoted apoptosis, implying that enzymatic activity may not be restricted to histone proteins (34). KDM1A has also been implicated in 17β-oestradiol (E-2)-induced interchromosomal interactions between specific gene loci within a network, whereby interacting loci demonstrate KDM1A-dependent interactions with interchromatin granules containing transcriptional elongation and pre-mRNA splicing factors (35). Pharmacological inhibition or small-interfering RNA-mediated knockdown of *KDM1A* has been associated with growth inhibition and down-regulation of proliferation-associated genes (32).

*SUV39H1* expression was significantly associated with increasing tumor grade, increasing TNM stage and showed a trend towards being associated with poorer DFS and OS, although these did not reach statistical significance. While lower transcript levels were noted in patients with distant recurrence compared to those who died of BC, higher transcript levels were also associated with patients who remained DF compared to those diagnosed with distant recurrence. SUV39H1 is specifically involved in the methylation of histone H3 lysine 9 (H3K9) and heterochromatin formation (36-38). Deleted in breast cancer 1 (DBC1) has been implicated in regulating chromatin modifications by disrupting SUV39H1–SIRT1 complex formation and inhibiting SUV39H1-dependent methylation of histone H3 (39). SUV39H1 also represents a component of two multimolecular complexes (pRb2/p130–E2F4/5–HDAC1–SUV39H1–p300 and pRb2/p130–E2F4/5–HDAC1–DNMT1–SUV39H1) which have been demonstrated to specifically bind the ER-α promoter in BC cells, potentially modulating its transcriptional regulation (40). Indeed, the epigenetic regulation of nuclear steroid receptors in general, and ER in particular, may be substantially mediated by interactions with HMG products (16, 41-44).

Limitations of the present study include the use of background parenchyma from BC specimens to provide 'normal tissue' for comparison. Ideally, such reference material should be derived from patient matched tissue remote from the cancer-bearing site in order to avoid any 'field change' which may exist. Although the sample size and follow-up period were substantial, it is possible that a larger cohort, particularly with regard to subgroup analysis, may have influenced several results which approached, but failed to reach, statistical significance. In addition to the detection of mRNA transcripts, semiquantitative immunohistochemistry and quantitative analysis of protein expression should be undertaken to ensure concordance. The genes evaluated were selected to be representative of the major classes of HMGs, rather than representing an exhaustive series or unbiased sample. Correlation with associated molecules and other markers of invasiveness and metastatic competence would also be of value.

Greater appreciation of chromatin-modifying/remodelling complexes may be of relevance to our understanding the mechanism of transcriptional repression associated with well established endocrine therapies, such as the tissue-selective oestrogen receptor modulator, tamoxifen (45). The inherent reversibility of epigenetic events, in contrast to their genetic counterparts, raises the intriguing possibility of novel targetable molecular alterations in human cancer (1, 46). Epigentic therapies directed at DNA methylation have been described in the context of myelodysplastic syndrome (47, 48). In addition to their potential prognostic utility, either as individual biomarkers or in combination as signatures, improved characterization of HMG expression profiles may yield rational interventions including histone modification-altering drugs (49). Indeed, HDAC inhibitors have been

demonstrated to be of utility in primary cutaneous T-cell lymphoma (50) and may sensitise solid tumors to topoisomerase inhibitors (51, 52).

## Conclusion

HMG expression levels in BC differ significantly from those in normal tissue. Differences in expression profiles exist between BC cases and are significantly associated with conventional pathological parameters and clinical outcomes. Further study is warranted to determine the consequences of altered expression for each specific HMG and the biological and clinical implications of combinatorial variations in expression profiles. Histone-modifier enzymes offer utility as biomarkers and potential for targeted therapeutic strategies.

## Conflicts of Interest

The Authors declare that they have no competing interests.

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## References

1 Lund AH and van Lohuizen M: Epigenetics and cancer. Genes Dev *18(19)*: 2315-2335, 2004.
2 Santos-Rosa H and Caldas C: Chromatin modifier enzymes, the histone code and cancer. Eur J Cancer *41(16)*: 2381-2402, 2005.
3 Ozdag H, Teschendorff AE, Ahmed AA, Hyland SJ, Blenkiron C, Bobrow L, Veerakumarasivam A, Burtt G, Subkhankulova T, Arends MJ, Collins VP, Bowtell D, Kouzarides T, Brenton JD and Caldas C: Differential expression of selected histone modifier genes in human solid cancers. BMC Genomics *7*: 90, 2006.
4 Kouzarides T: Chromatin modifications and their function. Cell *128(4)*: 693-705, 2007.
5 Wang GG, Allis CD and Chi P: Chromatin remodeling and cancer, Part I: Covalent histone modifications. Trends Mol Med *13(9)*: 363-372, 2007.
6 Khorasanizadeh S: The nucleosome: from genomic organization to genomic regulation. Cell *116(2)*: 259-272, 2004.
7 Jenuwein T and Allis CD: Translating the histone code. Science *293(5532)*: 1074-1080, 2001.
8 Jovanovic J, Ronneberg JA, Tost J and Kristensen V: The epigenetics of breast cancer. Mol Oncol *4(3)*: 242-254, 2010.
9 Lachner M, O'Sullivan RJ, and Jenuwein T: An epigenetic road map for histone lysine methylation. J Cell Sci *116(Pt 11)*: 2117-2124, 2003.
10 Kurdistani SK: Histone modifications as markers of cancer prognosis: a cellular view. Br J Cancer *97(1)*: 1-5, 2007.
11 Ronneberg JA, Fleischer T, Solvang HK, Nordgard SH, Edvardsen H, Potapenko I, Nebdal D, Daviaud C, Gut I, Bukholm I, Naume B, Borresen-Dale AL, Tost J and Kristensen V: Methylation profiling with a panel of cancer related genes: association with estrogen receptor, TP53 mutation status and expression subtypes in sporadic breast cancer. Mol Oncol *5(1)*: 61-76, 2011.
12 Soares J, Pinto AE, Cunha CV, Andre S, Barao I, Sousa JM and Cravo M: Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer *85(1)*: 112-118, 1999.
13 Li S, Rong M and Iacopetta B: DNA hypermethylation in breast cancer and its association with clinicopathological features. Cancer Lett *237(2)*: 272-280, 2006.
14 Elsheikh SE, Green AR, Rakha EA, Powe DG, Ahmed RA, Collins HM, Soria D, Garibaldi JM, Paish CE, Ammar AA, Grainge MJ, Ball GR, Abdelghany MK, Martinez-Pomares L, Heery DM and Ellis IO: Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. Cancer Res *69(9)*: 3802-3809, 2009.
15 Gayther SA, Batley SJ, Linger L, Bannister A, Thorpe K, Chin SF, Daigo Y, Russell P, Wilson A, Sowter HM, Delhanty JD, Ponder BA, Kouzarides T and Caldas C: Mutations truncating the EP300 acetylase in human cancers. Nat Genet *24(3)*: 300-303, 2000.
16 Kawai H, Li H, Avraham S, Jiang S and Avraham HK: Overexpression of histone deacetylase HDAC1 modulates breast cancer progression by negative regulation of estrogen receptor alpha. Int J Cancer *107(3)*: 353-358, 2003.
17 Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA and Chinnaiyan AM: EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA *100(20)*: 11606-11, 2003.
18 Berger SL: Histone modifications in transcriptional regulation. Curr Opin Genet Dev *12(2)*: 142-148, 2002.
19 Al Sarakbi W, Sasi W, Jiang WG, Roberts T, Newbold RF and Mokbel K: The mRNA expression of SETD2 in human breast cancer: correlation with clinicopathological parameters. BMC Cancer *9*: 290, 2009.
20 Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A and Ponte JF: Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci *983*: 84-100, 2003.
21 Jiang WG, Watkins G, Lane J, Cunnick GH, Douglas-Jones A, Mokbel K and Mansel RE: Prognostic value of rho GTPases and rho guanine nucleotide dissociation inhibitors in human breast cancers. Clin Cancer Res *9(17)*: 6432-6440, 2003.
22 Cameron EE, Bachman KE, Myohanen S, Herman JG and Baylin SB: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet *21(1)*: 103-107, 1999.
23 Suzuki J, Chen YY, Scott GK, Devries S, Chin K, Benz CC, Waldman FM and Hwang ES: Protein acetylation and histone deacetylase expression associated with malignant breast cancer progression. Clin Cancer Res *15(9)*: 3163-3171, 2009.
24 Wang Z, Zang C, Cui K, Schones DE, Barski A, Peng W and Zhao K: Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell *138(5)*: 1019-1031, 2009.
25 Taipale M, Rea S, Richter K, Vilar A, Lichter P, Imhof A and Akhtar A: hMOF histone acetyltransferase is required for histone H4 lysine 16 acetylation in mammalian cells. Mol Cell Biol *25(15)*: 6798-6810, 2005.

26 Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Perez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T and Esteller M: Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet *37(4)*: 391-400, 2005.

27 Pfister S, Rea S, Taipale M, Mendrzyk F, Straub B, Ittrich C, Thuerigen O, Sinn HP, Akhtar A and Lichter P: The histone acetyltransferase hMOF is frequently down-regulated in primary breast carcinoma and medulloblastoma and constitutes a biomarker for clinical outcome in medulloblastoma. Int J Cancer *122(6)*: 1207-1213, 2008.

28 Kapoor-Vazirani P, Kagey JD, Powell DR and Vertino PM: Role of hMOF-dependent histone H4 lysine 16 acetylation in the maintenance of TMS1/ASC gene activity. Cancer Res *68(16)*: 6810-6821, 2008.

29 Conway KE, McConnell BB, Bowring CE, Donald CD, Warren ST and Vertino PM: TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res *60(22)*: 6236-6242, 2000.

30 Parsons MJ, Patel P, Brat DJ, Colbert L and Vertino PM: Silencing of TMS1/ASC promotes resistance to anoikis in breast epithelial cells. Cancer Res *69(5)*: 1706-1711, 2009.

31 Li X, Wu L, Corsa CA, Kunkel S and Dou Y: Two mammalian MOF complexes regulate transcription activation by distinct mechanisms. Mol Cell *36(2)*: 290-301, 2009.

32 Lim S, Janzer A, Becker A, Zimmer A, Schule R, Buettner R and Kirfel J: Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. Carcinogenesis *31(3)*: 512-520, 2010.

33 Bradley C, van der Meer R, Roodi N, Yan H, Chandrasekharan MB, Sun ZW, Mernaugh RL, and Parl FF: Carcinogen-induced histone alteration in normal human mammary epithelial cells. Carcinogenesis *28(10)*: 2184-2192, 2007.

34 Huang J, Sengupta R, Espejo AB, Lee MG, Dorsey JA, Richter M, Opravil S, Shiekhattar R, Bedford MT, Jenuwein T and Berger SL: p53 is regulated by the lysine demethylase LSD1. Nature *449(7158)*: 105-108, 2007.

35 Hu Q, Kwon YS, Nunez E, Cardamone MD, Hutt KR, Ohgi KA, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MG and Fu XD: Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. Proc Natl Acad Sci USA *105(49)*: 19199-19204, 2008.

36 Aagaard L, Laible G, Selenko P, Schmid M, Dorn R, Schotta G, Kuhfittig S, Wolf A, Lebersorger A, Singh PB, Reuter G and Jenuwein T: Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. EMBO J *18(7)*: 1923-1938, 1999.

37 Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD and Jenuwein T: Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406(6796)*: 593-599, 2000.

38 Vaquero A, Scher M, Erdjument-Bromage H, Tempst P, Serrano L and Reinberg D: SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. Nature *450(7168)*: 440-444, 2007.

39 Li Z, Chen L, Kabra N, Wang C, Fang J and Chen J: Inhibition of SUV39H1 methyltransferase activity by DBC1. J Biol Chem *284(16)*: 10361-10366, 2009.

40 Macaluso M, Cinti C, Russo G, Russo A and Giordano A: pRb2/p130–E2F4/5–HDAC1–SUV39H1–p300 and pRb2/p130–E2F4/5–HDAC1–SUV39H1–DNMT1 multimolecular complexes mediate the transcription of estrogen receptor-alpha in breast cancer. Oncogene *22(23)*: 3511-3517, 2003.

41 Leader JE, Wang C, Popov VM, Fu M and Pestell RG: Epigenetics and the estrogen receptor. Ann NY Acad Sci *1089*: 73-87, 2006.

42 Yang X, Ferguson AT, Nass SJ, Phillips DL, Butash KA, Wang SM, Herman JG and Davidson NE: Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. Cancer Res *60(24)*: 6890-6894, 2000.

43 Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG and Davidson NE: Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res *61(19)*: 7025-7029, 2001.

44 Sun JM, Chen HY and Davie JR: Effect of estradiol on histone acetylation dynamics in human breast cancer cells. J Biol Chem *276(52)*: 49435-49442, 2001.

45 Liu XF and Bagchi MK: Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters *in vivo*. J Biol Chem *279(15)*: 15050-8, 2004.

46 Huang Y, Vasilatos SN, Boric L, Shaw PG and Davidson NE: Inhibitors of histone demethylation and histone deacetylation cooperate in regulating gene expression and inhibiting growth in human breast cancer cells. Breast Cancer Res Treat, 2011.

47 Issa JP: DNA methylation as a therapeutic target in cancer. Clin Cancer Res *13(6)*: 1634-1637, 2007.

48 Claus R and Lubbert M: Epigenetic targets in hematopoietic malignancies. Oncogene *22(42)*: 6489-6496, 2003.

49 Egger G, Liang G, Aparicio A and Jones PA: Epigenetics in human disease and prospects for epigenetic therapy. Nature *429(6990)*: 457-463, 2004.

50 Mann BS, Johnson JR, He K, Sridhara R, Abraham S, Booth BP, Verbois L, Morse DE, Jee JM, Pope S, Harapanhalli RS, Dagher R, Farrell A, Justice R and Pazdur R: Vorinostat for treatment of cutaneous manifestations of advanced primary cutaneous T-cell lymphoma. Clin Cancer Res *13(8)*: 2318-2322, 2007.

51 Marchion DC, Bicaku E, Turner JG, Schmitt ML, Morelli DR and Munster PN: HDAC2 regulates chromatin plasticity and enhances DNA vulnerability. Mol Cancer Ther *8(4)*: 794-801, 2009.

52 Munster P, Marchion D, Bicaku E, Lacevic M, Kim J, Centeno B, Daud A, Neuger A, Minton S and Sullivan D: Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. Clin Cancer Res *15(7)*: 2488-2496, 2009.

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