RASSF1A Methylation is Predictive of Poor Prognosis in Female Breast Cancer in a Background of Overall Low Methylation Frequency

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Abstract. *Breast cancer (BC) is the most common cancer worldwide. The Kingdom of Saudi Arabia is no exception, with ever increasing incidence rates. An interesting feature of this disease is the relatively young age of the affected women. The average age in the present cohort of 100 sporadic cases of invasive ductal carcinomas was 45 years, with a median of 46 years (range between 19-81 years). In an effort to understand the molecular signature of BC in the Saudi population, we undertook this study to profile the methylation events in a series of key genes including Ras association (RalGDS/AF-6) domain family member 1 isoform a (RASSF1A), hypermethylated in cancer 1 (HIC1), cyclin-dependent kinase inhibitor 2A (CDKN2A), retinoic acid receptor beta (RARB2), estrogen receptor 1 (ESR1), progesterone receptor (PGR), paired-like homeodomain 2 (PITX2), secreted frizzled-related protein 1 (SFRP1), myogenic differentiation 1 (MYOD1), and slit homolog 2 (SLIT2), using MethyLight analysis in archival tumour samples. Interestingly, the overall methylation levels were low in this cohort, with only 84% of the cases displaying methylation in one or more of the analysed genes. The frequency of RASSF1A methylation was the highest (65%), while there was almost complete absence of methylation of the ESR1 and the CDH1 genes (1% and 3%, respectively). Several statistically significant correlations were identified between specific methylation events and clinical parameters which gained more significance when analysis was limited to the estrogen receptor positive samples. Although there was no significant correlations between any methylation event and disease-specific survival, methylation of MYOD1 or RASSF1A was associated with lower disease-free survival and increased chance of disease recurrence. Furthermore, multivariate (Cox) regression analysis identified RASSF1A as an independent predictor of poor prognosis in terms of disease-free survival in this cohort. Our findings provide further evidence on the usefulness of RASSF1A methylation status as an informative prognostic biomarker in BC in a Saudi population.*

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Key Words: Breast cancer, methylation, *RASSF1A*, prognosis.

Breast cancer (BC) is the principal cause of cancer-related death among women world wide (1). The BC rates amongst females in the Middle Eastern and North African countries (MENA) range between 13-35% of all cancer cases, half of the patients being below 50 years of age, with a median of 49-52 years, as compared to 63 years in Western countries (2). Ethnic group-related differences in DNA methylation patterns are starting to emerge. Methylation of glutathione S-transferase pi 1 (*GSTP1*) reflected the higher incidence and mortality rates of prostate cancer in African-Americans compared to Caucasian or Asian populations (3). Similarly, DNA methylation levels are more prevalent in young Korean BC patients as compared to their Caucasian counterparts (4).

Detection of epigenetic changes in BC can be an additional tool in diagnosing the disease or in providing information regarding prognosis, recurrence or efficacy of therapeutic regimens. An ideal biomarker is one that can be detected from patient's samples obtained by non-invasive techniques. For BC, samples obtained by ductal lavage show particular promise as a template for detection of DNA hypermethylation. The ability to detect DNA methylation in patient's sera is a useful tool to monitor efficacy of adjuvant therapy, as shown with methylation of *RASSF1A* and tamoxifen treatment after surgery (5). Methylation of *PITX2* has been found to predict risk of distant metastasis and recurrence in tamoxifen-treated

and node-negative BC patients (6). It was suggested, therefore, that based on the extent of *PITX2* methylation, about half of hormone receptor-positive, node-negative BC patients receiving adjuvant tamoxifen therapy can be considered at low-risk regarding the development of distant recurrences and can thus be spared adjuvant chemotherapy (6).

The genes targeted for study here are mostly based on a study by Winschwendter *et al*. (7), who demonstrated that the methylation status of these genes can reflect an environmental element, and, in principle, can be used to study the risk of BC. Those genes used in this study fall into three groups: estrogen receptor (ER)-α target genes (including *ESR1, PGR1* and *PITX2*); polycomb target genes (including *HIC1, SLIT2, SFRP1* and *MYOD1*), which play a role in stem cell biology and are likely to be methylated in tumour-specific manner; genes shown to be commonly methylated in BC (including *RASSF1A, CDKN2A, CDH1* and *RARB2*).

Patients and Methods

The study was performed on Saudi female BC patients, diagnosed with invasive ductal carcinoma, at the Department of Pathology, King Abdul-Aziz University, Jeddah, Saudi Arabia during 2000-2008. Patients were excluded from this study on the basis of the following exclusion criteria: histopathological diagnosis was not invasive ductal carcinoma; patient history, and medical files, or specimens were not found. This left samples from 100 tumors available for DNA methylation analysis.

The pertinent clinicopathological features (age, menopausal status, stage, grade, and lymph node status), and the follow up and survival data were retrieved from the patients' records after obtaining all the relevant ethical approvals and are summarized in Table I. The average age in the present cohort was 45 years, with a median of 46 years (range 19-81 years).

Treatment and follow-up. The patients were seen at 3-6-month intervals until death or the end of follow-up which was mid-February, 2010. Some patients were lost from follow-up. The mean follow-up time for the whole series was 47 months (range: 4-118 months). During the follow-up period, 23 (23%) patients developed recurrence and 15 (15%) patients developed metastasis at different organs: liver, bone, lung, and others.

During the follow-up, patients were subjected to clinical examination every 6-12 months and bone isotope scan, chest, and abdominal-pelvic CAT scan were performed whenever needed. In most instances, the causes of death were obvious on clinical grounds alone. Autopsy was not performed in any case. Almost all patients were subjected to surgery in the form of lumpectomy, or radical or modified radical mastectomy with axillary node clearance. Postoperative early adjuvant systemic therapy in the form of chemotherapy, radiotherapy and hormonal therapy was given to 65%, 50%, and 39% of patients, respectively.

Analysis of DNA methylation. DNA was extracted from 10 μm-thin formalin-fixed paraffin-embedded slices using the Qiagen QIAMP Formalin-fixed Parafin-embedded Tissue DNA extraction kit, following the manufacturer's guidelines. Up to 0.5 μg of DNA was used for bisulfite conversion using the Qiagen Epitect Bisulfite

Table I. *Clinicopathological features of the cohort (100 patients) included in this study.*

Characteristic	No. of patients
Age (years)	
<50	67
≥50	33
Menopausal status	
Pre-	59
Post-	41
Localization	
Left	44
Right	55
Unknown	1
Margins	
Free	57
Involved	23
Unknown	20
Neurovascular invasion	
No	28
Yes	37
Unknown	35
Lymph node involvement	
No	29
Yes	49
Unknown	22
Metastasis	
No	66
Yes	15
Unknown	19
Histological grade	
I	21
II	51
III	22
Unknown	6
Stage	
I	14
II	58
III	5
IV	13
Unknown	10
Recurrence	
No	66
Yes	23
Unknown	11
Response to treatment	
Objective response	63
No response	15
Unknown	21
Status at end of follow-up	
Alive	75
Died of disease	11
Unknown	14
Hormonal status	
ER+	54
ER–	29
Unknown	17
PR+	49
PR-	34
Unknown	17
HER2 amplification	
Yes	23
No	49
Unknown	28

ER: Oesterogen receptor; PR: progesteron receptor.

Table II. *Primer and probe sequences used in this study.*

Gene	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
ESR1	GGCGTTCGTTTTGGGATTG	GCCGACACGCGAACTCTAA	6FAM-CGATAAAACCGAACGA CCCGACGA-BHQ1
PGR	TTATAATTCGAGGCG GTTAGTGTTT	TCGAACTTCTACTAACTC CGTACTACGA	6FAM-ATCATCTCCGAAAATCT CAAATCCCAATAATACG-BHQ1
PITX2	AGTTCGGTTGCGCGGTT	TACTTCCCTCCCCTACCTCGTT	6FAM-CGACGCTCGCCCGAACGCTA-BHQ1
SFRP1	CAACTCCCGACGAAACGAA	CGCGAGGGAGGCGATT	6FAM-CACTCGTTACCACGTCCGTCACCG-BHQ1
MYOD1	GAGCGCGCGTAGTTAGCG	TCCGACACGCCCTTTCC	6FAM-CTCCAACACCCGACTACTATAT CCGCGAAA-BHQ1
HIC1	GTTAGGCGGTTAGGGCGTC	CCGAACGCCTCCATCGTAT	6FAM-CAACATCGTCTACCCAACACAC TCTCCTACG-BHQ1
RASSF1A	ATTGAGTTGCGGGAGTT GGT	ACACGCTCCAACCGAATA CG	6FAM-CCCTTCCCAACGCGCCCA-BHQ1
CDKN2A	TGGAATTTTCGGTTGATT GGTT	AACAACGTCCGCACCTC CT	6FAM-ACCCGACCCCGAACCGCG-BHQ1
RARB2	TTTATGCGAGTTGTTTGAGGATTG	CGAATCCTACCCCGACGATAC	6FAM-CTCGAATCGCTCGCGTTCTCGA CAT-BHQ1
CDH1	AGGGTTATCGCGTTTATGCG	TTCACCTACCGACCACAA	6FAM-ACTAACGACCCGCCCACCCFA-BHQ1
SLIT2	CAATTCTAAAAACGCACGACTCTAAA	CGGGAGATCGCGAGGAT	6FAM-CGACCTCTCCCTCGCCCTCGACT-BHQ1
ALU	GGTTAGGTATAGTGGTTTATAT TTGTAATTTTAGTA	ATTAACTAAACTAATCTTAAA CTCCTAACCTCA	VIC-CCTACCTTAACCTCCC-MGBNFQ

Conversion kit. DNA methylation analysis was performed using MethyLight as described elsewhere (8). The methylation levels of *RASSF1A, HIC1, RARB2, CDKN2A, SLIT2, SFRP1, MYOD1, ESR1, PGR, PITX2* and *CDH1* were analysed using the primer-probe combinations listed in Table II which were made according to previously published reports (7, 9). A probe targeting bisulfite-modified Alu repeat sequences was used to normalise for input DNA. The specificity of the reaction was ascertained using sssI-treated and bisulfite-modified positive control DNA (Qiagen) and the negative control DNA (Qiagen). The percentage of fully methylated reference (PMR) was calculated by dividing the gene:Alu ratio of a sample by the gene:Alu ratio of the positive control DNA and multiplying by 100. Samples with PMR>10 were considered positive for methylation, whereas samples with PMR<10 were considered negative (*i.e.* unmethylated).

Statistical analysis. All statistical tests were performed using PASW Statistics 18.03. (SPSS, Inc., Chicago, IL USA) and STATA/SE 11.1 (StataCorp, TX, USA). Fisher's exact test was used to identify statistical significance of correlation between methylation events and clinicopathological factors. Pearson's correlation was used to test the significance of correlations between methylation events. The primary endpoints of the study included DFS and DSS calculated from the date of diagnosis to the appearance of disease recurrence, and the date last seen alive or when died of disease, respectively. In calculating DSS, patients who died of other or unknown causes were excluded. All survival times were calculated by univariate Kaplan-Meier analysis, and equality of the survival functions between the strata was tested by log-rank (Mantel-Cox) test. Multivariate Cox regression analysis was performed where all methylation markers were included in the model to disclose independent predictors of DFS and DSS. All tests were two-sided, and p-values <0.05 were considered statistically significant. Clustering was performed using the Gene CLUSTER 3.0 program and visualized using JavaTree software (http:// http://www.eisenlab.org/).

Results

Methylation frequency. Overall methylation frequency was that no sample (0%) was detected as having methylation in 9 or more genes, while 16 cases (16%) did not display any detectable methylation of any the markers used (Figure 1A). The majority (64%) of cases had one to few genes methylated. The methylation frequency of each gene used in this panel is shown in Figure 1B. *RASSF1A* was the most frequently methylated gene in this cohort (65%), followed by *HIC1* (41%). Surprisingly, *ESR1* promoter was methylated in only one sample and *CDH1* methylation frequency did not exceed 3%.

There was a strong correlation between the methylation events of the polycomb gene targets (PCGT). *SFRP1* methylation correlated with that of *MYOD1* and *SLIT2* (Pearson's two-tailed correlation, R=0.357, p=0.001 and R=0.302, p=0.002, respectively), while *SLIT2* methylation events were closely associated with HIC1 methylation events (R=0.238, p=0.018). *RASSF1A, CDKN2A, RARB2, PITX2,* and *PGR* are genes that are known to be methylated in BC. However, with few exceptions, the biological significance for such events is not clear. Identification of the pathways to which these genes segregate can help elucidate part of the mechanisms to their contribution to breast carcinogenesis (Figure 2). To this end, *RASSF1A* methylation correlates significantly with the methylation of the PCGT genes (Pearson's, R=0.470, p<0.0001). A similar level of association was found between the methylation status of PGR and PITX2 promoters (R=0.261, p=0.009 and R=0.229, p=0.023, respectively). *RARB2* methylation did not show statistically significant correlation with the methylation of

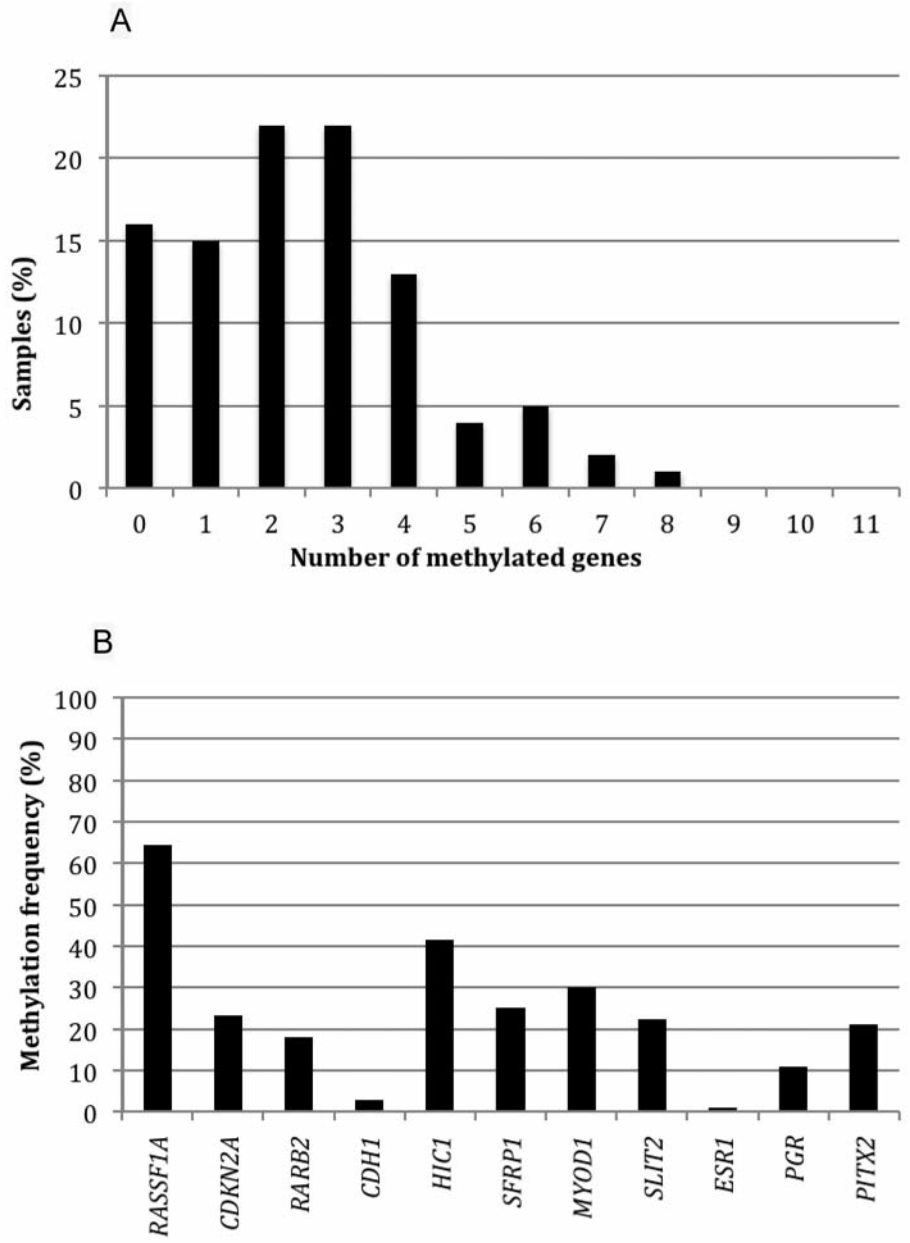

Figure 1. *A: Sample frequency of the number of methylated genes. B: Methylation frequency of individual genes analyzed in this study.*

PCGT, while *CDKN2A* methylation actually showed a negative correlation with such events (R=0.275, p=0.010).

Correlation with clinical parameters. There was no statistically significant association between methylation events and estrogen receptor (ER), progesterone receptor (PR), or v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER2) amplification status. However, *RASSF1A* methylation showed a trend towards being associated with positive expression of ER (p=0.051, odds ratio (OR)=1.47, 95% CI=0.98-2.22). Hypermethylation of the *CDKN2A* promoter appeared to be more prominent in pre-menopausal women (p=0.039, OR=2.34, 95% CI=0.93-5.85). Involvement of the surgical margins was significantly associated with *CDKN2A* methylation (p=0.003, OR=3.33, 95% CI=1.58-7.14).

The majority of this cohort displayed positive ER reactivity (65% of the cases with a known ER status). Therefore, their disease can be classified as BC, luminal type. The basal type, in which ER or PR are not expressed, constitutes less than 35% of this series. Therefore, we performed the statistical analysis of the association between

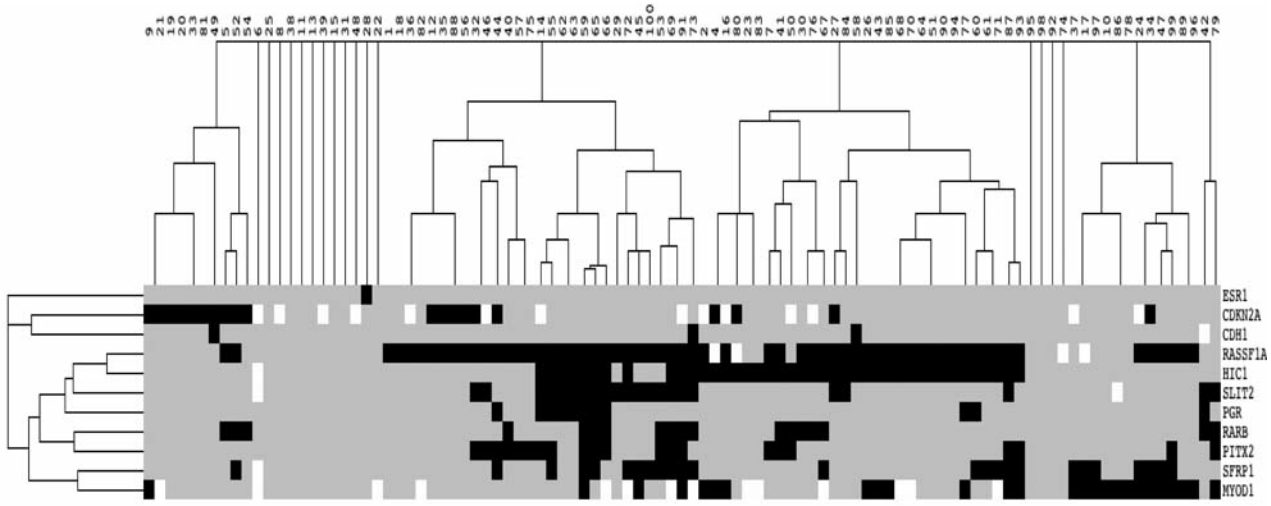

Figure 2. *Hierarchical clustering of the methylation events and breast cancer samples. Black shading represents positive methylation event while grey represents a negative methylation event. White areas indicate no data present for that particular sample. The numbers indicate sample numbers. Clustering was performed using the Gene CLUSTER 3.0 program and visualized using JavaTree software.*

single-gene methylation events and clinical parameters, with the samples dichotomized as ER± and ER±. This approach yielded a strong association between *RASSF1A* methylation and lymph node metastasis in ER± cases (p=0.008, OR=2.89, 95% CI=1.06-7.57). This association is further supported by the observation that 15 out of 15 samples showed complete concordance between *RASSF1A* methylation in surgically resected tumors and that in extracted lymph nodes. A similar pattern was observed for methylation of *HIC1* (p=0.013, OR=1.83, 95% CI=1.09-3.08), *SLIT2* (p=0.033, OR=1.67, 95% CI=1.16-2.40) and *SFRP1* (p=0.015, OR=1.79, 95% CI=1.22-2.62). *CDKN2A* methylation was found to be a strong indicator of lymphovascular invasion by the ER± tumors (p=<0.0001, OR=8.26, 95% CI=2.67-25.64). Conversely, *SLIT2* methylation was more frequent in samples where no lymphovascular invasion was detected in ER± patients (p=0.042, OR=1.49, 95% CI=1.19-1.93). *HIC1* methylation was predominant in postmenopausal, ER± patients (p=0.007, OR=2.15, 95% CI=1.20-3.89). Methylation of *MYOD1* and of *SFRP1* was significantly associated with involvement of the surgical margins (p=0.037, OR=2.33, 95% CI=1.30-4.16 and p=0.035, OR=1.94, 95% CI=1.18-3.19, respectively).

Next, we tested the value of all methylation markers as predictors of DFS and DSS using Kaplan-Meier analysis with log-rank statistics (Figure 3). None of the genes investigated showed any statistically significant association with DSS. However, when DFS was examined, *MYOD1* and *RASSF1A* methylation status was clearly associated with different DFS, being more favorable among patients with non-methylated genes (Figure 3). In addition, *MYOD1* methylation status demonstrated a statistically significant association with recurrent BC (p=0.036, OR=2.12, 95% CI=1.07-5.55, Fisher's exact test). Of all 8 markers entered in the multivariate (Cox) proportional hazards regression model, only *RASSF1A* methylation proved to be an independent predictor of DFS (p=0.026, HR=5.64, 95% CI=1.23-25.81).

Discussion

This is the first report on the role of gene hypermethylation and its association with invasive ductal BC in a cohort of Saudi Arab patients. BC is rapidly becoming a major cause of mortality among women in the Kingdom of Saudi Arabia. This is highlighted, by the fact that over 67% of the cases analysed were surgically resected samples from patients under 50 years of age. Although data on breast cancer 1 (*BRCA1*) and breast cancer 2 (*BRCA2*) mutation status were not available, patients with any familial history of any type of cancer were excluded to rule out the influence of inherited factors on the gene methylation profiles. The present study reports the methylation frequencies of the genes commonly hypermethylated in BC (*RASSF1A, CDKN2A, CDH1* and *RARB2*) as well as those recently described estrogen target genes (*ESR1, PGR, PITX2*) and polycomb gene targets (*MYOD1, SFRP1, HIC1* and *SLIT2*) (7).

There was no statistically significant association between methylation of any of the markers and age of the BC patients. Only hypermethylation of *CDKN2A* showed a significant association with the pre-menopausal status (p=0.039, OR=2.34, 95% CI=0.93-5.85). Early squamous cell carcinoma (OSCC) exhibits a significant increase in *CDKN2A* hypermethylation compared to late-onset OSCC

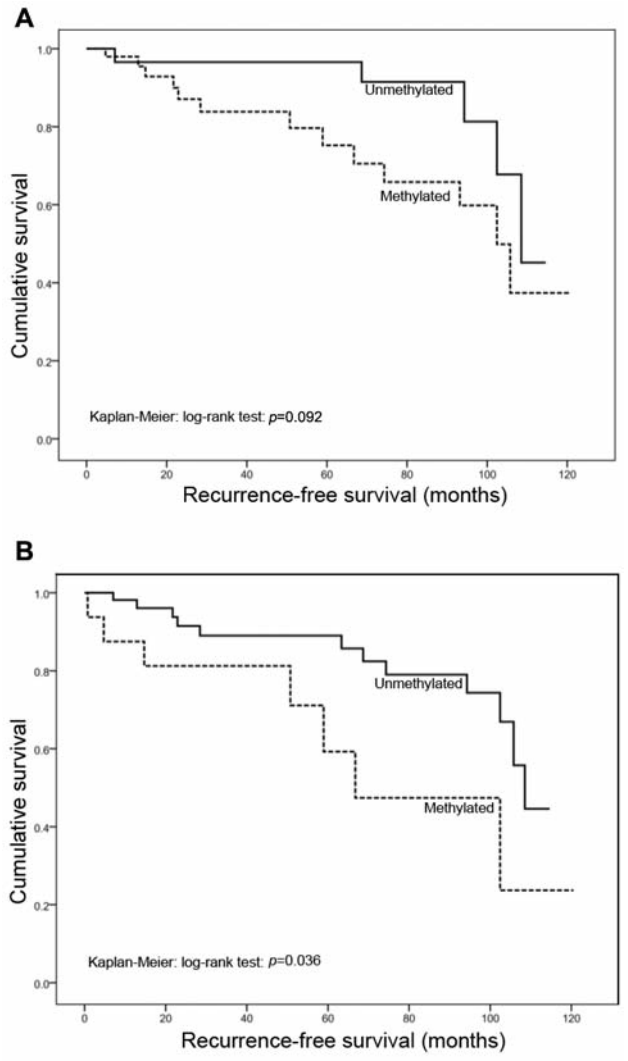

Figure 3. *RASSF1A (A) and MYOD1 (B) methylation status and recurrence-free survival in univariate (Kaplan-Meir) analysis. p-value for MYOD1 is calculated based upon using PMR>20 as a cut-off for positive methylation.*

with significant association with lymph node metastasis (10). CDKN2A promoter methylation in the colon appears to be modulated by differential exposure to bioactive food compounds (11). The observation that *CDKN2A* is hypermethylated in younger patients may represent a scenario that parallels the findings reported by Caragher *et al.*, who showed that Cdkn2a acts as a barrier to colon carcinogenesis induced by *Braf* mutations until its silencing by hypermethylation in the mouse colon (12). Therefore, there is a distinct possibility of the presence of a common genetic determinant in our *CDKN2A* methylation positive cases which would be able to act in a manner similar to *BRAF* mutations in the colon. Interestingly, *CDKN2A* methylation correlated negatively with the methylation of other genes analyzed in the present study (R=0.275, p=0.010), possibly representing an independent group of cases who are younger and carry an (epi)genetic determinant for early-onset BC.

We have detected overall lower methylation frequencies compared to other published reports using the same technology and materials. The average methylation frequency for *CDKN2A* is 41% compared to 20% identified in our cohort. Similarly, average methylation frequencies for *RARB2, SFRP1, SLIT2* and *MYOD1* are 28%, 54%, 49% and 85% respectively (http://www.pubmeth.org, (13); this is compared to 18%, 25%, 22% and 30%, respectively, in the present series. The most striking difference, however, was in the methylation frequencies of *ESR1, PGR* and *CDH1*. The average *ESR1* methylation frequency in BC is reported to be around 57%. However, we detected *ESR1* methylation in only one single sample (1%). Similarly, the reported methylation frequency of PGR is 100% in BC, but we detected PGR hypermethylation in 11% of cases only. In published series, CDH1 methylation frequency averages 57%, whereas in our cohort, only 3 samples displayed detectable CDH1 methylation. The reasons for such discrepancies are not clear. One reason could be the relatively younger age of our cohort which could play limiting factor in the accumulation of cancer-associated methylation events. Alternatively, the lack of *ESR1* and *PGR* methylation could reflect an overexposure to estrogen and progesterone in our population, as suggested by Winschwendter *et al.* (7). This could be influenced by the lower age at menarche and changes in socioeconomic characteristics of the Saudi population over the past 20 years.

Besides *RASSF1A* and *HIC1*, the PCGT genes were the most commonly methylated genes in this cohort. Methylation of the PCGT genes predicts the presence of BC regardless of the tumour heterogeneity (14). PCGT genes are suppressed in stem cells and their methylation is fast becoming an accepted hallmark of cancer. The methylation of the PCGT genes has been shown recently to increase as a function of age (15). Interestingly, *HIC1* methylation was predominant in post-menopausal, ER± women (p=0.007, OR=2.15, 95% CI=1.20-3.89). Another member of the PCGT gene group is *MYOD1*, the methylation of which was found to be significantly associated with disease-free survival (p=0.05, OR=2.12, 95% CI=1.07-5.55, Fisher's exact test).

The single most frequently methylated gene in this cohort was *RASSF1A* (65%). The methylation of *RASSF1A* correlated with the methylation of the PCGT genes. This suggests that *RASSF1A* may belong to such a group. In addition, *RASSF1A* methylation was detected in both surgically resected tumours and associated lymph nodes, further supporting the statistical observations. Interestingly, *RASSF1A* methylation was weakly associated with the ER

status in this cohort. However, Widschwendter *et al*. identified stronger association between *RASSF1A* methylation and hormonal status (16). Importantly, multivariate (Cox) regression analysis disclosed *RASSF1A* methylation as the only independent predictor of DFS, implicating a poor prognosis in this cohort. Other studies have identified *RASSF1A* methylation as an independent predictor for poor prognosis in breast cancer using a similar approach to DNA methylation analysis (17, 18).

Taken together, of the eight genes tested for methylation in the present study, *RASSF1A* methylation was found to be the only independent predictor of poor prognosis in this cohort of patients with operable BC in Saudi Arabia. In addition, we have found evidence suggesting that some as yet unknown genetic factor(s) can underline the oncogenesis of BC in a subset of our cohort that warrants further investigations.

Acknowledgements

This work was supported by the Ministry of Higher Education and King Abdulaziz City for Science and Technology (KACST Grant No. ARP-29-292).

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Received May 15, 2011
Revised June 21, 2011
Accepted June 22, 2011