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Original Article

Methylation of tumor suppressor genes is related with copy number aberrations in breast cancer

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Abstract: This study investigates the relationship of promoter methylation in tumor suppressor genes with copy-number aberrations (CNA) and with tumor markers in breast cancer (BCs). The study includes 98 formalin fixed paraffin-embedded BCs in which promoter methylation of 24 tumour suppressor genes were assessed by Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA), CNA of 20 BC related genes by MLPA and ER, PR, HER2, CK5/6, CK18, EGFR, Cadherin-E, P53, Ki-67 and PARP expression by immunohistochemistry (IHC). Cluster analysis classed BCs in two groups according to promoter methylation percentage: the *highly-methylated* group (16 BCs), containing mostly hyper-methylated genes, and the *sparsely-methylated* group (82 BCs) with hypo-methylated genes. *ATM, CDKN2A, VHL, CHFR* and *CDKN2B* showed the greatest differences in the mean methylation percentage between these groups. We found no relationship of the IHC parameters or pathological features with methylation status, except for Catherin-E ($p = 0.008$). However the highly methylated BCs showed higher CNA proportion than the sparsely methylated BCs ($p < 0.001$, OR = 1.62; IC 95% [1.26, 2.07]). *CDC6*, *MAPT*, *MED1, PRMD14* and *AURKA* showed the major differences in the CNA percentage between the two groups, exceeding the 22%. Methylation in *RASSF1*, *CASP8, DAPK1* and *GSTP1* conferred the highest probability of harboring CNA. Our results show a new link between promoter methylation and CNA giving support to the importance of methylation events to establish new BCs subtypes. Our findings may be also of relevance in personalized therapy assessment, which could benefit the hyper methylated BC patients group.

Keywords: Breast cancer, promoter methylation, copy number aberrations, immunochemistry

Introduction

Breast cancer (BC) is the most common cancer in women. Every year about one million women worldwide are diagnosed with BC [1]. Consequently, this pathology has been extensively investigated in terms of histopathology, immunohistochemistry (IHC) and genetic disorders. Carcinogenesis is the result of accumulating genetic alterations as mutations, copy number aberrations (CNA) and, recently, it has also been demonstrated that epigenetic alterations such as promoter methylation in tumor suppressor genes can drive to tumor development [2]. Epigenetic is defined as *changes in gene expression that are not due to any alteration in the DNA sequence* [3]. They are mediated by several molecular mechanisms including histone modifications, small non-coding RNAs and gene promoter methylation in CpG islands [4]. The understanding of these mechanisms is playing a relevant role in the diagnosis, prognosis and in the design of new treatment strategies.

Epigenetic deregulation, particularly altered DNA methylation patterns, is known to play a key role in the altered gene expression profiles

Table 1. Pathological and immunohistochemical parameters in breast cancer and breast cancer cluster methylated groups

	Parameter/Event		All Samples		Cluster Groups				x^2
			Event (%)	n	Highly methylated		Sparsely methylated		*Adjusted*
					Event (%)	n	Event (%)	n	*p*
Pathological characteristics	T	1	56 (58)	97	10 (62)	16	46 (57)	81	ns
		2	41 (42)	97	6 (38)	16	35 (43)	81	
	N	0	63 (67)	94	11 (69)	16	52 (67)	78	ns
		1	31 (33)	94	5 (31)	16	26 (33)	78	
	Histological Type	CDI	88 (90)	98	13 (81)	16	75 (91)	82	
		CLI	6 (6)	98	3 (19)	16	3 (4)	82	ns
		Other	4 (4)	98	0 (0)	16	4 (5)	82	
	HG	1	19 (20)	94	4 (29)	14	15 (19)	80	
		2	37 (39)	94	4 (29)	14	33 (41)	80	ns
		3	38 (40)	94	6 (43)	14	32 (40)	80	
	Tumor Stage	< III	79 (82)	96	14 (88)	16	65 (81)	80	ns
		≥ III	17 (18)	96	2 (12)	16	15 (19)	80	
Immunohistochemistry	ER	pos	68 (70)	97	11 (69)	16	57 (70)	81	ns
	PR	pos	57 (59)	97	6 (38)	16	51 (63)	81	ns
	HER2	pos	20 (21)	96	4 (25)	16	16 (20)	80	ns
	Cadherin-E	pos	84 (95)	88	10 (77)	13	74 (99)	75	0.008
	Ki-67	high	43 (47)	92	5 (36)	14	38 (49)	78	ns
	CK5/6	pos	18 (20)	89	2 (18)	14	16 (21)	78	ns
	EGFR	pos	12 (14)	85	0 (0)	10	12 (16)	75	ns
	CK18	pos	80 (91)	88	9 (75)	12	71 (93)	76	ns
	P53	pos	43 (57)	75	3 (30)	12	40 (62)	65	ns
	PARP	pos	67 (82)	82	9 (82)	11	58 (82)	71	ns

T: Tumor stage (T1 < 2 cm; T2 ≥ 2 cm); N: Node involvement (NO: Absence; N1: Presence); HG: Histological grade; CDI: Invasive ductal Carcinoma; CLI: Invasive lobular Carcinoma; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Erythroblastic leukemia viral oncogene homolog 2 receptor; EGFR: Epidermal growth factor receptor; PARP: Poly-ADP-Ribose-Polymerase; pos: Positivity; %: percentage of positives; n: total number of cases; *adjusted p*: Holm's adjusted χ^2 *p*-value associated to the differences between the cluster groups; ns: not significant.

found in all human cancers. The last advances in genome-wide approaches have contributed to BC molecular classification [5]. At this regard, methylation profiles have been widely studied in BC, finding different methylation patterns between normal breast and BC [6, 7]. Furthermore, methylation profiles are also associated with BC immunohistochemical features [8, 9] and they are able to differentiate new BC subtypes, not previously identified by conventional IHC [10].

Promoter alterations are also associated with follow-up parameters in BC. Thus, Xu *et al* [11] studied the methylation status of *BRCA1*, *APC* and *p16* in 800 archival retrieved BC and analyzed their relationship with mortality and disease free survival, finding that mortality is associated with *p16 p*romoter methylation, and that the number of methylated genes increases with the BC mortality risk [11].

Although the relevance of epigenetics in carcinogenesis is well established, little is known about the mechanism involved, particularly the relationship between DNA methylation in BC and molecular aberrations commonly detected in cancer. At this regard, recent advances in genome-wide approaches have contributed to BC molecular classification, finding that luminal B subtype is usually associated with chromosomal gains and promoter hyper-methylation [12].

We consider that it is necessary to deepen in the understanding of the molecular mechanisms involved in carcinogenesis in order to develop a personalized medicine based on the design of specific therapeutic agents. Therefore, this study aims to investigate the implications of DNA methylation of tumor suppressor gene promoters with CNA of genes related with BC, and with pathological and immunohistochemical parameters.

Material and methods

Patients

The study includes 98 formalin fixed paraffin-embedded (FFPE) BCs in which promoter methylation, CNA and IHC were assessed. The pathological and IHC features of these patients are summarized in **Table 1**.

All patients signed an informed consent elaborated by the Health Department following the recommendations of the Declaration of Human Rights, the Conference of Helsinki [http://www.wma.net/en/30publications/10policies/b3/index.html] and institutional regulations that was approved by the Hospital Ethics Committee.

Molecular studies

DNA was isolated from FFPE using Deparaffinization Solution and QuiAmp DNA Investigation Kit (Quiagen, Hilden, Germany). DNA quantity and quality was measured spectrophotometrically using NanoDrop 2000c (ThermoFisher).

Methylation studies were performed with the Methylation-Specific Multiplex Ligation Dependent Probe Amplification (MS-MLPA) technique [13]. We used ME001 Tumour Suppressor Mix 1 Kit (MRC Holland, Amsterdam, The Netherlands) [14]. This kit contains probes addressed to detect the methylation status in tumour suppressor gene promoters that are frequently silenced by methylation in cancer such as *TIMP3, APC, CDKN2A, MLH1, ATM, RARB, CDKN2B, HIC1, CHFR, BRCA1, CASP8, CDKN1B, PTEN, BRCA2, CD44, RASSF1, DAPK1, VHL, ESR1, TP73, FHIT, IGSF4, CDH13* and *GSTP1*.

To detect CNA we also employed the MLPA technique [15] with the P078B1 Breast Tumour Kit (MRC Holland, Amsterdam, The Netherlands) [16]. This kit includes probes to detect CNA of *HER2, BIRC5, MYC, TOP2A, ESR1, MTDH, CCND1, CCNE1, EGFR, EMSY, ADAM9, IKBKB, CDH1, CDC6, CPD, FGFR1, MED1, MAPT, PRMD14* and *AURKA* which are frequently altered in BC.

The amplicons generated on MS-MLPA and MLPA were analysed by capillary electrophoresis on an AB3130 Capillary Sequencer (Applied Biosystems) according to MLPA protocol, and fragment analysis was performed using Coffalyser.net software (MRC Holland, Amsterdam, The Netherlands). MLPA and MS-MLPA results were evaluated as previously reported [14, 17].

Immunohistochemistry

IHC was performed using tissue microarray (TMA). The slides were immuno-stained using primary antibodies against Ki-67, ER, PR, HER2, CK5/6, CK18, EGFR, Cadherin-E and P53, all from (DAKO, Glostrup, Denmark) and PARP from (ABCAM, Cambridge, UK). ER and PR expression was evaluated according to Allred scoring system [18]. HER2 expression was scored according to Hercep Test criteria [19]. In 2^+ HER2 expression samples fluorescent *in situ* hybridization was also performed [20]. For *EGFR* and Cadherin-E expression, the same criteria as for *HER-2* were applied [20-22]. Ki-67 and P53 expression was evaluated according to St Gallen International Expert Consensus [20, 23]. The criteria followed to assign CK5/6 and CK18 positivity was their cytoplasmic detection in more than 5% of cells. To evaluate PARP expression, nuclear staining percentage over 5% was considered positive. Finally, in order to define invasive BC subtypes we followed St. Gallen International Expert Consensus [20, 24].

Statistical analysis

Data were summarized by their mean and standard deviation and their median and 1^{st} and 3^{rd} quartiles (continuous variables) and by relative and absolute frequencies (categorical variables).

Unsupervised hierarchical analysis [25] was performed for clustering the samples according to the methylation grade, trying to achieve maximal homogeneity for each group and the highest difference between the groups.

Chi-square test applying Holm's correction [26] was used to compare pathological and IHC features between cluster groups.

The number of genes with CNA out of the total number of genes studied was computed for each individual creating a new variable. This new variable was used as a dependent variable in a binomial generalized linear model with methylation group as predictor. We also used the *lasso* ("*least absolute shrinkage and selection operator*") procedure [27] to find which

Table 2. Methylation status intensity in tumor suppressor genes in breast cancer

Gene Promoters		All Samples n = 98		Cluster Groups: Highly Methylated n = 16		Sparsely Methylated n = 82		Differences of the means
		¥ ± SD	(% MS)	¥ ± SD	(% MS)	¥ ± SD	(% MS)	Highly-Sparsely
APC		36.3 ± 27.4	83	53.8 ± 28.0	94	32.9 ± 26.1	80	20.8
ATM		15.7 ± 23.9	46	56.7 ± 20.3	100	7.6 ± 14.4	35	**49.0**
BRCA1		5.7 ± 16.2	15	13.5 ± 25.7	38	4.2 ± 13.3	11	9.3
BRCA2		3.2 ± 11.5	12	12.7 ± 25.3	25	1.4 ± 4.5	10	11.3
*CASP8**		7.1 ± 13.2	31	16.8 ± 15.9	69	5.2 ± 11.8	23	11.6
CD44		1.0 ± 7.2	2	2.4 ± 9.5	6	0.7 ± 6.7	1	1.6
CDH13		26.1 ± 21.9	78	36.1 ± 22.5	81	24.2 ± 21.3	77	11.9
CDKN1B		6.4 ± 18.6	15	33.1 ± 35.2	56	1.2 ± 4.3	7	32.0
CDKN2A		14 ± 23.1	45	51.1 ± 33.2	81	6.7 ± 10.4	38	**44.4**
CDKN2B		3.5 ± 11.7	11	17.3 ± 23.6	44	0.8 ± 4.0	5	16.5
CHFR		9.1 ± 18.9	26	38.5 ± 25.9	88	3.3 ± 9.9	13	**35.2**
*DAPK1**		9.6 ± 19.5	24	34.6 ± 27.6	69	4.8 ± 12.8	16	29.9
ESR1		3.3 ± 14.2	8	15.9 ± 31.8	25	0.8 ± 3.9	5	15.2
FHIT		4.3 ± 15.1	10	22.8 ± 31.1	44	0.7 ± 3.6	4	22.1
*GSTP1**		16.3 ± 26.9	40	28.4 ± 38.8	44	13.9 ± 23.5	39	14.6
HIC1		5.9 ± 20.1	12	31.4 ± 41.1	44	0.9 ± 3.9	6	30.4
IGSF4		3.2 ± 10.8	10	13.1 ± 20.9	31	1.2 ± 5.9	6	11.9
MLH1		3.9 ± 10.5	15	15.9 ± 16.6	62	1.6 ± 6.9	6	14.4
PTEN		3.9 ± 7.8	26	9.4 ± 14.3	38	2.8 ± 5.3	23	6.6
RARB		2.7 ± 10.0	8	3.8 ± 10.7	12	2.4 ± 9.9	7	1.3
*RASSF1**		37.5 ± 28.5	79	49.2 ± 35.0	81	35.3 ± 26.7	78	14.0
TIMP3		3.9 ± 12.7	12	5.8 ± 16.3	12	3.6 ± 12	12	2.2
TP73		4.0 ± 14.1	13	18.5 ± 30.5	31	1.2 ± 3.9	10	17.3
VHL		8.1 ± 23.8	13	41.3 ± 44.7	50	1.6 ± 6.8	6	**39.7**
TOTAL METHYLATED GENES PER PATIENT	Mean (SD)	5.97 (3.7)		12.1 (2.9)		4.8 (2.5)		
	Median (Q1-Q3)	5.0 (3.25-8.0)		11.5 (10.0-15.0)		4.0 (3.0-6.0)		

¥Mean methylation percentage; % MS: Percentage of Methylated samples; SD: Standard deviation; In bold are indicated genes that show major differences between highly and sparsely methylated; groups; *genes whose methylation is associated with an increased percentage of CNA in the tumors.

specific genes were most probably associated with these total CNA between both groups. 10-fold cross validation was used to select the regularization parameter for the *lasso*. All statistical analyses were performed using the R software (version 3.1.0) [http://www.R-project.org/].

Results

We detected methylation in the 24 promoters studied for the majority of samples studied (**Table 2**). The mean methylation percentage for each promoter ranged from 1.0 (*CD44)* to 37.5 (*RASSF1).* On average, we found a total of 5.97 (median: 5.0; Q1: 3.25; Q3: 8.0) promoter methylated genes per patient.

We found CNA, gains or losses, for the twenty studied genes in the majority of the BCs (**Table 3**). The incidence of CNA ranged from the 7% (*ESR1)* to 52% (*MED1)* and remained above 39% for *MYC, FGFR1, BIRC5, CCND1, HER2* and *MED1*. On average, we found a 31.0% (median: 30.0%; Q1: 20.0%; Q3: 40.0%) of total aberrations per patient.

Unsupervised clustering of promoters methylation percentage split the BCs into two groups (**Figure 1**), the first one (placed on the left) was named *highly-methylated* group and contained 16 BCs, many of them harboring hyper or moderately methylated genes. The second group (placed on the right) was called *sparsely-methylated* group*,* and contained 82 BCs mostly showing no or low methylation degree. Methylation status in both clusters is displayed in **Table 2** and represented in a *heatmap* (**Figure 1**). All promoters showed higher mean methylation percentage in the *highly-methylated* group (**Figure 2A**, **Table 2**). *ATM, CDKN2A, VHL* and *CHFR* were the ones that presented the widest differences in the mean methylation percentage between the two established groups with methylation percentages of 49.0%, 44.4%, 39.7% and 35.2%, respectively. We found on average 12.1 (median: 11.5; Q1: 10.0; Q3: 15.0) promoter methylated genes in the

Table 3. Incidence of abnormalities (gains or losses) in breast cancers

Gene	Chr	All Samples	Cluster Groups		Difference of Abnormalities
		n = 98	Highly Methylated n = 16	Sparsely Methylated n = 82	
		Abnormalities n (%)	Abnormalities n (%)	Abnormalities n (%)	Highly-Sparsely (%)
ESR1	06q25	7 (7)	0 (0)	7 (9)	-9
EGFR	07p11	15 (15)	3 (19)	12 (15)	4
FGFR1	08p12	40 (41)	4 (25)	36 (44)	-19
ADAM9	08p11	26 (27)	5 (31)	21 (26)	5
IKBKB	08p11	34 (35)	7 (44)	27 (33)	11
PRDM14	08q13	36 (37)	**9 (56)**	**27 (33)**	23
MYC	08q24	39 (40)	8 (50)	31 (38)	12
MTDH	08q22	33 (34)	8 (50)	25 (30)	20
CCND1	11q13	44 (45)	7 (44)	37 (45)	-1
EMSY	11q13	36 (37)	6 (38)	30 (37)	1
CDH1	16q22	18 (18)	5 (31)	13 (16)	15
CPD	17q11	19 (19)	4 (25)	15 (18)	7
MED1	17q11	51 (52)	**12 (75)**	**39 (48)**	27
HER2	17q12	47 (48)	9 (56)	38 (46)	10
CDC6	17q21	35 (36)	**10 (62)**	**25 (30)**	32
TOP2A	17q21	16 (16)	3 (19)	13 (16)	3
MAPT	17q21	35 (36)	**10 (62)**	**25 (30)**	32
BIRC5	17q25	44 (45)	8 (50)	36 (44)	6
CCNE1	19q12	13 (13)	4 (25)	9 (11)	14
AURKA	20q13	19 (19)	**6 (38)**	**13 (16)**	22
TOTAL CNA PER PATIENT	Mean (%)	31.0	40.0	29.2	
	Median (%) (Q1-Q3)	30 (20-40)	42.5 (30.0-50.0)	30.0 (16.3-40.0)	

Chr: chromosome location; CNA: Copy Number Aberration; Bold character indicate the genes that show major differences between highly and sparsely methylated groups.

highly-methylated cluster and 4.8 (median: 4.0; Q1: 3.0; Q3: 6.0) in the *sparsely-methylated* cluster.

With regard to the methylation percentage, we differentiated three groups (**Figure 1**); The first group contained *ATM, CHFR, CDKN2A* and *DAPK1,* was placed in the upper four rows of the *heatmap* and differentiated a 16 *highly-methylated* BCs; the second was formed by *RASSF1, APC, CDH13* and *GSTP1,* placed in the lower part of the *heatmap*, and showed a high methylation percentage in nearly all samples; finally, the central cluster included the remaining 16 genes showing low methylation degree.

We did not find statistical differences for the pathological and IHC features between the *highly* and the *sparsely-methylated* groups, except for Cadherin-E which was less expressed in the highly methylated samples (p = 0.008; **Table 1**).

We found a strong association between the total proportion of CNA detected in each BC sample and the corresponding methylation status. Hence, the *highly-methylated* group BCs showed a higher proportion CNAs than the *sparsely-methylated* group BCs. The total CNA mean percentage per sample was 40.0% (median: 42.5%; Q1: 30.0%; Q3: 50.0%) and 29.2% (median: 30.0%; Q1: 16.3%; Q3: 40.0%), in the *highly* and *sparsely-methylated* cluster, respectively (**Table 3**, **Figure 3**). This was confirmed applying a binomial model which showed that *highly-methylated* BCs was statistically linked with a higher probability of harboring genetic abnormalities (p < 0.001, OR = 1.62; IC 95% [1.26, 2.07]).

The proportion of BCs with aberrations for each gene in the methylated groups varied between the studied genes (**Figure 2B**, **Table 3**). Hence, *ESR1*, *FGFR1* and *CCND1* showed a higher proportion of abnormalities in the *sparsely-methylated* group. The remaining 17 genes presented a higher proportion of aberrations in the *highly-methylated* group. *CDC6*, *MAPT*, *MED1, PRDM14*, and *AURKA* showed the highest CNA

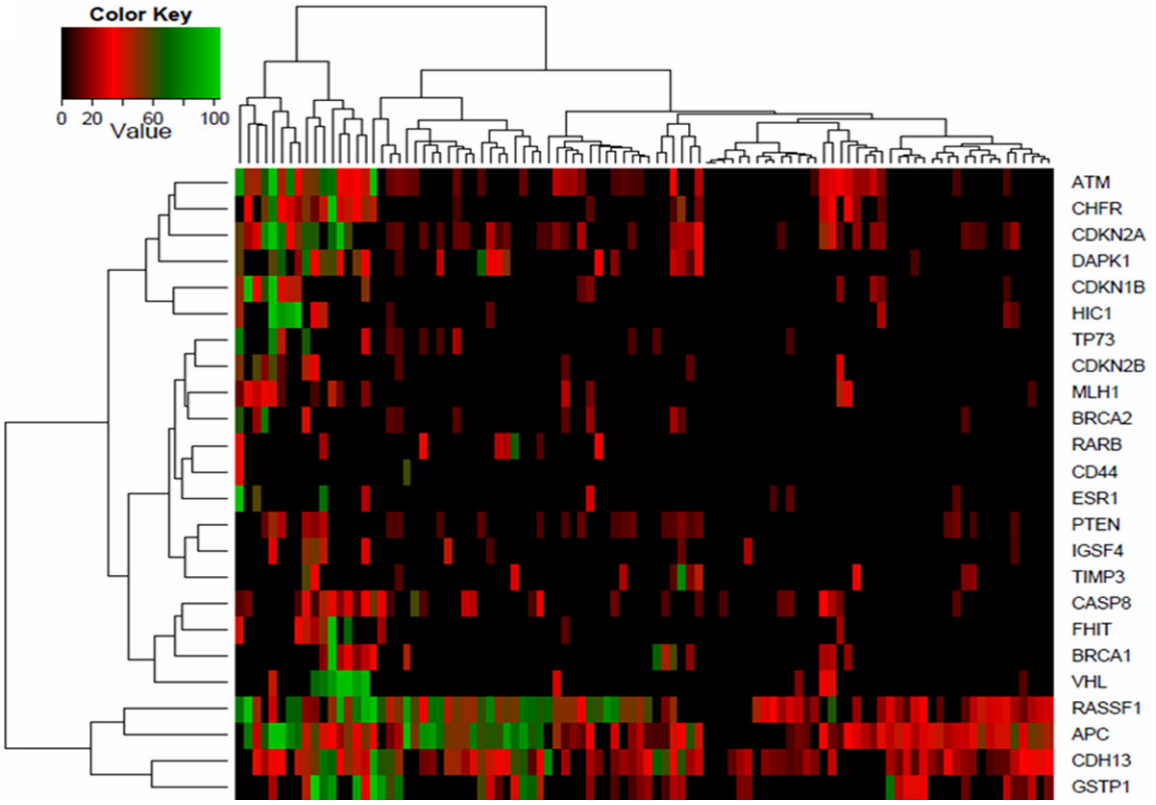

Figure 1. Clustering diagram of breast cancer methylation profile of 24 tumor suppressor genes. The *heatmap* depicts the percentage of promoter methylation (black, low methylation; red, medium methylation and green, high methylation).

percentage in the highly methylated group with a mean difference between groups of 32%, 32%, 27%, 23% and 22%, respectively.

We also analyzed the methylated genes that were more specifically associated with CNA in the two methylation groups. This study was performed by applying *lasso* analysis to the total patient series, using 10-fold cross validation. The analysis selected the genes *RASSF1*, *CASP8, DAPK1* and *GSTP1* as those whose methylation status showed higher probability of having CNA.

Discussion

It is well known that promoter methylation is related with gene expression and tumor subtypes, which suggest that altered methylation signatures in BC could play an important role in phenotype establishment [2].

In the present study we detected methylation in all promoters, although the methylation percentage varied greatly among the genes. At this regard our results for *BRCA1*, *RASSF1*, *RARB* and *CDKN2B* were similar with those previously reported [28-30]. However, for the remaining genes we observed a great disparity [31] that could be attributed to the variability in the methylation assays, sample types (FFPE samples or fresh tumor), different CpG islands studied, etc. [31].

Our CNA results are in full agreement with those reported by Moelans *et al* [16] in a series of 104 BC using MLPA assays. They found similar gain percentages for *EGFR, FGFR, IKBKB, PRMD14, HER2, CCND1, CDH1, CCNE1* and *AURKA*. Here, we observed that *MED1*, *HER2*, *BIRC5*, *CCND1*, *MYC* and *FGFR1* showed the highest CNA incidences. In this regard, abnormalities in *MED1*, *HER2* and *BIRC5*, like many other genes located in chromosome 17 [32], could have a role in cancer initiation, progression or in targeted therapy such as *HER2* amplifications in BC.

Cluster analysis revealed that the methylation profile of 24 tumor suppressor gene promoters

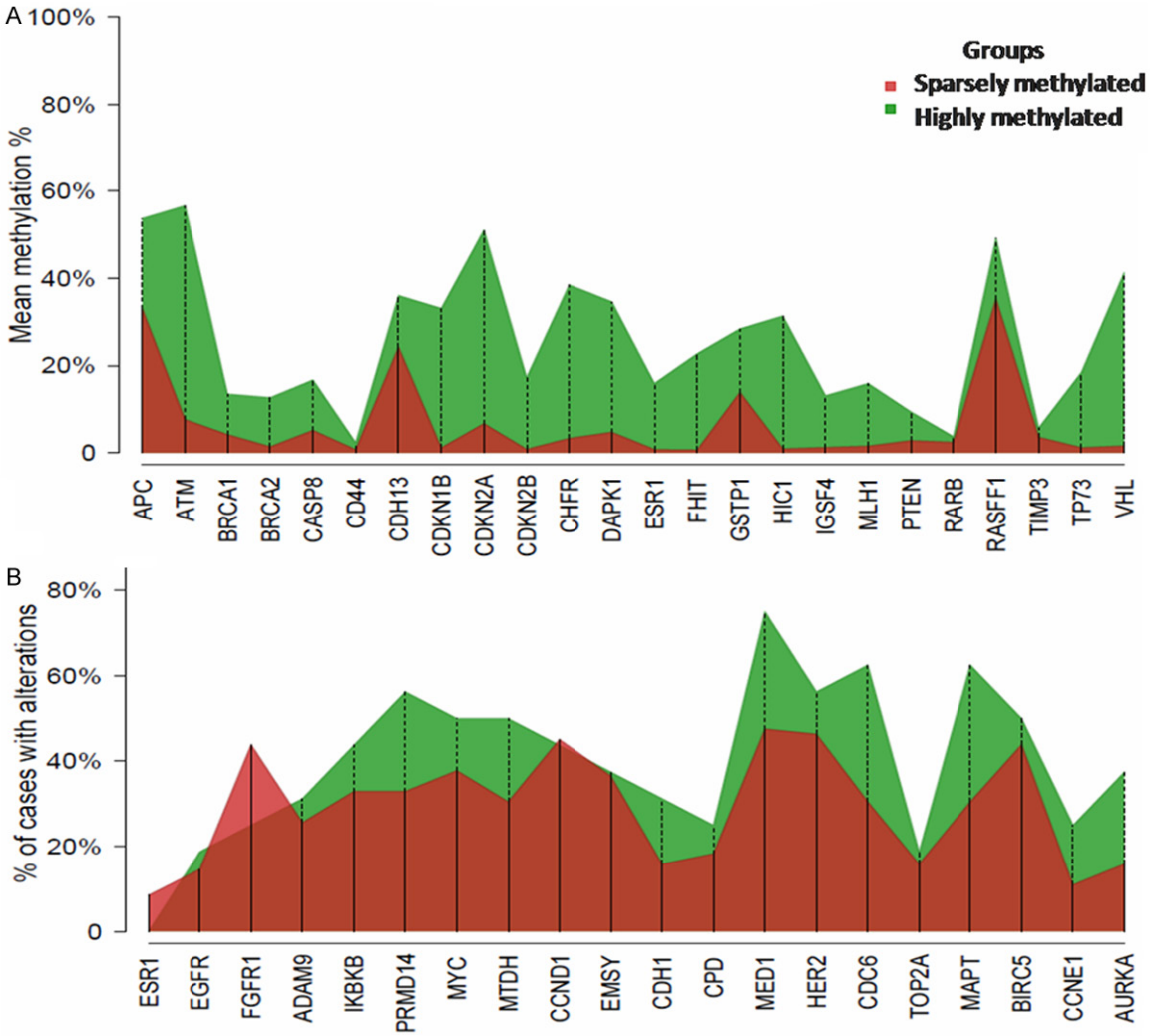

Figure 2. Percentage of promoter methylation (Panel A) for each gene and proportion of BCs with genetic aberrations (Panel B) by methylation group.

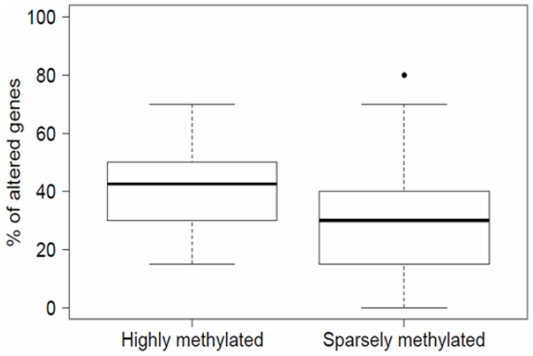

Figure 3. Proportion of genetic aberrations by methylation status. BCs in the *highly-methylated* group show a greater proportion of genetic aberrations than BCs in the *sparsely-methylated* group.

enabled the differentiation of two BCs sets, the *highly* and *sparsely-methylated* BCs. Of these 24 genes *ATM, CDKN2A, VHL* and *CHFR* were the ones that showed the highest differences between the mean methylation percentages of the groups. These particular genes are mainly involved in cell cycle regulation and are associated with poor prognosis and tumor progression. Specifically, for *ATM*, it is widely reported that mutation carriers present an increased risk of BC development [33]. On the other hand, the association of *CDKN2A*, methylation and BC development has been broadly studied; however the results are still controversial [34]. pVHL is the central component of an ubiquitin ligase complex, that interacts with HIF protein. In its absence, as it would be the case of VHL methylation, HIF becomes stabilized and is free to induce the expression of its target genes, many of which are relevant in the angiogenesis process, cell growth or cell survival regulation

[35]. And, *CHFR* has been found poorly expressed in BC cell lines and its reduced expression was reactivated with the use of demethylating agents [36]. In addition, its reduced expression was associated with an increase of aneuploidy [37].

We found that samples belonging to the highly methylated group showed less Cadherin-E expression. This fact would confer to this group of samples an advantage in the dedifferentiation and invasiveness [38], although no association with these pathological features was here found.

Samples in the highly methylated group presented higher CNA. This finding identifies a subgroup of tumors that combines epigenetic and genetic aberrations. The major differences in the CNA percentage between the two groups were found in *CDC6*, *MAPT*, *AURKA, PRDM14* and *MED1.* These genes are implicated in carcinogenesis [39-43] and are frequently amplified in BC. *CDC6* is essential for DNA replication and mitosis regulation [42], and *AURKA* is crucial for proper chromosomes segregation during mitosis [41]. These genes are associated with poor differentiated tumors and reduced survival.

Here we found that of 24 studied genes *CASP8*, *GSTP1*, *RASSF1* and *DAPK1* promoter methylation were the most specifically related with CNA in the BC. To our knowledge, there are no reports relating the methylation status with copy number variations in cancer, and more specifically in BC. The hitherto existing reports related methylation events with genetic instability in colorectal cancer, [44] suggesting that methylation might play an important role in chromosomal segregation processes during the clonal evolution of tumors. Furthermore it has been reported that genetic silencing caused by methylation of genes involved in apoptosis, metabolism detoxification, and cell cycle regulation [45-47] may be a factor that could impair the prognosis of these BCs.

Of the four methylated genes, *GSTP1* plays an essential role in carcinogenesis detoxification and its absence leads to toxic accumulation in the cell and cancer progression [45]. *GSTP1* may act as a caretaker and its methylation could lead to additional somatic genome alterations promoting tumor growth [48]. On other hand, *CASP8* plays a relevant role in the induction of apoptosis by external death signals or in response to DNA damage. Its epigenetic silencing caused by DNA promoter methylation leads to an increased risk of tumor formation and cancer progression [46, 47]. *DAPK1*, a pro-apoptotic serine/threonine protein kinase gene is also essential for the execution of the apoptotic process [49], so its silencing by methylation is also related with an increased risk of BC development. Consequently, *CASP8* and *DAPK1* methylation could contribute to BC progression and to the maintenance of cells carrying genetic abnormalities. Finally, epigenetic *RASSF1* silencing has also been widely reported in BC. RASSF1 protein acts at level of G1/S-phase cell cycle progression regulating cyclin D1 protein accumulation [50]. When the RASSF1 protein is not expressed there is an imbalance in favor of cell division. Therefore these cells can continue dividing increasing genetic instability instead of arresting the cell cycle. Likewise, it has been observed in knockout mice that re-expression of *RASFF1* inhibits tumorigenesis [51].

In summary, our results support that tumor suppressor genes promoter methylation is associated with genetic aberrations. Furthermore, the methylation profile of tumor suppressor genes identifies two types of BCs, being the *highly-methylated* associated with a greater number of aberrations in genes involved in development, progression and response to therapy in BC. Finally, our results reveal a new link between methylation and CNA which gives support to the importance of methylation events to establish new subtypes of BCs. Our findings may be of significance in personalized therapy assessment that could benefit the hyper-methylated BC patients group.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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