Methylation profiling in promoter sequences of *ATM* and *CDKN2A* (*p14^{ARF}*/*p16^{INK4a}*) genes in blood and cfDNA from women with impalpable breast lesions

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Abstract. The objective of the present study was to evaluate the epigenetic changes occurring in early stages of breast cancer. The present study investigated the methylation profile of the *ATM*, *p14^{ARF}* and *p16^{INK4a}* promoters in total blood and plasma cell-free DNA (cfDNA) from women with impalpable breast lesions compared with in total blood of a control cohort of women without breast lesions. The samples were evaluated using the methylation-specific PCR method. The Fisher's exact test was used to evaluate statistical significance between the methylation and clinical variables. A total of 111 women were evaluated, including 56 women with impalpable breast cancer (39/56 also had paired plasma cfDNA) and 55 women in the control cohort (55 blood DNA). For blood DNA from women with malignant impalpable breast lesions, *p16^{INK4a}* exhibited the greatest percentage of methylation (48%), followed by *ATM* (37.5%) and *p14^{ARF}* (27%) promoters, regardless of age variation. For plasma cfDNA, the methylation rates for *ATM*, *p14^{ARF}* and *p16^{INK4a}* were 26, 26 and 10%, respectively. The methylation rates for the blood DNA of controls were the lowest for *ATM* (9%), *p14^{ARF}* (7%) and *p16^{INK4a}* (7%). The women with impalpable breast lesions (benign and malignant lesions) exhibited the highest methylation rate, regardless of age, compared with the paired plasma cfDNA and controls. This epigenetic change was statistically significant for the promoters of *ATM* (P=0.009) and *p16^{INK4a}* (P=0.001) (impalpable breast lesions vs. control). The present study demonstrated that epigenetic changes occurring in the *ATM* and *CDKN2A* genes detectable in liquid biopsy were associated with the development of impalpable breast lesions.

Introduction

In recent decades, the detection of impalpable breast lesions has increased due to the dissemination of mammographic screening programs and the improved resolution and accuracy of imaging tests (1,2). In general, impalpable breast lesions are small (<2 cm) with an initial histopathologic phenotype, for example, lesions *in situ* associated with infiltrative lesions, and positive estrogen and/or progesterone receptors (luminal) (3,4). Although the pathological scenario is favorable, surgical management has become a challenge. With conservative surgery, the residual tumor cells became a risk, increasing the possibility of relapses over the years. There is no estimation of the impalpable breast lesion relapse rate (4-7), however, for initial breast lesions, the risk of relapse for treated and not treated radiotherapy cases may reach 7 to 26%, respectively. Also, mortality over 15 years may reach 36% (8) which is higher than expected given the favorable prognosis.

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Shedding of tumor cells in the blood circulation can occur simultaneously with primary tumor. This process which is part of metastasis may take weeks, or even decades, to develop and varies depending of the tumor type (9). During this period, tumor cells initiate the process of cellular plasticity and motility promoting their detachment from the primary site. On the other hand, an immune response is initiated to eliminate not only the tumor cells but possible circulating tumor cells (CTCs). In this battle between immune system and tumor, many apoptotic and necrotic tumor cells are phagocytized, increasing the concentration of cell-free DNA (cfDNA) in the blood which can be used as liquid biopsy. In some cases, this process starts very early, even in the absence of the primary site formed (10-13). In this context, in the course of carcinogenesis and invasion of tumor cells in the bloodstream, white blood cells (WBC) are constantly undergoing molecular alterations.

Hypomethylation and silencing of tumor suppressor gene expression by hypermethylation have been recognized as important markers for different cancers (14). Especially hypermethylation has been studied in DNA from WBC and revealed potential signatures to detect and predict breast cancer evolution (15,16). In this way, the *CDKN2A* ($p14^{ARF}/p16^{INK4a}$) and *ATM* genes are potential targets for epigenetic study, as they are described as hypermethylated in breast carcinogenesis (17-23). *ATM* plays a critical role in DNA double-break repair, involved in DNA damage recognition, recruitment of repair proteins, cell signaling for checkpoints, transcriptional regulation, and apoptosis activation (24). Hypermethylation in the *ATM* promoter has been reported in different types of cancers, including breast (20,24-26), glioma (27), gastric lymphoma (28), and colorectal neoplasia (29). The $p14^{ARF}$ and $p16^{INK4a}$ tumor suppressor genes (TSGs) are encoded within the *CDKN2A* locus on chromosome 9q21 (29). The encoded proteins are kinase-dependent inhibitors, and regulate the cell cycle under interference with the actions of p53 and Rb (29). Genetic and epigenetic alterations have been described in these genes in some cancers, including breast cancer (30), cervical intraepithelial neoplasia (31), follicular lymphoma (32), non-small cell lung cancer (33), and others (34,35).

To contribute to the understanding of the epigenetic changes detected by liquid biopsies of women with impalpable breast lesions, we analyzed the methylation pattern of *ATM* and *CDKN2A* ($p14^{ARF}/p16^{INK4a}$) promoter genes in total blood DNA and plasma cfDNA from women with impalpable breast lesions, and compared this with the blood DNA from a control cohort of women without breast lesion.

Materials and methods

Study population. The women with impalpable breast lesions were recruited in 2015-2016 at Americas Barra Medical City, in the city of Rio de Janeiro, Brazil. The control cohort of women with nipple aspirate fluid (NAF) without breast lesions were recruited in 2008-2012 from the Radiology Service at Hospital Universitário Gafrée-Guinle (HUGG). The subjects enrolled in this study signed an informed consent and protocols were approved by ethics committee approval, Rio de Janeiro State University Hospital, no. CAAE:43560115.5.0000.5259 and HUGG-07/2007-80/2012. The control cohort was part of a previous study from our group (36) and were followed up to the year 2015, and none of them had developed benign or malignant breast lesions. All participants were subject to clinical evaluation, mammography, and/or breast ultrasonography. The patients with impalpable breast lesions were classified as Breast Imaging Reporting and Data System (BIRADS)3 or 4. The NAF control subjects were classified by their macroscopic characteristics, including whether they were watery, citrine, serous, bloody, or mixed (seropurulent). Subjects were excluded from the study if they showed immunodeficiency or genetic syndromes or were previously diagnosed as cancer patients and in treatment. The patients' clinical data were obtained from hospital/clinic records. The lesion histological classification was graded according to current (2012) World Health Organization (WHO) criteria (37), and nuclear grade was defined as grades I-III according to Elston and Ellis (38). For more details, the social demographic profile and clinical data of the cases are shown in Table I.

DNA preparation. Blood-4 ml of blood from controls and impalpable breast lesion patients was collected in EDTA and transferred to a 15 ml tube and then centrifuged at room temperature for 10 min at 2,000 x g. The plasma was discarded and 10 ml of erythrocyte lysing solution (4˚C) was added to the cells (10 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM NaCl). The DNA extraction was performed by the Phenol-Chloroform method. The DNA samples were stored at -20˚C until further analysis. For cfDNA extraction, 10 ml of blood from impalpable breast lesion patients were collected in EDTA before surgery, and centrifuged at room temperature for 10 min at 2,000 g. Supernatants were centrifuged at 16,000 x g for 10 min at 20˚C to remove debris. Plasma was harvested and stored at -80˚C. When DNA was to be analyzed, 2 ml was used to obtain cfDNA using the QIAamp® Circulating Nucleic Acid Kit (Qiagen), according to the manufacturer's protocol. The blood DNA and cfDNA samples were quantified with the Qubit dsDNA HS Assay Kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Evaluation of ATM and CDKN2A promoter methylation. For DNA modification reaction, the EpiTect Bissulfite (Qiagen) and EZ DNA Methylation (Zymo Research) kits were used according to the manufacturer's protocol. PCR amplification was performed in a reaction mixture containing 50 ng of modified genomic DNA, STR 1X buffer, 200 mM dNTPs; 3 mM of $MgCl_2$, primers for each promoter (10 pmol/μl each), and 0.2 units *Platinum® Taq DNA Polymerase* (all from Invitrogen; Thermo Fisher Scientific, Inc.) in a final volume of 25 μl. The primers used for methylation specific polymerase chain reaction (MSP-PCR) have been previously described (39-41). Universal Methylated DNA Standard (Zymo Reseach) and *DLD-1* cell line were used as positive controls for *ATM* and *CDKN2A* genes, respectively. PCR assays were performed in the Veriti™ DX Thermal Cycler (Thermo Fisher Scientific, Inc.). The PCR program consisted of a pre-denaturation at 94˚C for the first 10 min, followed by 35 cycles at 94˚C for 45 sec, 60˚C (methylated and non-methylated primers) for 45 sec, and 72 for 1 min. The final extension was performed at 72˚C for 7 min. MSP products were resolved in 10% polyacrylamide

Table I. Social demographic and clinical data of the cases available.

Characteristic	Patients, n (%) (n=111)
Age, years	
Benign cases	
Mean	45.5
SD	7.12
Malignant cases	
Mean	61
SD	11.6
NAF cases	
Mean	50
SD	11.3
NAF classification	N=55
Watery	4 (7)
Bloody	4 (7)
Mixed (serupurulent)	4 (7)
Serous	18 (33)
Citrine	18 (33)
NI	7 (13)
Malignant lesions	N=48
IDC	19 (40)
DCIS	5 (10)
IDC-DCIS	17 (35)
LCIS	1 (2)
ILC-LCIS	4 (8)
Micropapillary carcinoma	2 (5)
Benign lesions	N=8
Fibroadenoma	1 (12)
Ductal ectasia/apocrine metaplasia	4 (50)
Hyperplasia of columnar cells with and without atypia	3 (8)
Nuclear grade	N=48[a]
I	11 (23)
II	26 (54)
III	10 (21)
Unknown	1 (2)
TNM/Stage	N=48[a]
T1N0M0 (stage I)	35 (73)
T1N1M0 (stage IIa)	6 (13)
T2N1M0 (stage IIa)	1 (2)
TisN0M0 (stage IIa)	6 (12)
ER status	N=48[a]
Positive	40 (83)
Negative	2 (4)
Unknown	6 (13)
PR status	N=48[a]
Positive	33 (69)
Negative	9 (19)
Unknown	6 (12)
HER2 status	N=48[a]
Positive	7 (14)
Negative	33 (69)
Unknown	8 (17)
Ki 67	N=48[a]
Low (<20%)	21 (44)
Intermediate/High (≥20%)	10 (21)
Unknown	17 (35)
BC subtype	N=48[a]
Luminal A	33 (69)
Luminal B	7 (15)
Triple negative	2 (4)
Unknown	6 (12)

[a]Malignant lesions. NAF, nipple aspirate fluid; NI, not informative; IDC, infiltrative ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, infiltrative lobular carcinoma; LCIS, lobular carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal receptor 2; BC, breast cancer.

gels at 160 V in 1 X TBE buffer (Tris-Borate-EDTA), stained by silver.

Statistical analysis. Contingency tables were used to associate the hypermethylation of each promoter (*ATM*, $p14^{ARF}$, $p16^{INK4a}$) with the specimens evaluated herein. For the purpose of statistical analysis and evaluation of the correlation between age and methylation, women were divided into age groups of ≤50 and >50 years old. The Fisher's exact test was adopted to test the statistical significance. The receiver operating characteristic (ROC) curve was used to evaluate the correlation between positive methylation and age for each gene analyzed. The survey data were processed in Predictive Analytics Software (PASW), version 20. In all statistical tests, a 5% significance level was considered. Thus, statistically significant associations were considered as those whose P-value was <0.05.

Results

Clinical data. One hundred and eleven women were included in this study, being 55 and 56 (plus 39 cfDNA) women with NAF and impalpable breast lesions, respectively. The women with benign breast lesions ranged in age from 27 to 49 years (M=45.5 years, SD=7.12), while the patients with malignant breast lesions ranged in age from 33 to 90 (M=61 years, SD=11.62). The NAF patients ranged in age from 30 to 82 years (M=50 years, SD=11.32). Following surgery, the histopathological diagnosis revealed 8/56 (14%) benign lesions and 48/56 (86%) malignant lesions. Malignant lesions were 40% infiltrative ductal carcinomas (IDC) and 35% mixed lesions (IDC with ductal carcinoma *in situ*). Immunohistochemistry revealed 83% ER-positive, 69% PR-positive, and 69% HER2-negative tumors. Thus, 69% of the malignant tumors were Luminal A

Table II. DNA methylation pattern in promoters of *ATM, $p14^{ARF}$* and *$p16^{INK4a}$* genes.

DNA type	Gene	Groups: Benign lesions, n (N=8) (%)	Groups: Malignant lesions, n (N=48) (%)
Blood DNA from women with impalpable breast lesions	*ATM*	0	18/48 (37.5)
	$p14^{ARF}$	4/8 (50)	13/48 (27)
	$p16^{INK4a}$	3/8 (37.5)	23/48 (48)
DNA type	Gene	Benign lesions, n (N=6) (%)	Malignant lesions, n (N=39) (%)
cfDNA from women with impalpable breast lesions	*ATM*	2/6 (33.3)	10/39 (26)
	$p14^{ARF}$	0	10/39 (26)
	$p16^{INK4a}$	0	4/39 (10)
DNA type	Gene	Cases, n (N=55) (%)	
Blood DNA from women with NAF	*ATM*	5/55 (9)	
	$p14^{ARF}$	4/55 (7)	
	$p16^{INK4a}$	4/55 (7)	

cfDNA, circulating free DNA; NAF, nipple aspirate fluid.

Table III. Distribution of DNA methylation pattern in promoters of *ATM*, *$p14^{ARF}$* and *$p16^{INK4a}$*, according to the two different age groups (≤50 and >50 years old).

DNA type	Gene	Groups: ≤50 years Benign (N=8) (%)	Groups: ≤50 years Malignant (N=9) (%)	Groups: >50 years Malignant (N=39) (%)
Blood DNA from women with impalpable breast lesions	*ATM*	0	4/9 (44)	14/39 (36)
	$p14^{ARF}$	4/8 (50)	3/9 (33.3)	10/39 (26)
	$p16^{INK4a}$	3/8 (37.5)	5/9 (55.5)	18/39 (46)
DNA type	Gene	≤50 years Benign (N=6) (%)	≤50 years Malignant (N=6) (%)	>50 years Malignant (N=27) (%)
cfDNA from women with impal pable breast lesions	*ATM*	2/6 (33.3)	1/6 (16.7)	9/27 (33)
	$p14^{ARF}$	0	1/6 (16.7)	9/27 (33)
	$p16^{INK4a}$	0	1/6 (16.7)	3/27 (11)
DNA type	Gene	≤50 years (N=28) (%)	>50 years (N=27) (%)	
DNA from women with NAF	*ATM*	3/28 (11)	2/27 (7.4)	
	$p14^{ARF}$	2/28 (7.1)	2/27 (7.4)	
	$p16^{INK4a}$	2/28 (7.1)	2/27 (7.4)	

NAF, nipple aspirate fluid.

and 15% Luminal B. In relation to the staging, 83% of lesions were initials (T1N0M0) (Table I).

Methylation analysis. The DNA methylation pattern was assessed in the promoters of *ATM*, *$p14^{ARF}$*, and *$p16^{INK4a}$* in

Table IV. Association of the variable methylation of the *ATM, $p14^{ARF}$* and *$p16^{INK4a}$* genes among the groups of women with impalpable breast lesions and nipple aspirate fluid.

	Groups (>50 years old)		
Genes/Methylation	Cases, n (%)	NAF, n (%)	P-value
ATM			
No	25 (64.1)	25 (92.6)	0.009
Yes	14 (35.9)	2 (7.4)	
$p14^{ARF}$			
No	29 (74.3)	25 (92.6)	0.102
Yes	10 (25.7)	2 (7.4)	
$p16^{INK4a}$			
No	21 (53.8)	25 (92.6)	0.001
Yes	18 (46.2)	2 (7.4)	

NAF, nipple aspirate fluid.

Table V. Association of the variable methylation of the *ATM*, *$p14^{ARF}$* and *$p16^{INK4a}$* genes among the groups of women with IBLs (blood and cfDNA).

	Group (>50 years old)		
Genes/Methylation	IBL, n (N=39) (%)	cfDNA, n (N=27) (%)	P-value
ATM			
No	25 (64.1)	18 (66.7)	>0.999
Yes	14 (35.9)	9 (33.3)	
$p14^{ARF}$			
No	29 (74.3)	18 (66.7)	0.584
Yes	10 (25.7)	9 (33.3)	
$p16^{INK4a}$			
No	21 (53.8)	24 (88.9)	0.003
Yes	18 (46.2)	3 (11.1)	

IBL, impalpable breast lesions; cfDNA, circulating free DNA.

56 blood DNA samples and 39 paired plasma cfDNA samples from the women with impalpable lesion and 55 blood DNA samples from women without breast lesion were. Further, in relation to blood DNA from women with malignant impalpable breast lesions, among the 3 promoter genes assayed, *$p16^{INK4a}$* showed the greatest percentage of methylation, regardless of age variation, followed by *ATM* and *$p14^{ARF}$* promoters. However, the *$p14^{ARF}$* gene had the highest rate of methylation for benign cases (Table II). This same result can be observed in the distributions by age (Table III). For the cfDNA samples, the methylation rates showed a pattern of positivity for all age groups and genes analyzed here (Table II). However, there was a slight increase in methylation rates for the promoters of *ATM* and *$p14^{ARF}$* in cases older than 50 years old (Table III). Regarding the control subjects, there was no difference in the methylation positivity for *$p14^{ARF}$* and *$p16^{INK4a}$* (Table II). Two cases were methylated, for each age group or gene analyzed (Table III). For the *ATM* gene three cases showed methylation positivity for the group with ≤50 years, and two for >50 years old (Table III). For details of the histological types of hypermethylated cases shown in Tables II and III and SI.

The hypermethylation present in the group of malignant cases (blood DNA from cases with impalpable breast lesions) was higher than that found in the group of women without lesions (Tables II and III). From this association between groups, the promoters of *ATM* and *$p16^{INK4a}$* presented significant P-values of 0.001 and 0.009, respectively (Table IV). When comparing the same group of malignant cases with their respective cfDNA, only hypermethylation of the *$p16^{INK4a}$* promoter showed a significant P-value of 0.003 (Table V).

Nine cases had a methylation correlation between blood DNA and the respective cfDNA: Among these cases, 7/9 (77%), 4/9 (44%), 2/9 (22%), and 1/9 (11%) for *ATM*, *$p14^{ARF}$*, *$p14^{ARF}$/ATM*, and *$p16^{INK4a}$*, respectively. For clinical and histopathological details (Table SII).

The individual correlations (benign, malignant lesions, cfDNA of benign cases, cfDNA of malignant cases, and control subjects) between the methylation of each promoter (*ATM*, *$p14^{ARF}$*, and *$p16^{INK4a}$*) and age (ROC curve) did not reveal statistically significant values. For all analyses, P-value was >0.05 (data not shown).

Discussion

In the present study, we describe epigenetic changes occurring in liquid biopsies from women with impalpable breast lesions, compared to a control cohort of women without lesions. The women with impalpable breast lesions (benign and malignant lesions) had the highest methylation rate, regardless of age, compared to the cfDNA and control groups (Tables II and III). This change was statistically significant for the promoters of *ATM* (P=0.009) and *$p16^{INK4a}$*

(P=0.001) (P=0.003) (impalpable breast lesions vs. control and cfDNA) (Tables IV and V).

In our previous study (3), the methylation rates in 39 blood DNA samples from women with impalpable breast lesions were similar to those found here: Frequencies for the *ATM*, *p14^{ARF}* and *p16^{INK4a}* genes in the previous study were 41, 26, and 41%, respectively, whereas in the current study frequencies were 37.5, 27 and 48% (Table II), respectively. Both studies show the *ATM* and *p16^{INK4a}* genes with high hypermethylation rate in malignant cases, suggesting the silencing in the repair pathways, senescence, and cell cycle control in the impalpable breast lesions establishment.

The *ATM* gene involvement in mammary carcinogenesis has been described by several studies, but presented with controversies (3,19,20,25,26). In the study by Cao *et al* (19) the authors evaluated more than 30 CpG islands of *ATM* gene (using MassARRAY Epithelial Assay and Infinium HumanMethylation450 BeadChip array) in peripheral blood from women with breast lesions, similar to those analyzed here, and reported 62% of mammary tumor stage I/II (*N*=229) (78% IDC, 69% ER positive, 63% PR positive, and 72% HER negative); interestingly, the authors did not find any significant difference in the *ATM* methylation levels between the breast cancer patients and the healthy controls. Brennan *et al* (20), evaluating *ATM* intragenic regions (ATMmvp2a and ATMmvp2b) in sporadic breast cancer cases (*N*=501), familial breast cancer cases (*N*=166), and controls (*N*=769), found a strong association of *ATM* methylation levels in the family group ($P=4.87 \times 10^{-6}$), and also in cases up to 59 years old (P=0.01). In relation to the young and familiar cases, it is not possible to compare with the data described here, because we analyzed sporadic breast cancer and the number of women under 50 years old is small, making statistical analysis unfeasible.

Regarding *ATM* hypermethylation in breast tissue, although not evaluated in this study, methylation rates have been described ranging from 36 to 78% (3,25,39). In the study by Begam *et al* (26) the frequency of methylation in sporadic mammary tumors was 59%, while in adjacent non-tumor tissue 4%. Further, the authors found association between promoter hypermethylation and lower *ATM* mRNA expression (P=0.035). For malignant and benign impalpable lesions, we found proximal frequencies of 63.4 and 33.3%, respectively, in our previous study (3).

In the study by Askari *et al* (22) *p14^{ARF}* and *p16^{INK4a}* hypermethylation in blood from women with breast cancer was 11 and 22%, respectively. Further, the authors found a significant association between hypermethylation for *p14^{ARF}* (P=0.004) and *p16^{INK4a}* (P=0.000) in women over 50 years old. The hypermethylation found in our study was superior, with 27 and 48% of methylated cases for *p14^{ARF}* and *p16^{INK4a}*, respectively. In addition, the significant association between hypermethylation of *p16^{INK4a}* and *ATM* was revealed in women over 50 years old (Tables IV and V). These data show that hypermethylation of *p14^{ARF}* and *p16^{INK4a}* promoters demonstrate significant association with breast cancer, hence indicating involvement in the breast tumor pathogenesis.

The methylation findings found in cfDNA in our study should be interpreted with caution. Despite the presence of circulating tumor DNA (ctDNA) in the bloodstream and metastases in the bone marrow in early cases (11,12), the concentration of cfDNA in these cases is lower than in metastatic cases (42). Hypothetically, in malignant cases, methylation in blood DNA may be a result of CTCs. In this study we found nine cases with cfDNA that coincided with the methylation pattern of the WBCs. Of these cases, seven were from women with malignant lesions, and only one with lymph node infiltration (Table SII). In this context, it is not possible to affirm that the hypermethylation found in these cases originated from the CTCs.

The *p16^{INK4a}* gene has been reported methylated in the cfDNA of mammary tumors at a rate of 22% (23). In our study, this rate was 10% lower. In the study by Shan *et al* (23) the *p16^{INK4A}* methylation in cfDNA associated with five other genes (*SFN*, *hMLH1*, *HOXD13*, *PCDHGB7* and *RASSF1a*) reached sensitivity (79.6-72.4%) and specificity (82.4-78.1%) for the distinction of initial malignant lesions (*N*=268) of controls (*N*=245) and benign lesions (*N*=236), respectively. However, the authors emphasized the importance of study expansion, since the methylation found in the genes was associated with cases with a family history (P=0.0249), low proliferation index (Ki67) (P=0.0356), and luminal tumors (P=0.0314). These data corroborate with those presented here, since both studies used similar populations (Table I) except for cases with a family history.

Further, the evaluation of methylation through high technology platforms (MassARRAY Epityper assay, Illumina Infinium array, Infinium HumanMethylation450 BeadChip array, and Pyrosequencing) has shown higher sensitivity values (20,23,25,31,32,35), when compared to other conventional methods, as performed here. Thus, larger multicenter prospective study cohorts are needed to validate the findings here.

To our knowledge this is the first study that evaluates the methylation positivity in the promoters of the *ATM* and *CDKN2A* genes in liquid biopsies from women with impalpable breast lesions compared to women without lesions. This study is in progress and further analyses should be performed for molecular description of the factors involved with the development of impalpable lesions. Although methylation levels may be associated with environmental (43) and social (44,45) factors, the percentages disclosed here and by other studies (17,18,20,22,23,25) show that methylation levels of WBC are high in the presence of breast lesions.

In conclusion, we found high rates of methylation in blood from women with benign and malignant breast lesions. Regardless of nature, the breast lesions presence is capable of promoting epigenetic responses in liquid biopsies. The alterations detected here represent the systematic heterogeneity of every woman in front of the impalpable breast lesion installation. We believe that epigenetic changes in liquid biopsy may reveal potential biological biomarkers capable of complement biopsy results and predict the risk of lesion invasion or tumor response to treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LD, MHFO and GA conceived and designed the study. LD, RJG, PDOV and ABPDS conducted the experiments. LD, MASMC, MVF, CMDA and GA recruited the cases, acquired pathological and radiological reports, and analyzed and interpreted the data. LD, LRS, MVF and GA obtained statistical data, and analyzed and interpreted data. LD, MVF, MHFO and GA were involved in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final version.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of the Hospital Universitário Gafrée-Guinle (HUGG) and Rio de Janeiro State University Hospital (approval nos. H UGG-07/2007-80/2012 and CAAE:43560115.5.0000.5259). The subjects enrolled in the present study signed the informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hortobagyi GN, de la Garza SJ, Pritchard K, Amadori D, Haidinger R, Hudis CA, Khaled H, Liu MC Martin M, Namer M, *et al*: The global breast cancer burden: Variations in epidemiology and survival. Clin Breast Cancer 6: 391-401, 2005.
2. Kohler BA, Sherman RL, Howlader N, Jemal A, Ryerson AB, Henry KA, Boscoe FP, Cronin KA, Lake A, Noone AM, *et al*: Annual report to the nation on the status of cancer, 1975-2011, featuring incidence of breast cancer subtypes by race/ethnicity,poverty and state. J Natl Cancer Inst 107: djv048, 2015.
3. Delmonico L, Moreira Ados S, Franco MF, Esteves EB, Scherrer L, Gallo CV, do Nascimento CM, Ornellas MH, de Azevedo CM and Alves G: CDKN2A (p14(ARF)/p16(INK4a)) and ATM promoter methylation in patients with impalpable breast lesions. Hum Pathol 46: 1540-1547, 2015.
4. Delmonico L, Costa MASM, Fournier MV, Romano SO, Nascimento CMD, Barbosa AS, Moreira ADS, Scherrer LR, Ornellas MHF and Alves G: Mutation profiling in the PIK3CA, TP53, and CDKN2A genes in circulating free DNA and impalpable breast lesions. Ann Diagn Pathol 39: 30-35, 2019.
5. Cady B, Stone MD, Schuler JG, Thakur R, Wanner MA and Lavin PT: The new era in breast cancer: Invasion, size, and nodal involvement dramatically decreasing as a result of mammographic screening. Arch Surg 131: 301-308, 1996.
6. Peters NH, Borel Rinkes IH, Mali WP, van den Bosch MA, Storm RK, Plaisier PW, de Boer E, van Overbeeke AJ and Peeters PH: Breast MRI in nonpalpable breast lesions: A randomized trial with diagnostic and therapeutic outcome-MONET-study. Trials 8: 40, 2007.
7. Ribelles N, Perez-Villa L, Jerez JM, Pajares B, Vicioso L, Jimenez B, de Luque V, Franco L, Gallego E, Marquez A, *et al*: Pattern of recurrence of early breast cancer is different according to intrinsic subtype and proliferative index. Breast Cancer Res 15: R98, 2013.
8. Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans V, Godwin J, Gray R, Hicks C, James S, *et al*: Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15 year survival: An overview of the randomised trials. Lancet 366: 2087-2106, 2005.
9. Gomis RR and Gawrzak S: Tumor cell dormancy. Mol Oncol 11: 62-78, 2017.
10. Naume B, Borgen E, Beiske K, Herstad TK, Ravnås G, Renolen A, Trachsel S, Thrane-Steen K, Funderud S and Kvalheim G: Immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood. J Hematother 6: 103-114, 1997.
11. Krishnamurthy S, Cristofanilli M, Singh B, Reuben J, Gao H, Cohen EN, Andreopoulou E, Hall CS, Lodhi A, Jackson S and Lucci A: Detection of minimal residual disease in blood and bone marrow in early stage breast cancer. Cancer 116: 3330-3337, 2010.
12. Bidard FC, Mathiot C, Delaloge S, Brain E, Giachetti S, de Cremoux P, Marty M and Pierga JY: Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer. Ann Oncol 21: 729-733, 2010.
13. Hall CS, Karhade MG, Bowman Bauldry JB, Valad LM, Kuerer HM, DeSnyder SM and Lucci A: Prognostic value of circulating tumor cells identified before surgical resection in nonmetastatic breast cancer patients. J Am Coll Surg 223: 20-29, 2016.
14. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
15. Parashar S, Cheishvili D, Mahmood N, Arakelian A, Tanvir I, Khan HA, Kremer R, Mihalcioiu C, Szyf M and Rabbani SA: DNA methylation signatures of breast cancer in peripheral T-cells. BMC Cancer 18: 574, 2018.
16. Guan Z, Yu H, Cuk K, Zhang Y and Brenner H: Whole-Blood DNA methylation markers in early detection of breast cancer: A systematic literature review. Cancer Epidemiol Biomarkers Prev 28: 496-505, 2019.
17. Tang Q, Cheng J, Cao X, Surowy H and Burwinkel B: Blood-based DNA methylation as biomarker for breast cancer: A systematic review. Clin Epigenetics 8: 115, 2016.
18. Sturgeon SR, Pilsner JR, Arcaro KF, Ikuma K, Wu H, Kim SM, Chopra-Tandon N, Karpf AR, Ziegler RG, Schairer C, *et al*: White blood cell DNA methylation and risk of breast cancer in the prostate, lung, colorectal, and ovarian cancer screening (PLCO). Breast Cancer Res 19: 94, 2017.
19. Cao X, Tang Q, Holland-Letz T, Gündert M, Cuk K, Schott S, Heil J, Golatta M, Sohn C, Schneeweiss A and Burwinkel B: Evaluation of promoter methylation of RASSF1A and ATM in peripheral blood of breast cancer patients and healthy control individuals. Int J Mol Sci 19: E900, 2018.
20. Brennan K, Garcia-Closas M, Orr N, Fletcher O, Jones M, Ashworth A, Swerdlow A, Thorne H; KConFab Investigators: Intragenic ATM methylation in peripheral blood DNA as a biomarker of breast cancer risk. Cancer Res 72: 2304-2313, 2012.
21. Xu Z, Bolick SC, DeRoo LA, Weinberg CR, Sandler DP and Taylor JA: Epigenome-wide association study of breast cancer using prospectively collected sister study samples. J Natl Cancer Inst 105: 694-700, 2013.
22. Askari M, Sobti RC, Nikbakht M and Sharma SC: Promoter hypermethylation of tumor suppressor genes (p14/ARF and p16/INK4a): Case control study in north Indian population. Mol Biol Rep 40: 4921-4928, 2013.
23. Shan M, Yin H, Li J, Li X, Wang D, Su Y, Niu M, Zhong Z, Wang J, Zhang X, *et al*: Detection of aberrant methylation of a six-gene panel in serum DNA for diagnosis of breast cancer. Oncotarget 7: 18485-18494, 2016.
24. Ahmed M and Rahman N: ATM and breast cancer susceptibility. Oncogene 25: 5906-5911, 2006.
25. Flanagan JM, Munoz-Alegre M, Henderson S, Tang T, Sun P, Johnson N, Fletcher O, Dos Santos Silva I, Peto J, Boshoff C, *et al*: Gene-Body hypermethylation ATM in peripheral blood DNA of bilateral breast cancer patients. Hum Mol Genet 18: 1332-1342, 2009.
26. Begam N, Jamil K and Raju SG: Promoter hypermethylation of the ATM gene as a novel biomarker for breast cancer. Asian Pac J Cancer Prev 18: 3003-3009, 2017.

27. Majchrzak-Celinska A, Paluszczak J, Szalata M, Barciszewska AM, Nowak S, Kleszcz R, Sherba A and Baer-Dubowska W: The methylation of a panel of genes differentiates low-grade from high-grade gliomas. Tumour Biol 36: 3831-3841, 2015.
28. Huang Q, Su X, Ai L, Li M, Fan CY and Weiss LM: Promoter hypermethylation of multiple genes in gastric lymphoma. Leuk Lymphoma 48: 1988-1996, 2007.
29. Bai AH, Tong JH, To KF, Chan MW, Man EP, Lo KW, Lee JF, Sung JJ and Leung WK: Promoter hypermethylation of tumor-related genes in the progression of colorectal neoplasia. Int J Cancer 112: 846-853, 2004.
30. Serrano M, Hannon GJ and Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366: 704-707, 1993.
31. Spitzwieser M, Entfellner E, Werner B, Pulverer W, Pfeiler G, Hacker S and Cichna-Markl M: Hypermethylation of CDKN2A exon 2 in tumor, tumor-adjacent and tumor-distant tissues from breast cancer patients. BMC Cancer 17: 260, 2017.
32. Wijetunga NA, Belbin TJ, Burk RD, Whitney K, Abadi M, Greally JM, Einstein MH and Schlecht NF: Novel epigenetic changes in CDKN2A are associated with progression of cervical intraepithelial neoplasia. Gynecol Oncol 142: 566-573, 2016.
33. Alhejaily A, Day AG, Feilotter HE, Baetz T and Lebrun DP: Inactivation of the CDKN2 tumor-suppressor gene by deletion or methylation is common at diagnosis in follicular lymphoma and associated with poor clinical outcome. Clin Cancer Res 20: 1676-1686, 2014.
34. Wang BH, Li YY, Han JZ, Zhou LY, Lv YQ, Zhang HL and Zhao L: Gene methylation as a powerful biomarker for detection and screening of non-small cell lung cancer in blood. Oncotarget 8: 31692-31704, 2017.
35. Ding K, Chen X, Wang Y, Liu H, Song W, Li L, Wang G, Song J, Shao Z and Fu R: Plasma DNA methylation of p16 and shp1 in patients with B cell non-Hodgkin lymphoma. Int J Clin Oncol 22: 585-592, 2017.
36. Delmonico L, Areias VR, Pinto RC, Matos Cda S, Rosa MF, De Azevedo CM and Alves G: Protein identification from dried nipple aspirate fluid on guthrie cards using mass spectrometry. Mol Med Rep 12: 159-164, 2015.
37. Lakhani SR, Ellis IO, Schnitt SJ, Tan PH and van de Vijver MJ: WHO Classification of Tumours of the Breast. 4th edition. IARC Press, Lyon, p240, 2012.
38. Elston CW and Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. Histopathology 19: 403-410, 1991.
39. Herman JG, Graff JR, Myöhänen S, Nelkin BD and Baylin SB: Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
40. Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB and Herman JG: Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res 60: 129-133, 2000.
41. Vo QN, Kim WJ, Cvitanovic L, Boudreau DA, Ginzinger DG and Brown KD: The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene 23: 9432-9437, 2004.
42. Heitzer E, Haque IS, Roberts CES and Speicher MR: Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet 20: 71-88, 2019.
43. Herceg Z, Ghantous A, Wild CP, Sklias A, Casati L, Duthie SJ, Fry R, Issa JP, Kellermayer R, Koturbash I, *et al*: Roadmap for investigating epigenome deregulation and environmental of cancer. Int J Cancer 142: 874-882, 2018.
44. Ambatipudi S, Cuenin C, Hernandez-Vargas H, Ghantous A, Le Calvez-Kelm F, Kaaks R, Barrdahl M, Boeing H Aleksandrova K, Trichopoulou A, *et al*: Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. Epigenomics 8: 599-618, 2016.
45. Weihrauch-Blüher S, Richter M and Staege MS: Body weight regulation, socioeconomic status and epigenetic alterations. Metabolism 85: 109-115, 2018.