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Original Article

Immunohistochemical, genetic and epigenetic profiles of hereditary and triple negative breast cancers. Relevance in personalized medicine

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Abstract: This study aims to identify the profile of immunohistochemical (IHC) parameters, copy number aberrations (CNAs) and epigenetic alterations [promoter methylation (PM) and miR expression] related to hereditary (H) and triple negative (TN) breast cancer (BC). This profile could be of relevance for guiding tumor response to treatment with targeting therapy. The study comprises 278 formalin fixed paraffin-embedded BCs divided into two groups: H group, including 88 hereditary BC (HBC) and 190 non hereditary (NHBC), and TN group, containing 79 TNBC and 187 non TNBC (NTNBC). We assessed IHC parameters (Ki67, ER, PR, HER2, CK5/6, CK18 and Cadherin-E), CNA of 20 BC related genes, and PM of 24 tumor suppressor genes employing MLPA/MS-MLPA (MRC Holland, Amsterdam). MiR-4417, miR-423-3p, miR-590-5p and miR-187-3p expression was assessed by quantitative RT-PCR (Applied Biosystems). Binary logistic regression was applied to select the parameters that better differentiate the HBC or TN groups. For HBC we found that, ER expression, *ERBB2* CNA and PM in *RASSF1* and *TIMP3* were associated with NHBC whereas; *MYC* and *AURKA* CNA were linked to HBC. For TNBC, we found that *CDC6* CNA, *GSTP1* and *RASSF1* PM and miR-423-3p hyperexpression were characteristic of NTNBC, while *MYC* aberrations, *BRCA1* hypermethylation and miR-590-5p and miR-4417 hyperexpression were more indicative of TNBC. The selected markers allow establishing BC subtypes, which are characterized by showing similar etiopathogenetic mechanisms, some of them being molecular targets for known drugs or possible molecular targets. These results could be the basis to implement a personalized therapy.

Keywords: Sporadic breast cancer, hereditary breast cancer, miR expression profile, BRCA1, BRCA2, mutations, molecular markers

Introduction

Every year about one million women worldwide are diagnosed with breast cancer (BC) [1], a heterogeneous disease that includes distinct biological entities associated with specific pathological features and clinical evolution. In 5-10% of BCs, the disease occurs as part of a hereditary cancer susceptibility syndrome [2].

A substantial proportion of hereditary breast and ovarian cancers (HBC/HOC) can be attributed to mutations in *BRCA1* [3] or *BRCA2* genes [4], representing 16-25% of high risk familial BCs [5, 6]. Women with triple negative breast cancer (TNBC), represent 15-20% of all BCs [7], and are approximately five and a half times more likely to have *BRCA1* mutations compared to non-TNBC (NTNBC). Moreover, approximately two out of nine women with TNBC harbor *BRCA1* mutations [8].

Somatic acquired copy number aberrations (CNAs) are an important mechanism for onco-

Table 1. Anatomo-clinical parameters in breast cancer samples

Features		n
Age	Mean (SD) n	
	49.28 (12.02) 278	
T	<2 cm	143
	>2 cm	117
N	0	160
	1	94
M	0	146
	1	5
	1	45
GH	2	85
	3	113
Histopathology	CDI	229
	CLI	19
	In situ	8
	Other	14
Hereditary	HBC	88
	NHBC	190
TN	TNBC	79
	NTNBC	187

T: Tumor stage; N: Node involvement (N0: Absence; N1: Presence); M: Metastasis (M0: Absence; M1: Presence); HG: Histological grade (1: Differentiated; 2: Medium differentiation; 3: Undifferentiated); TN: Triple negative; SD: Standard deviation.

gene activation, a crucial step in carcinogenesis [9]. Furthermore epigenetic alterations such as promoter methylation (PM) and miR expression are known to have a key role in the altered gene expression profiles found in all human cancers, playing a relevant role in carcinogenesis and disease progression [10]. These alterations may act as modifiers of carcinogenesis affecting proliferation pathways, DNA repair mechanisms and cell cycle control.

One of the most recurrent CNAs are *AURKA* aberrations, detected in more than 12% of BCs [11] and has emerged as a great prognostic marker [12] conferring sensitivity to the PARP [13] and AURKA inhibitors [11]. In addition, *MYC* amplification has been detected in 14.6% of BCs [14, 15] being consistently observed in aggressive forms of the disease and is correlated with poor prognosis and distant metastases [16]. Also, amplification and overexpression of human epidermal growth factor receptor type 2 (HER2) occurs in 20 to 30% of invasive BCs. In general, patients with BC cells overexpressing this receptor or carrying a high copy number of this gene have decreased overall survival and may have differential responses to chemotherapy and targeted monoclonal antibodies [17].

Altered DNA methylation of CpG islands is known to play a key role in the altered gene expression patterns in all human cancers. Thus, a DNA methylation study performed in 70 candidate gene loci in 140 BCs and matched normal tissues revealed six DNA methylation patterns in breast tumors relative to matched normal tissue [18]. Methylation profiles are associated with BC immunohistochemical features [19-22], being able to differentiate new BC subtypes, not previously identified by conventional immunohistochemistry (IHC) [23]. Promoter hypermethylation of *APC*, *ATM*, *CDH13*, *GSTP1* and *RASSF1* have been frequently detected in SBC being absent in normal tissues [20, 24]. In addition, *RASSF1* methylation is related to tumor size and associated with ER+ and PR+ BCs [22]. The *BRCA1* silencing caused by its promoter hypermethylation supports the role of this gene in breast and ovarian tumorigenesis [25].

MiRNAs expression arrays in BC have identified specific patterns associated with the expression of HER2 and ER [26]. MiR are expressed in a tissue-specific manner and changes in miR expression within a tissue type can be correlated with disease status [27, 28]. It has also been found that the expression profile of miRNA enables differentiation of luminal A, luminal B, basal-like, HER2 BCs and normal breast [26]. The potentiality shown by miRNAs profiles opens up the possibility to detect the tumors with defects in homologous recombination.

But leaving aside SBC and HBC, little is known about TNBC, a heterogeneous and aggressive disease with different molecular subtypes, in which the lack of known biomarkers limit the development of therapeutic strategies for the disease [29].

The relevance of CNAs and epigenetic alterations, promoter methylations and miR expression, in the etiopathogenesis has been poorly studied in BC [9, 24, 30, 31], and only somewhat for Hereditary and TN BCs.

The aim of the present study is to identify the patterns of IHC parameters and genetic and epigenetic alterations linked to the etiopatho-

genic mechanisms and cancer progression in Hereditary and TN BCs. These patterns could be of great relevance to guide tumor response to treatment with anti-neoplastic agents or targeting therapy, which could support the basis for a personalized medicine.

Materials and methods

Patients

The study includes 278 formalin fixed paraffin-embedded (FFPE) BCs, 88 hereditary BCs (HBC) and 190 non-hereditary BCs (NHBC). Moreover, we considered 79 TNBC and 187 NTNBC. The pathological characteristics and histopathology of patients included are summarized in **Table 1**.

All the samples were assessed for IHC markers, PM, CNA and four microRNA expressions (miR-4417, miR-423-3p, miR-590-5p and miR-187-3p) [32].

Patients signed the informed consent elaborated by the Health Department following the recommendations of the Declaration of Human Rights, the Conference of Helsinki (http://www.wma.net/e/policy/pdf/17c.pdf).

Study of BRCA1 and BRCA2 mutations

BRCA1/2 mutation status in HBOC patients was assessed on genomic DNA extracted from peripheral blood. The entire BRCA1/2 exons and exon-intron boundaries were amplified by PCR using primer pairs and PCR conditions reported in the Breast Cancer Information Core (http://research.nhgri.nih.gov/bic/Member/index.shtml). Mutational screening was carried out by pre-screening the heteroduplex formed in the PCR products by conformation sensitive gel electrophoresis [33] followed by direct sequencing of the PCR products in which heteroduplexes were identified [34].

Immunohistochemistry

IHC analyses were performed using tissue microarray. The slides were immunostained using primary antibodies against Ki67, ER, PR, HER2, CK5/6, CK18 and Cadherin-E (DAKO, Denmark). ER and PR expression were evaluated according to the Allred scoring system [35]. HER2 expression was scored according to HercepTest criteria. In 2+ HER2 expression, fluorescent *in situ* hybridization was performed [36]. For Cadherin-E expression, the same criteria were applied as for HER2 [37]. Ki67 expression was evaluated according to the St. Gallen International Expert Consensus [38, 39]. For CK5/6 and CK18 cytoplasmatic expression, the cut off score was 5% of cells. Finally, in order to define invasive BC subtypes we followed the St. Gallen International Expert Consensus [38, 40].

Molecular studies

Two selected areas from FFPE were deparaffinized using Deparaffinization Solution (Quiagen). DNA was isolated using QuiAmp DNA Investigation Kit (Quiagen) and total RNA using Recover All TM Total Nucleic Acid Isolation Kit (Applied Biosystems, Ambion) according to manufacturer's protocols.

We detect CNA by employing the multiple ligation probes amplification (MLPA) technique with P078B1 Breast Tumor Kit [41] (MRC Holland, Amsterdam). This kit includes probes to detect CNA of *ERBB2, BIRC5, MYC, TOP2A, ESR1, MTDH, CCND1, CCNE1, EGFR, C11orf30 (EMSY), ADAM9, IKBKB, CDH1, CDC6, CPD, FGFR1, MED1, MAPT, PRMD14* and *AURKA*.

Methylation studies were performed using the methylation-specific (MS) MLPA technique [42]. We used ME001 Tumor Suppressor Mix 1 Kit [43] (MRC Holland, Amsterdam) which contains probes to detect methylation in promoter regions of tumor suppressor genes (*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*).

Amplicons generated on MLPA and MS-MLPA were separated by capillary electrophoresis on AB3130 Capillary Sequencer (Applied Biosystems), fragment analysis was performed using Coffalyser.net software (MRC Holland, Amsterdam, The Netherlands) and the results were evaluated as previously reported [42, 44].

Expression of miR-4417, miR-423-3p, miR-590-5p and miR-187-3p was assessed by quantitative RT-PCR (qRT-PCR) using the TaqMan miRNA Reverse Transcription kit, TaqMan miRNA Assays (specific for each miR)

Table 2. Anatomo-clinical features in HBC/NHBC and TNBC/NTNBC

Features		HBC	NHBC	χ^2	TNBC	NTNBC	χ^2
		n (%)	n (%)	*P*	n (%)	n (%)	*P*
Age [Mean (SD) n]		47.5 (11.2) 88	50.1 (12.3) 190	n.s.	47.5 (11.6) 79	49.8 (11.9) 187	ns
T	<2 cm	42 (51)	101 (57)	n.s.	33 (44)	110 (61)	0.014
	>2 cm	40 (49)	77 (43)		42 (56)	71 (39)	
N	0	47 (59)	113 (69)	n.s.	49 (69)	109 (61)	n.s.
	1	33 (41)	61 (35)		70 (39)	22 (31)	
M	0	56 (98)	90 (96)	n.s.	41 (98)	102 (96)	n.s.
	1	1 (2)	4 (4)		1 (2)	4 (4)	
HG	1	10 (14)	35 (21)	0.04	3 (4)	41 (24)	<0.001
	2	20 (27)	65 (38)		16 (22)	68 (40)	
	3	43 (59)	70 (41)		52 (73)	60 (35)	

T: Tumor stage; N: Node involvement (N0: Absence; N1: Presence); M: Metastasis (M0: Absence; M1: Presence); HG: Histological grade (1: Differentiated; 2: Medium differentiation; 3: Undifferentiated); TN: Triple negative; SD: Standard deviation; Groups are compared using the mean comparison test t student in the case of age and proportion comparison test χ^2 in the other parameters; n.s.: Not significant.

Table 3. Immunohistochemical parameters in HBC/NHBC and TNBC/NTNBC

IHC Marker	HBC	NHBC	χ^2	TNBC	NTNBC	χ^2
	Pos (%) n	Pos (%) n	*P*	Pos (%) n	Pos (%) n	*P*
ER	44 (50) 88	133 (70) 190	0.001	-	168 (90) 187	-
PR	33 (38) 88	114 (61) 188	<0.001	-	142 (76) 187	-
HER2	5 (6) 88	34 (18) 188	0.003	-	37 (20) 187	-
Ki67	47 (53) 88	71 (39) 183	0.015	54 (68) 79	63 (34) 183	<0.001
CK5/6	22 (25) 87	32 (18) 178	n.s.	37 (47) 79	17 (10) 179	<0.001
CK18	75 (94) 80	159 (93) 171	n.s.	64 (82) 78	163 (98) 166	<0.001
Cadherin-E	79 (95) 83	168 (96) 176	n.s.	77 (100) 77	163 (93) 175	0.02

IHC: immunohistochemical; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Erythroblasticleukaemia viral oncogene homolog 2 receptor; Pos: number of positive cases for the expression of each immunohistochemical marker. In Ki67 indicates the number of cases with high proliferation index; n: total number of cases; n.s.: Not significant. *p*: χ^2 *p*-value associated to the differences between groups.

and TaqMan Universal PCR Master Mix (Applied Biosystems) [32]. Small nucleolar RNA U44 was used for normalization and relative expression was calculated using the ΔΔCT (Delta-Delta CT) method [45].

Statistical analysis

Categorical data (TNM, IHC and molecular results of CNA and methylation) were expressed in percentages and chi-square was applied to compare the proportions between the study groups. The quantitative data (miR expression) was summarized by their mean and standard deviation and univariated ANOVA was applied to compare the means between the established groups.

Multivariate binary logistic regression with stepwise backwards Wald option was applied to compare categorical data and quantitative covariates between the study groups, selecting those independent variables most strongly linked with the group. We applied the multifactorial ANOVA to analyze the influence of the factors on the variability of quantitative parameters. All the analyses were performed using SPSS ver. 20 package.

Results

Anatomo-clinical parameters in hereditary and triple negative breast cancers

HBC show higher prevalence of tumors with advanced histological grade (HG) in comparison to NHBC (*P*=0.04, **Table 2**). TNBC present advanced HG and, in addition, TNBC are usually large tumors (>2 cm) in comparison to NTNBC (**Table 2**).

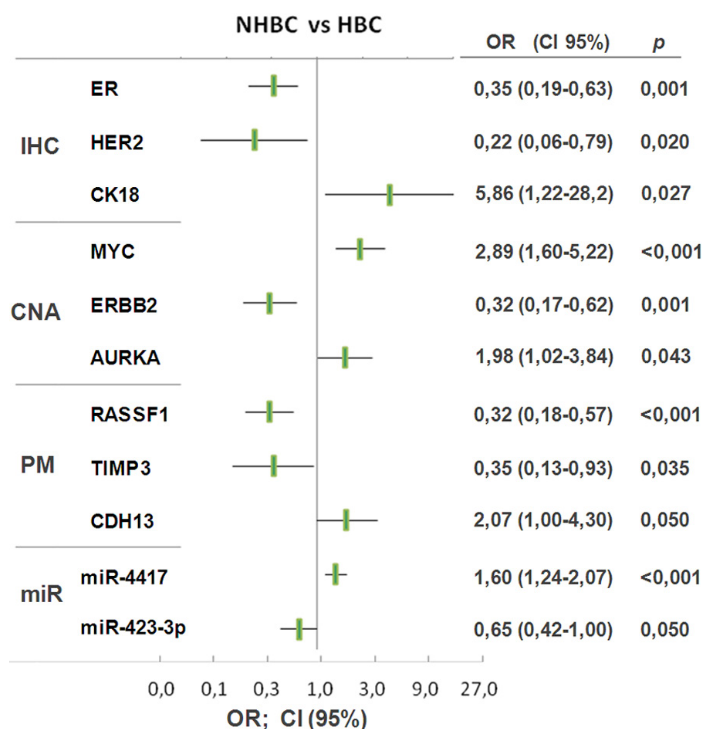

Figure 1. Immunohistochemical, CNA, PM and miR expression profiles for the best differentiation of HBC and NHBC. Binary stepwise backward Wald logistic regression. IHC: immunohistochemical markers; CNA: Copy number aberration; PM: Promoter methylation; NHBC: No hereditary breast cancer; HBC: Hereditary breast cancer.

Immunohistochemical parameters in hereditary and triple negative breast cancers

HBC show a lower proportion of ER, PR and HER2 positivity than NHBC, and a larger proportion of Ki67 (**Table 3**). However, TNBC are characterized by showing a greater proportion of Ki67, CK5/6 and Cadherin-E but a lower proportion of CK18 in comparison with NTNBC (**Table 3**).

The binary stepwise backward Wald logistic regression selects the IHC parameters HER2, ER and CK18 as the ones that better differentiate HBC from NHBC (**Figure 1**). The largest proportion of HER2 and ER positive tumors are presented in NHBC while the higher proportion of CK18 positive are related to HBC.

Copy number aberrations in hereditary and triple negative breast cancers

HBC present the greatest proportion of aberrations, in *AURKA* (mainly losses), and *MYC* (gains), while NHBC show aberrations, mostly gains, in *ERBB2* and *MED1* (**Table 4**). TNBC present higher incidence of gains in *MYC*, *BIRC5* and *MTDH*, however, NTNBC show higher proportions of gains in *CDC6* and *MED1* (**Table 4**).

The binary stepwise backward Wald logistic regression selects *AURKA*, *ERBB2* and *MYC* as genes that better differentiate HBC and NHBC (**Figure 1**). *AURKA* and *MYC* aberrations are linked to HBC while *ERBB2* aberrations are related to NHBC. The TNBC are more prone to show aberrations on *CCNE1* and *MYC,* while the aberrations in *CCND1* and *CDC6* are more likely in NTNBC (**Figure 2**).

Promoter methylation in hereditary and triple negative breast cancers

NHBC show a higher prevalence of methylated *APC* and *RASSF1* than the HBC (**Table 5**). TNBC present a greater proportion of methylation in *BRCA1* and *ESR1* than in NTNBC, while in this latter group the highest prevalence of methylation occurs in *DAPK1*, *GSTP1*, *HIC1* and *RASSF1* (**Table 5**).

The binary stepwise backward Wald logistic regression selects the profile of *CDH13*, *TIMP3* and *RASSF1* methylated genes as those that better differentiate HBC and NHBC (**Figure 1**). *CDH13* methylated is more prevalent in HBC, whereas *RASSF1* and *TIMP3* methylated show a higher prevalence in NHBC. Regarding the TNBC, the logistic regression selects the methylation profile of *BRCA1*, *CHFR*, *DAPK1*, *GSTP1*, *IGSF4*, *RARB* and *RASSF1* as the methylated genes that better differentiate TNBC and NTNBC. *BRCA1*, *RARB* and *IGSF4* methylated are strongly associated with TNBC whereas *CHFR*, *DAPK1*, *GSTP1* and *RASSF1* methylated are more linked with NTNBC (**Figure 2**).

MiR expression and IHC parameters

The four miR studied here are strongly related with ER status. MiR-187-3p, miR-590-5p and miR-4417 are overexpressed in ER negative BCs, whereas miR-423-3p is overexpressed in ER positive BCs. We also observed that miR-4417 and miR-423-3p are related to PR status; the first is overexpressed in PR negative BCs while the second is overexpressed in PR positive BCs (**Table 6**).

Table 4. Prevalence of genetic alterations (CNAs) between HBC/NHBC and TNBC/NTNBC

GENES	CNA	HBC (n=81)	NHBC (n=172)	χ^2	TNBC (n=75)	NTNBC (n=174)	χ^2
		n (%)	n (%)	*P*	n (%)	n (%)	*P*
ADAM9	Gain	15 (18)	37 (22)	n.s.	14 (19)	37 (21)	n.s.
	Loss	8 (10)	10 (6)		6 (8)	12 (7)	
AURKA	Gain	13 (16)	27 (16)	0.001	8 (11)	25 (14)	n.s.
	Loss	14 (17)	7 (4)		11 (15)	10 (6)	
BIRC5	Gain	38 (47)	62 (36)	n.s.	39 (52)	61 (35)	0.05
	Loss	0 (0)	6 (4)		0 (0)	6 (3)	
CCND1	Gain	29 (36)	60 (35)	n.s.	24(32)	65 (37)	n.s.
	Loss	5 (6)	13 (8)		3 (4)	15 (9)	
CCNE1	Gain	22 (13)	14 (17)	n.s.	16 (21)	20(12)	n.s.
	Loss	1 (1)	1 (1)		0 (0)	2 (1)	
CDC6	Gain	17 (21)	50 (29)	n.s.	12 (16)	55 (32)	0.005
	Loss	1 (1)	6 (4)		1 (1)	6 (3)	
CDH1	Gain	14 (17)	28 (16)	n.s.	17 (23)	25 (14)	n.s.
	Loss	4 (5)	9 (5)		3 (4)	10 (6)	
CPD	Gain	10 (12)	32 (19)	n.s.	8 (11)	33 (19)	n.s.
	Loss	4 (5)	5 (3)		3 (4)	6 (3)	
EGFR	Gain	6 (7)	19 (11)	n.s.	13 (18)	12(7)	n.s.
	Loss	6 (7)	6 (4)		2 (3)	10 (6)	
EMSY	Gain	17 (21)	41 (24)	n.s.	19 (25)	38 (22)	n.s.
	Loss	5 (6)	19 (11)		7 (9)	17 (10)	
ERBB2	Gain	16 (20)	62 (36)	0.002	18 (24)	60 (35)	n.s.
	Loss	2 (3)	10 (6)		3 (4)	9 (5)	
ESR1	Gain	9 (11)	14 (8)	n.s.	9 (12)	14 (8)	n.s.
	Loss	0 (0)	1 (1)		1 (1)	0 (0)	
FGFR1	Gain	18 (22)	47 (27)	n.s.	19 (25)	46 (26)	n.s.
	Loss	3 (4)	11 (7)		5 (7)	9 (5)	
IKBKB	Gain	19 (24)	43 (25)	n.s.	24 (32)	38 (22)	n.s.
	Loss	1 (1)	9 (5)		4 (5)	6 (3)	
MAPT	Gain	22 (27)	45 (26)	n.s.	18 (24)	48 (28)	n.s.
	Loss	2 (3)	7 (4)		3 (4)	6 (3)	
MED1	Gain	18 (22)	67 (39)	0.021	18 (24)	66 (38)	0.04
	Loss	5 (6)	8 (5)		4 (5)	9 (5)	
MTDH	Gain	28 (35)	52 (30)	n.s.	30 (40)	49 (28)	0.05
	Loss	0 (0)	2 (1)		1 (1)	1 (1)	
MYC	Gain	46 (57)	61 (35)	0.001	47 (63)	59 (34)	<0.001
	Loss	1 (1)	1 (1)		1 (1)	0 (0)	
PRMD14	Gain	24 (30)	56 (33)	n.s.	26 (35)	53(31)	n.s.
	Loss	0 (0)	1 (1)		1 (1)	0 (0)	
TOP2A	Gain	5 (6)	21 (12)	n.s.	3 (4)	23 (13)	n.s.
	Loss	3 (4)	7 (4)		3 (4)	7 (4)	

CNA: Copy Number Aberrations; n: number of cases with alterations (gains or losses) for each gene. The proportions between groups are compared using χ^2 test; n.s.: No significant.

We have also seen that miR-590-5p and miR-423-3p expression levels are related to the immunophenotype. MiR-590-5p shows minimum expression levels in luminal A BCs and maximum expression in basal/plus fivefold negative. The miR-423-3p, presents the higher expression in luminal B and the minimum at basal/plus fivefold negative (**Table 6**).

The miR expression is strongly linked with TNBC. Thus, miR-4417 and miR-590-5p show a high average expression in TNBC while miR-423-3p presents the lowest expression in NTNBC (**Table 6**). The multifactorial ANOVA, including the miRs as independent variables and ER, PR, HER2, CK5/6, Cadherin-E, Ki67, CK18, as factors, selects as independent variables linked to miR expression, the TN status (*P*=0.006) and ER status (*P*=0.057).

The binary stepwise backward Wald logistic regression selects miR-4417 and miR-423-3p as those that better differentiate HBC and NHBC (**Figure 1**). The first miR is overexpressed in the HBC while the second miR does it in NHBC. Regarding TNBC, the binary stepwise backward Wald logistic regression selects the miR-590-5p, miR-4417 and miR-423-3p as those that better differentiate TNBC and NTNBC. The first two miRs were overexpressed in TNBC whereas the third does it in NTNBC (**Figure 2**).

Logistic regression summary with all the selected parameters in hereditary and triple negative breast cancers

The binary stepwise backward Wald logistic regression, including the IHC, molecular (CNA and PM) and miR expression, selects the parameters that better differentiate the group status of HBC or TN groups (**Figure 3**).

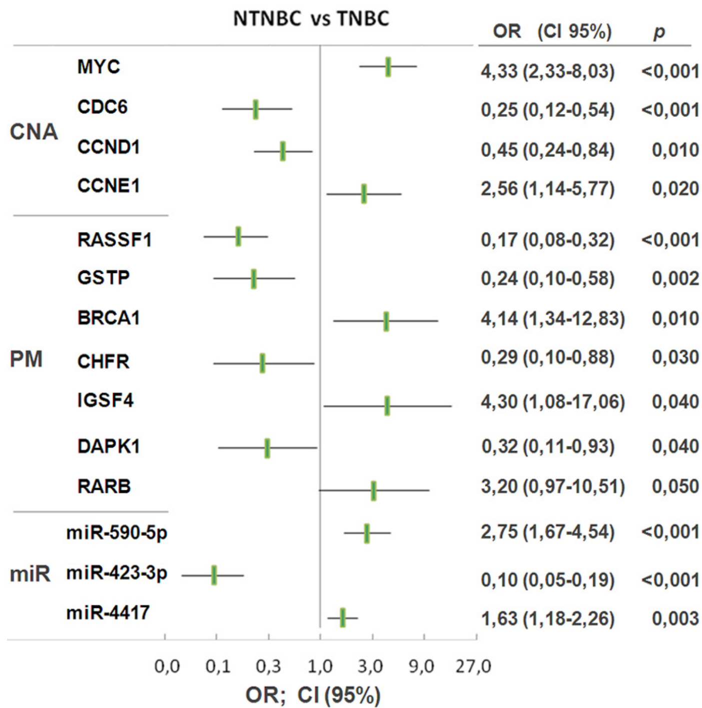

Figure 2. Immunohistochemical, CNA, PM and miR expression profiles for the best differentiation of TNBC and NTNBC. Binary stepwise backward Wald logistic regression. IHC: immunohistochemical markers; CNA: Copy number aberration; PM: Promoter methylation; NTNBC: No Triple negative breast cancer; TNBC: Triple negative breast cancer.

For H groups, ER positivity, *ERBB2* CNAs and *RASSF1* and *TIMP3* PM are linked to NHBC (**Figure 3A**), whereas the presence of *MYC* and *AURKA* CNAs are more prone in HBC. The regression correctly classified 37.1% of HBC (26/70) and 89.2% (132/148) of NHBC.

For TNBC, *CDC6* CNA, *GSTP1* and *RASSF1* PM, and miR-423-3p hyperexpression are more prone in NTNBC, whereas *MYC* aberrations, *BRCA1* PM, miR-590-5p and miR-4417 hyperexpression are more characteristic of TNBC (**Figure 3B**). The regression correctly classified 69.0% (49/71) of TNBC and 93.9% (155/165) of NTNBC.

Discussion

MYC CNA and *RASSF1* PM are the parameters that better differentiated Hereditary and TNBC groups. *MYC* CNA present the highest prevalence in Hereditary and TNBC, whereas methylated *RASSF1* showed the highest prevalence in NHBC and NTBC. However, the rest of the selected variables that differentiated the groups are characteristic of each one of the study groups.

MYC is a central regulator of cell growth, proliferation and apoptosis. Its overexpression enhances DNA double-strand breaks (DSBs), genetic instability and tumorigenesis [46]. *MYC* amplification has been detected in 14.6% of BCs [14]. The present study shows that *MYC* exhibit the highest CNA prevalence (57%, 46/81) in HBC, principally in *BRCA1* mutation carriers (67%, 31/46), and in 63% (47/75) of TNBC. It has also been reported that TNBC exhibit elevated *MYC* expression associated with altered expression of *MYC* regulatory genes, resulting in increased activity of the *MYC* pathway [47]. The increased *MYC* expression found in TNBC could be exploited using a synthetic-lethal approach based on cyclin-dependent kinase (CDK) inhibition [15].

Methylation of *RASSF1* and other tumor suppressor genes such as *APC*, *ATM*, *CDH13*, *GSTP1* were frequently detected in SBC being absent in normal tissues [20]. A positive correlation between *RASSF1* methylation and ER+/PR+ BCs has been reported [22]. We observed here higher prevalence of *RASSF1* PM in NHBC and NTNBC (72% and 78%, respectively) associated with the higher frequency of ER and PR positive in these tumors. *RASSF1* methylation provides the opportunity to assay in these tumors treatments with demethylating agents or histone deacetylase inhibitors.

One relevant difference between HBC and NHBC found here is CNA in *AURKA*, the gene involved in mitosis and meiosis processes as well as in DNA damage response modulating DSB repair [13]. Our results, in correspondence with previous reports [48], have shown that *AURKA* aberrations, mainly losses, are more prevalent in HBC, (mainly BCs of *BRCA1* carriers) than in NHBC. Hence, we have observed a prevalence of 41% for *AURKA* CNA in *BRCA1* BCs, 23% in *BRCA2*, and 14% and 16% for BRCAX and SBC, respectively (P=0.002). *AURKA* CNA is associated with a poorer prognosis in ER+ BCs [49]. However, these patients might benefit from targeted therapies based on Aurora-A inhibitors [11, 12].

Table 5. Prevalence of methylation between HBC/NHBC and TNBC/NTNBC

GENE PM	HBC (n=78)	NHBC (n=162)	χ^2	TNBC (n=71)	NTNBC (n=166)	χ^2
	n (%)	n (%)	*P*	n (%)	n (%)	*P*
APC	49 (63)	122 (75)	0.045	46 (65)	122 (74)	n.s.
ATM	31 (40)	77 (48)	n.s.	25 (35)	80 (48)	n.s.
BRCA1	9 (12)	20 (12)	n.s.	14 (20)	15 (9)	0.02
BRCA2	10 (13)	27 (17)	n.s.	11 (16)	25 (15)	n.s.
CASP8	21(27)	39 (24)	n.s.	15 (21)	45 (27)	n.s.
CD44	1 (1)	5 (3)	n.s.	3 (4)	3 (2)	n.s.
CDH13	64 (82)	123 (76)	n.s.	51 (72)	133 (80)	n.s.
CDKN1B	9 (12)	27 (17)	n.s.	9 (13)	26 (16)	n.s.
CDKN2A	36 (46)	70 (43)	n.s.	29 (41)	76 (46)	n.s.
CDKN2B	12 (15)	21 (13)	n.s.	8 (11)	23 (14)	n.s.
CHFR	11 (14)	35 (22)	n.s.	9 (13)	37 (22)	n.s.
DAPK1	24 (31)	44 (27)	n.s.	10 (14)	56 (34)	0.002
ESR1	4 (5)	15 (9)	n.s.	10 (14)	6 (5)	0.02
FHIT	9 (12)	13 (8)	n.s.	9 (10)	15 (9)	n.s.
GSTP1	28 (36)	59 (36)	n.s.	12 (17)	75 (45)	<0.001
HIC1	10 (13)	23 (14)	n.s.	5 (7)	28 (17)	0,05
IGSF4	5 (6)	16 (10)	n.s.	7 (10)	13 (8)	n.s.
MLH1	7 (9)	24 (15)	n.s.	8 (11)	21 (13)	n.s.
PTEN	16 (21)	44 (27)	n.s.	15 (21)	45 (27)	n.s.
RARB1	10 (13)	14 (9)	n.s.	8 (11)	16 (10)	n.s.
RASSF1	38 (49)	117 (72)	<0.001	24 (34)	130 (78)	<0.001
TIMP3	6 (8)	26 (16)	n.s.	11 (16)	21 (13)	n.s.
TP73	15 (19)	21 (13)	n.s.	12 (17)	23 (14)	n.s.
VHL	9 (12)	16 (10)	n.s.	5 (7)	19 (11)	n.s.

n: number of cases with methylation in the promoter of the gene; PM: Promoter methylation; n.s.: Not significant. The proportions between groups are compared using the χ^2 test.

We here found the higher prevalence of *ERBB2* amplification in NHBC with regard to HBC, 36% vs. 20%, respectively. Patients harboring high *ERBB2* CNA show decreased overall survival although HER2-targeted therapies have significantly improved the survival of these patients [50].

TIMP3 is an inhibitor of the matrix metalloproteases, a group of endopeptidases involved in the degradation of the extracellular matrix. We show here that *TIMP3* methylation is more frequent among NHBC than HBCs (16% vs. 8%). Its hypermethylation has been reported in gastric cancer and pancreatic endocrine tumors [51, 52].

We have shown here that *CDC6* CNA, *BRCA1* and *GSTP1* PM and miR-590-5p, miR-4417 and miR-423-3p expression are specific parameters that differentiated TN and NTNBCs.

Studies conducted in series between 77 and 450 TNBCs (median of 137 patients) reported between 2011 and 2014 [53-57] showed a prevalence of 17.35% (range: 6.10-24.6) of *BRCA1/2* mutation, which increases to 38.12% (range: 25.7-66.0) when family history of BC/OC was present. In our series that includes 156 BCs with family history, we detected 39 (49.4%) *BRCA1/2* mutation carriers among 79 TNPBC and 45 (24.1%) amid 187 NTNBC.

GSTP1 participates in detoxification processes protecting cells from carcinogens. It is expressed in normal breast epithelial cells and its methylation is associated with hormone receptor expression in BCs [58-60]. We have seen that the percentage of *GSTP1* methylation shows a neat difference between TNBC and NTNBC, with NTNBC displaying the highest prevalence.

Week *et al* reported that 36.7% of NH TNBC presented *BRCA1* PM [61]. In this respect we also found that TNBCs showed higher prevalence of *BRCA1* methylation in comparison to NTNBC (20% vs. 9%). *BRCA1* promoter methylation presents a particular interest since these patients are responsive to PARP inhibitors [62] and, conversely, the unmethylated could achieve a pathological complete response to anthracycline-based therapy [63].

We have seen that *CDC6* shows the higher percentage of gains in NTNBC (32%) in comparison to TNBC (16%). *CDC6* is overexpressed in human cancers, where it might play a role in DNA replication. Gonzalez et al [64] reported that high levels of *CDC6* result in RD (INK4/ARF)-dependent transcriptional repression, recruitment of histone deacetylases and heterochromatinization of the INK4/ARF locus, with concomitant decrease in the expression of the three tumor suppressors encoded by this locus. Furthermore, *CDC6* shows cellular

Table 6. Mean expression of microRNAS and immunohistochemical markers and immunophenotype

IHC Marker		n	miR-187-3p	P	miR-590-5p	P	miR-4417	P	miR-423-3p	P
			X̄ (SD)		X̄ (SD)		X̄ (SD)		X̄ (SD)	
ER	-	98	10.4 (1.3)	0.008	9.4 (0.8)	<0.001	10.7 (1.1)	0.02	9.1 (0.6)	<0.001
	+	170	9.9 (1.4)		9.0 (0.7)		10.3 (1.2)		9.5 (0.6)	
PR	-	125	10.3 (1.4)	ns	9.2 (0.9)	ns	10.6 (1.2)	0.04	9.2 (0.7)	<0.001
	+	143	9.9 (1.4)		9.1 (0.7)		10.3 (1.1)		9.5 (0.6)	
HER2	-	233	10.0 (1.4)	0.03	9.1 (0.8)	ns	10.4 (1.2)	ns	9.4 (0.7)	ns
	+	37	10.6 (1.4)		9.2 (0.7)		10.6 (1.1)		9.5 (0.7)	
Ki67	h	149	10.0 (1.41)	ns	9.0 (0.8)	ns	10.5 (1.1)	ns	9.5 (0.6)	ns
	l	117	10.2 (1.40)		9.2 (0.8)		10.4 (1.3)		9.3 (0.7)	
CK5-6	-	208	10.1 (1.4)	ns	9.1 (0.8)	ns	10.4 (1.2)	ns	9.5 (0.6)	0.01
	+	54	10.3 (1.4)		9.3 (0.8)		10.7 (1.3)		9.2 (0.8)	
CK18	-	17	10.2 (1.5)	ns	9.5 (0.8)	0.03	10.4 (0.7)	ns	9.3 (0.4)	ns
	+	231	10.1 (1.4)		9.1 (0.8)		10.4 (1.2)		9.4 (0.7)	
EGFR	-	209	10.2 (1.4)	ns	9.1 (0.8)	ns	10.4 (1.2)	0.02	9.4 (0.7)	ns
	+	27	10.1 (1.5)		9.3 (0.2)		11.0 (1.4)		9.4 (0.8)	
Cad-E	-	12	9.9 (0.8)	ns	8.8 (0.7)	ns	10.0 (0.9)	ns	9.2 (0.5)	ns
	+	244	10.1 (1.5)		9.2 (0.8)		10.5 (1.2)		9.4 (0.7)	
PHENOTYPE	LA	112	9.9 (1.3)	0.05	9.0 (0.7)	0.005	10.4 (1.1)	ns	9.5 (0.6)	<0.001
	LB	46	9.9 (1.6)		9.0 (0.6)		10.2 (1.4)		9.6 (0.8)	
	HER2	33	10.6 (1.5)		9.2 (0.6)		10.5 (1.2)		9.5 (0.7)	
	TN	79	10.3 (1.3)		9.4 (0.8)		10.7 (1.1)		9.0 (0.6)	

X: mean expression; SD: standar deviation; h: high; l: low; n: number of cases; Cad-E: Cadherin-E; LA: Luminal A; LB: Luminal B; TN: Triple Negative (includes basal tumors and fivefold negative tumors); n.s.: Not significant. Means are compared using ANOVA test.

immortalization activity and neoplastic transformation capacity in cooperation with oncogenic *RAS*.

We have shown that miR-590-5p, miR-4417 were significantly hyperexpressed in TNBC, while miR-423-3p showed higher expression in NTNBC.

We observed that miR-590-5p was hyperexpressed in ER negative BCs (P<0.001), TN (P=0.001) and in basal/plus fivefold negative BCs (P=0.005). *In silico* studies employing the target prediction software microT-cds indicate that this microRNA can interact with two mRNA sequences of the *ESR1* gene located on two 3'UTR regions, which has also been confirmed by the TargetScan. Experimental studies confirmed that miR-590 interacts with *ESR1* mRNA in its 3p form [65]. Hongfei et al [66] analyzed the expression pattern of miR-590 in liver cancer specimens and cell lines by miRNA microarrays and qPCR, he reported that miR-590 is an important tumorigenic factor for hepatocellular carcinoma and its two forms, 3p and 5p, can both promote tumorigenesis by regulating the expression of their target tumor suppressor genes, *PDCD4* and *PTEN*, promoting hepatocellular carcinoma, cell proliferation and survival.

MiR-4417 shows higher expression in TNBC (P=0.043) and also in ER negative (P=0.019), PR negative (P=0.04), EGFR positive (P=0.02) and HBC (*P*=0.001). Target prediction software micro-cds indicates that this miR can establish connections with 3 different regions of *BRCA1* mRNA.

We have seen that miR-423-3p is overexpressed in NTNBC (P<0.001) and in ER+ (P<0.001), PR+ (P<0.001), CK5/6 negative (P=0.005), and luminal A and B immunophenotypes (P<0.001). Target prediction software micro-cds and miRanda identified a strong bond of this miR with mRNA of *ESRRA* (Related Estrogen Receptor Alpha) gene [67]. This suggests that ER positive tumors could have a diminished protein synthesis of *ESRRA*, in cor-

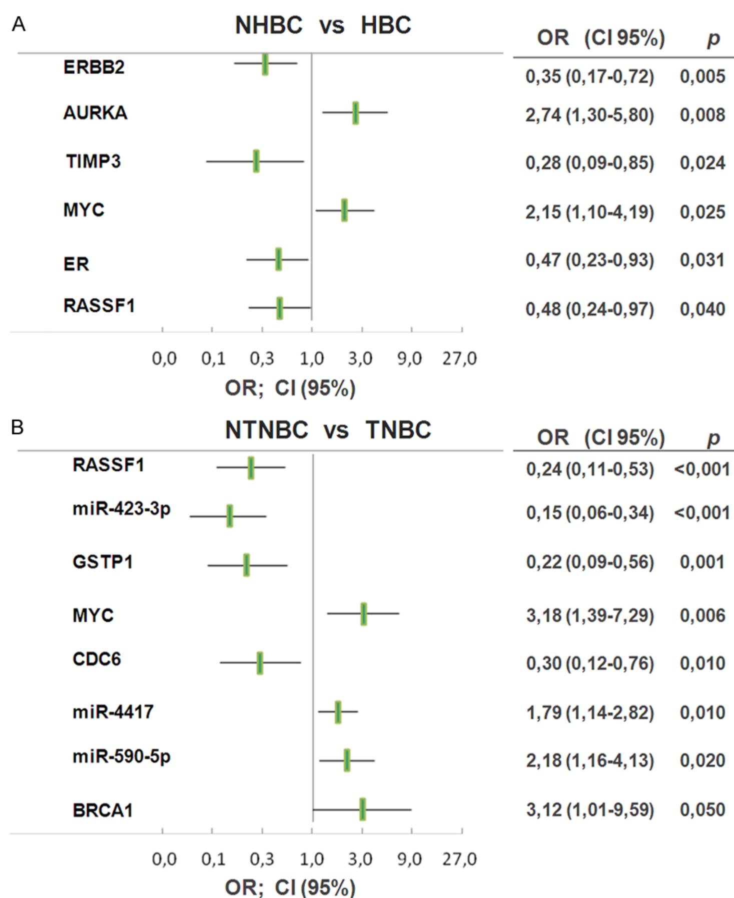

Figure 3. Immunohistochemical and molecular profiles for the best differentiation of the HBC/NHBC (A) and TNBC/NTNBC phenotypes (B). Binary stepwise backward Wald logistic regression.

respondence with this hypothesis, Ariazi *et al* [68] showed that the high expression of *ESRRA* is correlated with the absence of expression of ER and PR.

With regard to miR-4417 and miR-423-3p, we have previously reported that co-expression of both miR enabled the differentiation in 70% of cases of HBC and NHBC [32].

Our results indicate that BCs present a different spectrum of altered markers, mostly specific of the groups with the exception of *MYC* CNAs and *RASSF1* PM that are shared by the two study groups. The markers selected are involved in the pathogenesis of the tumors, many of them being targets for drug therapy or predictors of response to adjuvant therapy. Hence *MYC* CNA is highly prevalent in HBC and TNBC and associated with aggressive forms of BC and correlated with poor prognosis and distant metastases. Likewise *RASSF1* methylation, chiefly detected in NHBC and NTNBC, has been associated with a high risk of relapse and a short survival.

The parameters characteristic of each group are also relevant in the etiopathogenesis and BC progression. Hence, *AURKA* and *ERBB2* CNA are very relevant in HBC groups, *AURKA* CNA is associated with a poorer prognosis in ER positive BCs, and *ERBB2* is a reliable biomarker and a drug target. In the TNBC group, *BRCA1* PM shows a high prevalence in TNBC, conferring sensitivity to PARP inhibitors, while the unmethylated forms could achieve pathological complete response to anthracycline-based therapy. *GSTP1* methylation, prevalent in NTNBC, reduces the detoxification capacity of the enzyme. And of the microRNAs miR-590-5p and miR-4417 both hyperexpressed in TNBC, the first one interacts with two ESRA sequences of *ESR1*, and miR-4417 could be implicated in the regulation of *BRCA1* mRNA.

In summary the markers selected could allow us to establish subtypes of BCs characterized by showing similar etiopathogenetic mechanisms, some of them being molecular targets for known drugs or possible molecular targets. The results found here could be a basis to implement a personalized therapy.

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Disclosure of conflict of interest

None.

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