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ORIGINAL ARTICLE

Molecular evaluation of a multiplex methylation panel for epigenetic analysis of FNAB samples from Greek patients with suspicious breast lesions

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Summary

Purpose: *Aberrant DNA methylation in promoter regions has been found in many cancers, including breast cancer (BC). A Methylation Specific PCR (MSP) was applied in breast Fine Needle Aspiration Biopsy (FNAB) material, which has been rarely used in the literature, to estimate the methylation frequencies of CND2, APC, HIN1 & CDH13 and to assess whether this multiplex methylation panel can be possibly used as an indicator-biomarker for BC detection in a Greek population.*

Methods: *A total of 104 participants were subjected to FNAB and both cytological evaluation and epigenetic analysis were carried out. DNA was extracted from FNAB samples and was subjected to bisulfite conversion. MSP was carried out with primers specific for either the methylated or unmethylated status for each gene. The final MSP products were analyzed in 2% agarose gels with electrophoresis.*

Results: *Hypermethylation was observed in 74%, 69.2%, 59.6% and 63.4% of the samples for CND2, HIN1, APC and CDH13, respectively. CND2 was the most hypermethylated in C5 cases (90%) and APC and HIN1 in C4 cases (88.2%). A significant correlation between histologic evaluation and the methylation frequencies for all 4 genes was calculated ($p<0.001$). Odds ratio for breast malignancy was 8.267 for CND2, 5.235 for APC, 7.852 for HIN1 and 22.920 for CDH13, underlying that their methylation is positively related to breast malignancy. Also, it seems that the combination of all genes into a multiplex methylation panel has significantly higher SP and PPV than any single gene methylation.*

Conclusions: *Our study shows that breast FNAB combined with methylation data from the collected aspirates has a promising potential as a biomarker for the early detection of BC risk in women with suspicious lesions.*

Key words: *breast fine needle aspiration biopsy, DNA promoter hypermethylation, epigenetic analysis, methylation specific PCR, multiplex methylation panel*

Introduction

Breast cancer (BC) remains a crucial health issue, impacting over 2 million new women while causing over 620,000 deaths in 2018 [1,2]. Early diagnosis and screening strategies are essential to increase the number of BCs identified at an early stage, providing timely access to more effective treatment and leading to reduced mortality and improved survival [3]. Mammographic screen-

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ing helps towards this direction despite its false-positive and false-negative rates [4]. It seems that further improvement in diagnosis and prognosis of BC patients implies the need for alternative approaches for BC detection.

It is known that BC is highly heterogeneous with multiple histological and molecular subtypes associated with different clinical behaviors and treatment responses [5]. With the advent of high-throughput technologies, molecular genetics has provided a more comprehensive view of BC and has gradually gained ground in the field of BC diagnostics especially by casting light on the epigenetics of BC progression [6]. It has been proved that epigenetic modifications including aberrant DNA methylation play a pivotal role since the early stages of BC progression [7].

Aberrant DNA methylation in cancer usually appears as increasing levels of 5-methylcytosine in DNA sequence, especially in CG dinucleotides located in the promoter regions of tumor suppressor genes (TSGs) [8]. Promoter regions of TSGs are unmethylated in normal tissues but when cancer-linked promoter hypermethylation occurs, it gradually leads to the transcriptional silencing of these TSGs causing cumulative disturbances in cellular growth, cellular adhesion and genetic expression/signaling, resulting at the end in malignant transformation [9]. Promoter hypermethylation is a very common molecular alteration in numerous cancers including, among others, BC [10,11] with studies based mainly on histological samples revealing high methylation frequencies of TSGs in cancerous tissues in comparison with normal ones [12-15]. These TSGs are associated with cell cycle regulation (Cyclin D2-CND2 [16], High In Normal1-HIN1 [14]), signal transduction (Adenomatous Polyposis Coli-APC [17]) and cellular adhesion (Cadherin 13-CDH13 [15]).

However, most epigenetic studies in breast pathology are based on bioptic material obtained through invasive procedures [18,19]. Fine needle aspiration biopsy (FNAB), on the other hand, is a minimally invasive, quick, inexpensive procedure to remove cytologic material from suspicious breast lesions without the need for more invasive approaches [20-22], offering this way alternative source of material for molecular cancer testing [23,24].

The significance of DNA methylation biomarkers for early cancer detection is reflected in the literature with studies estimating the diagnostic parameters of specific epigenetic panels associated with various cancers [25]. However, due to the heterogeneity of BC, most methylation studies fail to come to an agreement concerning the methylation status as well as the genes composing the methylation panel [26].

In this study, a Methylation Specific PCR (MSP) protocol was designed and applied in breast FNAB material to estimate the promoter hypermethylation frequencies of CND2, APC, HIN1 & CDH13 genes and to assess whether this multiplex methylation panel can be possibly used as an indicator-biomarker for BC detection in a Greek population.

Methods

Patients & sample collection

All 104 participants were patients of the Breast Clinic of the 2nd Department of Obstetrics & Gynecology Medical Faculty Aristotle University of Thessaloniki, located at Hippokration General Hospital, between January 2013 and June 2019. Before participation, all patients were informed about the purpose and aims of the study and gave their informed consent afterwards. The Aristotle University Bioethics Research Committee on Human Research has officially approved the protocol of the study.

FNAB was performed pre-operatively using a 23-gauge needle attached to a 10 ml syringe and inserted into a syringe holder under ultrasound guidance. The aspirates were collected in vials containing PreservCyt/ThinPrep solution (Hologic, Marlborough, USA) from which both cytological evaluation and epigenetic analysis were carried out at the Molecular Cytopathology Lab of the Clinic. When there were cytological findings either inconclusive for malignancy, suspicious for malignancy or malignant, depending on the severity of the diagnosis, core-needle biopsy or surgical biopsy (incisional or excisional) were taken for histopathological confirmation. FNAB samples with normal findings were included as control group.

Cytological and histopathological evaluation

FNAB cytological slides were prepared using the Thin Prep 2000 Processor (Hologic, Marlborough, MA, USA) according to the manufacturer's instructions. The prepared slides were stained by the Pap method and assessed by 2 cytopathologists of the Lab according to the criteria set out in the 2019 Yokohama System for Reporting Breast FNAB Cytopathology [27] which categorizes the cytological findings in 5 categories: 1.Insufficient/adequate(C1) 2.Benign(C2) 3.Atypical(C3) 4.Suspicious of malignancy(C4) and 5.Malignant(C5).

Histopathological specimens from core needle and surgical biopsies were diagnosed by 2 pathologists of the Hospital according to the 2019 WHO classification of tumors of the breast (5th Edition)[28]. Also, they provided additional information focusing on the grade of the lesion and the expression status of estrogen receptors (ER), progesterone receptors (PR), HER2/neu and Ki67 protein.

DNA extraction

DNA was extracted with Quick-gDNA MiniPrep (Zymo, Irvine, CA, USA) which is suitable for efficient extraction and purification from various samples. Zymo-Spin Columns enable high-quality DNA purification

while removing PCR inhibitors. DNA extraction was performed according to the manufacturer's instructions (Zymo, Irvine, CA USA). Nucleic acid concentration and quality were assessed using a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, USA). The DNA extracts were stored at -30°C until used.

Bisulfite conversion

Bisulfite conversion of unmethylated cytosine residues to uracil of the DNA extracts was performed using the EZ DNA Methylation-Gold (Zymo, Irvine, CA, USA) which uses heat denaturation and optimizes spin columns for desulphonation and recovery of bisulfite-treated DNA. Bisulfite conversion was performed according to the manufacturer's instructions (Zymo, Irvine, CA, UsSA). The bisulfite-converted products were stored at -60°C until used.

Methylation specific PCR (MSP)

MSP was designed with primers specific for either the methylated or unmethylated status for CND2, APC, HIN-1 & CDH13 genes. The primer sequences were obtained from previous studies [29-32] and presented in Table 1. The primers were synthesized by Eurofins Genomics, Ebersberg, Germany).

MSP reactions were prepared using ZymoTaq PreMix (Zymo, Irvine, CA, USA) which is a ready-to-use concentrated PCR master mix with polymerase optimized for the amplification of bisulfite-treated DNA. Modified DNA was amplified in a total volume of 50 μl solution containing 1x ZymoTaq PreMix (2U ZymoTaq DNA polymerase, 1.75mM $MgCl_2$), 1 μM of each primer and up to 200 ng of bisulfite-modified genomic DNA. MSP conditions were an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 30 sec at 95°C, 30 sec at the relevant annealing temperature (Table 1) and 45 sec at 72°C. The reaction was terminated with a 7-min final extension at 72°C. The MSP was performed with 5341 Mastercycler Ep Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). To avoid false positive and false negative results, the human methylated & non-methylated DNA set (Zymo, Irvine, CA, USA) was used as positive and negative controls. The set contains one CpG-methylated human DNA standard which is purified HCT116 DKO DNA enzymatically methylated at CpG sites and one non-methylated human DNA standard purified from the HCT116 DKO cell line, which contains genetic knockouts of both DNA methyltransferases DNMT1 (-/-) and DNMT3b (-/-). PCR products were resolved on a 2% agarose gel containing DNA-Dye NonTox (AppliChem, Darmstadt, Germany) and visualized under UV illumination.

Statistics

The correlation between the categorical (qualitative) variables of the sample and the incidence of breast malignancy was examined by Pearson's x^2 test (association is significant if p value<0.05). Furthermore, binary logistic regression was used to assess the association between breast malignancy incidence with the genes as well as with other demographics. Data analysis was carried out with SPSS® Statistics version 24 statistical package (IBM, USA).

Results

Demographics

The study sample consisted of 104 patients, most of whom were Greek (95%) with a median age of 55 years, height of 159 cm and weight of 68 kg. Almost half the participants (50 out of 104) were

Table 1. List of gene names, primer names, primer sequences, size of MSP products, and annealing MSP temperatures

Gene	*Primer name*	*Primer sequence (5'-3')*	*Size of MSPproduct (bp)*	*Annealing temperature for MSP (°C)*
CND2	FORWARD-METH	TACGTGTTAGGGTCGATCG	276	56.5
	REVERSE-METH	CGAAATATCTACGCTAAACG		
	FORWARD-UNM	GTTATGTTATGTTTGTTGTATG	222	51.5
	REVERSE-UNM	TAAAATCCACCAACACAATCA		
APC	FORWARD-METH	TATTGCGGAGTGCGGGTC	100	60
	REVERSE-METH	TCGACGAACTCCCGACGA		
	FORWARD-UNM	GTGTTTTATTGTGGAGTGTGGGTT	110	57.5
	REVERSE-UNM	CCAATCAACAAACTCCCAACAA		
CDH13	FORWARD-METH	TCGCGGGGTTCGTTTTTCGC	243	60
	REVERSE-METH	GACGTTTTCATTCATACACGCG		
	FORWARD-UNM	TTGTGGGGTTGTTTTTTGT	242	57.5
	REVERSE-UNM	AACTTTTCATTCATACACACA		
HIN1	FORWARD-METH	GGTACGGGTTTTTTACGGTTCGTC	136	62
	REVERSE-METH	AACTTCTTATACCCGATCCTCG		
	FORWARD-UNM	GGTATGGGTTTTTTATGGTTTGTT	136	57.5
	REVERSE-UNM	CAAAACTTCTTATACCCAATCCTCA		

Table 2. Demographics

Parameters	*Median (minimum - maximum)*
Age (yrs)	55 (22-95)
Height (cm)	159 (141-178)
Weight (kg)	68 (39-110)
Parameter	*n (%)*
No. of births (n=104)	
0	19 (18.3)
1	10 (9.6)
2	50 (48.1)
3	17 (16.3)
4	8 (7.7)
Smoking habits (n=104)	
Smoker	50 (48.1)
Non-smoker	54 (51.9)
Family history of cancer (n=104)	
Yes	20 (19.2)
No	84 (80.8)
Position of the breast lesion (n=104)	
Right breast	52 (50)
Left breast	52 (50)

smokers and had two births. The vast majority (84 out of 104) had no family history of cancer (80.8%), while in 20 individuals with history, BC was the most common cause (50%). In terms of position of the breast lesion, the sample numbers were equal. Age was the only demographic correlated with breast malignancy (p=0.003) since smoking habits (p=0.285), weight (p=0.739), height (p=0.665), history of family cancer (p=0.288), position of lesion (p=0.562) and number of births (p=0.261) did not show any consistent associations. The demographics are summarized in Table 2.

Cytological and histological findings

Most patients (64.4%; n=67), had crucial abnormalities in their FNAB smear (C4 or C5), with 17 diagnosed as suspicious for malignancy and 50 as malignant. Atypical breast lesion (C3) was diagnosed in 14 (13.4%) patients and benign breast lesions (C2) in 21 (20.2%) patients used as control group. Finally, in two cases (2%) the FNAB material was characterized as insufficient for cytological results (C1).

Table 3. Cytological and histological findings

	n (%)
Cytological diagnosis (n=104)	
C1	2 (2)
C2	21 (20.2)
C3	14 (13.4)
C4	17 (16.3)
C5	50 (48.1)
Histological diagnosis (n=81)	
C3 (N=14)	
Malignancy / Without malignancy	3 (21.4) / 11 (78.6)
C4 (N=17)	
Malignancy / Without malignancy	17 (100) / 0 (0)
C5 (N=50)	
Malignancy / Without malignancy	50 (100) / 0 (0)
Histological classification of the histologically confirmed breast lesions (n=70)	
Invasive ductal carcinoma	55 (78.6)
Invasive lobular carcinoma	3 (4.3)
Carcinoma *in situ*	1 (1.4)
Mixed type and other special types of breast carcinoma	11 (15.7)
Grade	
1	7 (10)
2	34 (48.5)
3	29 (41.5)
ER expression	
+ / -	56 (80) / 14 (20)
PR expression	
+ / -	44 (62.8) / 26 (37.2)
Ki67 expression	
+ / -	47 (67.1) / 23 (32.9)
HER2/neu expression	
+ / -	12 (17.2)/58 (82.8)

All 81 patients with cytology C3+ were scheduled for core needle or excisional biopsy for histological confirmation. Malignancy was confirmed in all the C4+ cases (n=67) and only 3 out of 14 (21.4%) C3 cases. From the 70 histologically confirmed malignancies, the vast majority (78.6%) were invasive ductal carcinomas, followed by mixed type and other special types of BC (15.7%), while only few cases were invasive lobular carcinoma or carcinoma *in situ* (4.3% and 1.4%, respectively). Concerning the grade, most of the BCs were either grade 2 (48.1%) or grade 3 (41.5%). In terms of hormone receptors, 56 out of 70 BCs were ER-positive (80%) and 44 out of 70 were PR-positive (62.8%). Ki67 and HER2/neu expression was detected in 47 (67.1%) and 12 (17.2%) of 70 BCs, respectively. Pearson's x^2 test was applied between cytological and histological diagnosis and revealed a statistically significant relationship between them ($p<0.001$). The results are summarized in Table 3.

MSP results

On agarose gels, a band corresponding to U primer set (Unmethylated) underlies the absence of DNA methylation while a band corresponding to M primer set (Methylated) states the presence

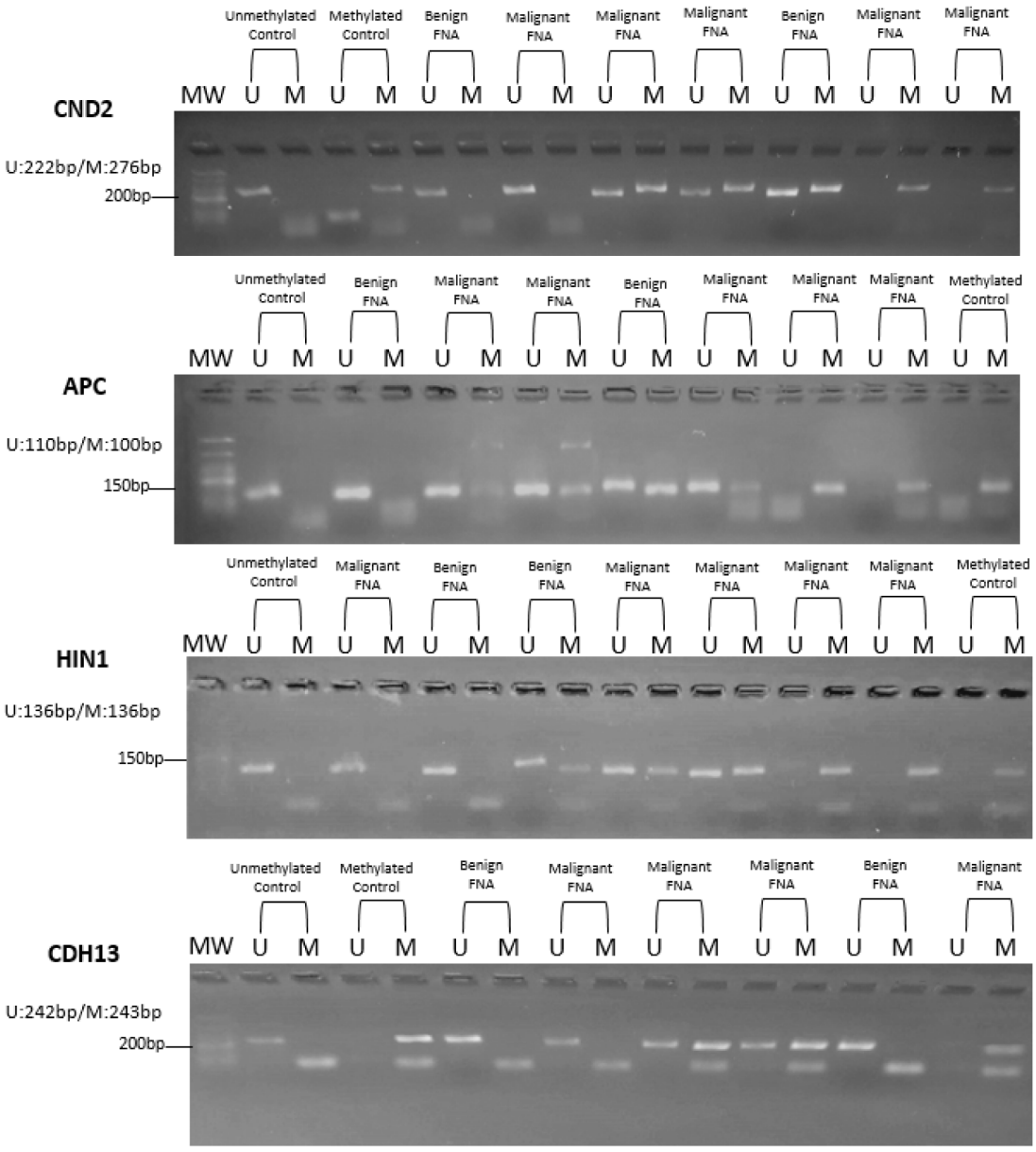

Figure 1. Electrophoretic MSP analysis of CND2, HIN-1, APC and CDH13 viewed from left to right shows a 200-bp ladder as molecular weight marker. Methylated and Unmethylated controls have been used in all MSP reactions. A band corresponding to U primer set (Unmethylated) underlies the absence of DNA methylation while a band corresponding to M primer set (Methylated) states the presence of DNA methylation. When both U and M bands are present, there is partial DNA methylation.

Table 4. CND2, HIN-1, APC & CDH13 methylation frequencies

	CND2 gene		*APC gene*		*HIN1 gene*		*CDH13 gene*	
Cytological diagnosis	*U n (%)*	*M n (%)*	*U n (%)*	*M n (%)*	*U n (%)*	*M n (%)*	*U n (%)*	*M n (%)*
C1 (n=2)	1 (50)	1 (50)	1 (50)	1 (50)	2 (100)	0 (0)	2 (100)	0 (0)
C2 (n=21)	14 (66.7)	7 (33.3)	14 (66.7)	7 (33.3)	18 (85.7)	3 (14.3)	18 (85.7)	3 (14.3)
C3 (n=14)	3 (21.5)	11 (78.5)	6 (42.8)	8 (57.2)	6 (42.8)	8 (57.2)	6 (42.8)	8 (57.2)
C4 (n=17)	4 (23.5)	13 (76.5)	2 (11.8)	15 (88.2)	2 (11.8)	15 (88.2)	5 (29.5)	12 (70.5)
C5 (n=50)	5 (10)	45 (90)	9 (18)	41 (82)	10 (20)	40 (80)	11 (22)	39 (78)
Total	27	77	32	72	38	66	42	62

Table 5. Diagnostic parameters for single CND2, single APC, single HIN1, single CDH13 and multiplex CND2/APC/HIN1/CDH13 hypermethylation

	Breast malignancy as threshold			
Type of methylation	*Sensitivity (%)*	*Specificity (%)*	*Positive predictive value (%)*	*Negative predictive value (%)*
Single CND2	87.14	52.94	79.22	66.67
Single APC	84.29	61.76	81.94	65.62
Single HIN1	82.86	76.47	87.88	68.42
Single CDH13	77.14	76.47	87.10	61.90
Multiplex CND2/APC/HIN1/CDH13	45.21	87.10	89.19	40.30

of DNA methylation. When both U and M bands are present, there is partial DNA methylation. As shown in Table 1, the size of U and M products are: 222bp/276bp for CND2, 110bp/100bp for APC, 242bp/243bp for CDH13 and 136bp/136bp for HIN1. Electrophoresis pictures of MSP products for all 4 genes are shown in Figure 1.

Aberrant methylation was observed in all CND2, HIN1, APC and CDH13 promoters. In fact, from 104 samples, hypermethylation was observed in 77 (74%), 72 (69.2%), 62 (59.6%) and 66 (63.4%) for APC, CDH13 and HIN1, respectively. CND2 was the most hypermethylated from all genes since it was methylated in 90% of C5 cases, followed by APC (82%), HIN1 (80%) and CDH13 (78%). APC and HIN1 had the highest methylation frequency (88.2%) in the C4 cases, followed by CND2 (76.5%) and CDH13 (70.5%). In the "grey zone" of the C3 samples (n=14), CND2 was the most hypermethylated (78.5%), followed by HIN1, APC and CDH13 (57.2%). In C2 cases (n=21), CND2 and APC methylation frequency was 33.3%, while HIN-1 and CDH13 had 14.3%. In 37 samples (35.6%) all genes were methylated, while in 29 (27.8%) three genes were methylated, in 15 (14.4%) two genes were methylated and in 10 (9.6%) one gene was methylated. Finally, only 13 out of 104 (12.5%) showed no methylation at all. Table 4 summarizes the methylation frequencies for all genes.

Pearson's x^2 test was applied to find potential correlations between gene methylation in FNAB samples and histological findings. A significant correlation between histologic evaluation and the methylation frequencies of all four genes was calculated ($p<0.001$). Risk estimate calculation revealed that the odds ratio (OR) for breast malignancy was 8.267 (95% CI, 3.106-22.003) for CND2, 5.235 (95% CI, 2.105-13.015) for APC, 7.852 (95% CI, 3.085-19.985) for HIN1 and 22.920 (95% CI, 3.368-21.891) for CDH13 ($p<0.001$), underlying that their methylation is positively related to breast malignancy. Demographics, ER, PR, Ki67 and HER2/neu expression and type of BC did not show any consistent associations with any gene promoter methylation and were considered statistically insignificant (data not shown).

Considering breast malignancy as a threshold, sensitivity (SV), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) were calculated for single CND2, APC, HIN1 or CDH13 methylation and multiplex CND2/APC/HIN1/CDH13 methylation. It appears that CND2 methylation (87.14%) was more sensitive than the other genes, while at the same time was the least specific (52.94%). On the other hand, HIN1 and CDH13 methylation were the most specific ones (76.47%). In terms of predictive values, HIN1 methylation had the highest PPV (87.88%) and NPV

(68.42%). However, it seems that the combination of all genes in a multiplex methylation panel had significantly higher SP and PPV than any single gene (87.10% and 89.19%, respectively). All diagnostic parameters are included in Table 5.

Discussion

FNA is a quick, cost-effective, minimally invasive, safe and reliable cancer diagnostic preoperative procedure indicated for patients with a mammographic abnormality or palpable breast lesions [33]. Cytological evaluation of breast FNAB smears has wide applicability as an initial triage option to identify malignant breast lesions from benign ones, offering valuable assistance to clinicians [34], while overcoming some core-needle biopsy associated complications such as hematoma or pneumothorax [35]. Despite being a critical component in the investigation of breast masses, FNAB cytology is an operator-dependent technique limited by parameters such as the clinician's technical ability to sample representative material from the lesion, resulting many times in high rates of non-diagnostic insufficient samples, as well as the cytopathologist's proficiency to make the correct diagnosis especially when cellularity is limited [36,37].

Previous methylation studies of CND2, APC, HIN1 & CDH13 promoters used mostly histological material from breast tissues for molecular epigenetic analysis [12,29,31,38-51]. In this study, we extended the spectrum of methylation analysis of CND2, APC, HIN1 & CDH13 promoters in BC via evaluation of breast FNAB material which has been rarely used in the literature [52-56].

Regarding CND2 methylation, hypermethylation was detected in 86.2% of malignant cases, a result in agreement with other breast FNAB studies [52-56] where methylation frequency was up to 87.9%. On the other hand, we found that CND2 promoter was methylated in 33.3% of benign cases, while the relative percentage in the literature is almost double (67%) [52-56]. For comparison with studies based on histological breast samples, CND2 methylation frequencies were up to 71% for malignant [12,29,43,44,46,48,51], and up to 45% for benign tissues [12,43,44,46,49,51], showing slight differences from our results. In our study, the OR for breast malignancy was 8.267 (95%CI: 3.106-22.003) in patients with CND2 hypermethylation, while Euhus et al [52] calculated an OR of 4.54 (95%CI: 1.37-15.07), far less than ours with the difference possibly explained by the fact that that study was based on different molecular technique (real-time MSP, RT-MSP).

In terms of APC methylation, promoter hypermethylation was found in 86.5% of all malignant and in 33.3% of all benign cases, whereas the relative methylation frequencies in other breast FNAB studies [52-56] were up to 83.3% and up to 64.5%, respectively, showing that our methylation frequencies are similar for malignant cases and almost half for benign ones. In studies with histological breast samples, APC methylation frequencies were up to 52.5% for malignant [31,45-47] and up to 44.4 % for benign tissues [12,43,44,46,49,51]. In our study, the OR for breast malignancy was 5.235 (95%CI: 2.105-13.015) in patients with APC hypermethylation which is slightly higher from the result of a meta-analysis from Zhou et al [57] who estimated an OR of 3.95 (95%CI: 2.10-7.42) for breast malignancy for FNAB, with the difference possibly explained by the fact that the authors included numerous techniques such as RT-MSP, Pyrosequencing (PS), Methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA), Methylation-sensitive high-resolution melting analysis (MS-HRMA) in comparison with the conventional MSP used in our study. Also, a meta-analysis from He et al [58] estimated the OR for breast malignancy to be 5.92 (95%CI: 3.16-11.07), very similar to ours.

HIN1 promoter was found hypermethylated in 82.1% of all malignant cases, a result not in agreement with the other two breast FNAB studies [52,53] where methylation frequencies were either 33.3% or 97%. Also, we found that HIN1 promoter was methylated in 14.3% of the benign cases which is far smaller than the results from Jeronimo et al [53] who calculated a methylation frequency of 91.7%. For comparison with studies based on histological breast samples, HIN1 methylation frequencies were up to 75% for malignant [38-42,46,48] and up to 70.4% for benign tissues [38-40,46,48], showing slight differences from our results for benign cases with the results for malignant cases being very close. In our study, the OR for breast malignancy was 7.852 (95%CI: 3.085-19.985) in patients with HIN1 hypermethylation while Euhus et al calculated the OR to be 2.77 (95%CI: 0.85-9.02) [52], far less than ours with the difference possibly explained by the fact that in that study RT-MSP was used.

In terms of CDH13 methylation, promoter hypermethylation was found in 76.1% of all malignant and in 14.3% of all benign cases, whereas the relative methylation frequencies in the study by Lewis et al [54] were 36% and 17%, respectively, showing that our methylation frequencies are similar for benign cases and almost double for malignant cases. In studies with histological breast

samples, CDH13 methylation frequencies were up to 97.2% for malignant [41,42,59-62] and up to 19 % for benign tissues [41,42,59-62]. In our study, the OR for breast malignancy was 22.920 (95%CI: 3.368-21.891) in patients with CDH13 hypermethylation which is slightly higher from the result of a meta-analysis from Yang et al [57] who estimated the OR for breast malignancy to be 14.23 (95%CI:5.06-40.05) with the difference possibly explained by the fact for that study the authors included various molecular techniques (RT-MSP, PS, MS-MLPA, MS-HRMA) and calculated both histological and FNAB samples.

We assessed the diagnostic parameter of the methylation status of all genes as a potential indicator for breast malignancy. It seems that the combination of all genes into a multiplex methylation panel has significantly higher SP and PPV than any single gene methylation. High SV is desirable since missing cases of cancer could lead to a delayed correct diagnosis and, in cancer cases where early treatment offers improved chances of recovery, there is increased risk of morbidity and mortality [63]. However, SP is more important in terms of cancer diagnostics since false-positive results lead to the misleading impression of a disease status and thus having unnecessary psychological consequences in patients as well as having to undergo unnecessary possibly invasive diagnostic or treatment procedures [64].

Concerning the differences among our results and the methylation frequencies in the literature, it should be noted that, as all epigenetic mechanisms, DNA methylation has been proved to be significantly affected from various environmental factors such as nutrition, stress, working habits, smoking, alcohol consumption and lifestyle [65-71] in combination with the genome of the study sample for which many studies have revealed that certain genetic methylation profiles (methylomes) appear in specific populations, different from others [72-74] while in this study we focused on Greek population with its geographic, environmental and lifestyle characteristics distinct from study samples in other epigenetic studies. Furthermore, another possible reason for the appeared discrepancies might me that MSP and other related methodologies used in former studies require particular gene sequence information for the design of PCR primers and so, the different primers applied in each study might have impacts on methylation results [75]. Finally, it has been demonstrated that FNAB specimens are significantly enriched in tumor cells compared with surgical resection specimens, resulting in potential discrepancies between them in terms of genetic testing [76,77].

Our study shows that breast FNAB, a safe, well tolerated, cost-efficient, preoperative process combined with methylation epigenetic data from the analysis of the collected cytological material has a promising potential as a biomarker for the early detection of BC risk in women with suspicious breast lesions. Our proposed multiplex methylation panel should be enriched with more TSGs associated with breast carcinogenesis in order to further maximize its diagnostic parameters in a more extensive population, setting possibly the implementation of predictive algorithms for pre-operative staging and therapy response, in combination with the validated testing assays, aiming to optimized BC personalized management.

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Conflict of interests

The authors declare no conflict of interests.

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