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Promoter methylation and expression changes of CDH1 and P16 genes in invasive breast cancer and adjacent normal breast tissue

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We studied the promoter methylation status and expression levels of P16 and CDH1 genes in breast cancer and their adjacent normal tissues with normal control breast tissues, to correlate with their histopathological parameters. Hundred twenty four samples (tumor and adjacent nonmalignant tissues) from 62 breast cancer patients and 4 normal control breast tissues were included in the study. We used methylation specific PCR to evaluate methylation status and quantitative RT-PCR to measure the gene expression levels. Methylation incidence of P16 gene and CDH1 gene in tumor tissues were 24.2 % and 33.9 %, respectively. CDH1 and P16 gene were not methylated in normal control tissues. CDH1 underexpression is found to be significant in correlation with advanced stage, histologic type, high tumor grade and lymph node involvement. P16 expression is found not to be significantly related with any histopathological parameters. But 60% of cases which overexpresses P16 were estrogen negative, and 40% of them were histologic grade 3. Both P16 and CDH1 had different expression levels in tumor tissues compared to the adjacent normal tissues and in adjacent normal tissues compared to the normal non-tumor tissues.

Key words: Breast cancer, P16, CDH1, methylation

Changes in the status of DNA methylation represent one of the most common molecular alterations in human neoplasia [1], including breast cancer [2]. Methylation in breast cancer has been related to clinical and pathologic characteristics evident at presentation and clinical outcomes. A higher prevalence of HIN-1 and RAR β2 methylation was found in the lymph nodes, bone, brain, and lung metastases than the primary tumor [3]. Widschwendter and colleagues [4] reported that the methylation of certain genes was associated with hormone receptor status, in addition to the response to treatment with tamoxifen.

An uncontrolled cell division requires further progression through G1 phase in the cell cycle, where P16/Retinoblastoma(RB) pathway is the target for molecular genetic and epigenetic changes. P16, a cyclin dependent kinase inhibitor which binds to inhibit cyclin dependent kinases 4 and 6 to negatively regulate cell cycle, is one of the most inactivated tumor suppressor genes in human cancers [5]. It was shown that inhibition of P16 can cut loose an epithelial cell of breast tissue from a temporarily trapped phase in cell cycle [6].

The CDH1(E Cadherin) gene encodes the transmembrane glycoprotein E-cadherin that is important in maintaining homophilic cell-cell adhesion in epithelial tissues. CDH1 (E Cadherin), a Ca++ dependent transmembrane glycoprotein functioning in cell to cell adhesion placed in 16q22.1, is one of the cardinal regulators of morphogenesis [7]. Decreased levels of CDH1 expression related with the advanced stage and poorly differentiated cancers [8].

We aimed to find out any possible relationship and concordance between promoter methylation status and expression levels of CDH1 and P16 genes, two critical genes in the carcinogenesis, with the histopathological parameters in sporadic breast cancer and adjacent normal breast tissue.

Materials and methods

Breast cancer primary tissues. Breast tumors and adjacent nonmalignant tissues from resection margin are obtained from the Dokuz Eylül Breast Tumor Biobank (DEBTB) under permission of local clinical and laboratory research ethical council for analysis of patient samples. All the breast tissue samples were collected from patients, who had neither chemotherapy nor radiotherapy before operation. Tissue samples were constituted of tumor cells at average of 60% of the whole specimens

area. True normal control breast tissues were obtained from breast reduction surgeries of otherwise healthy patients. All carcinomas were classified according to the criteria of the World Health Organization [9]. Tumors with lobular and ductal features intermixed in a single tumor.we classified as being 100% mixed, meaning that lobular and ductal features were intermixed in the same tumor. The Elston-Ellis modification of the Scarff-Bloom-Richardson grading system (Nottingham grading system) is based on a microscopic evaluation of morphologic and cytologic features of tumor cells [10]. The clinical stage of the disease was determined according to American Joint Committee on Cancer staging manual [11]. All of the macroscopic and microscopic examinations were performed by the same pathologist.

DNA extraction and methylation analyses. Fresh tumor, adjacent nonmalignant and true normal tissues were homogenized, and genomic DNA was extracted via digestion of homogenized tissues. About 10-20 µg DNA was isolated from tissue pieces of about 1 mg by spin colon technique (RBC Bioscience, Genomic DNA Extraction, Mini-Tissue). About 10-20 µg DNA was isolated from tissue pieces of about 1 mg by spin colon technique (RBC Bioscience, Genomic DNA Extraction, Mini-Tissue) after mechanical homogenization of the tissue. DNA concentration and purity was measured with an UV spectrophotometer (UV-3600, Shimadzu). Bisulfite conversion of DNA was carried out by using the MethylDetector kit (MethylDetector, Active Motive). One microgram purified genomic DNA was converted with bisulfite and purified according to the instructions of the manufacturer. Methylation specific PCR (MSP) was performed using CpG P16 and CDH1 WIZ amplification kit (Chemicon, International). With a complete chemical modification reaction, U primers amplified only unmethylated DNA, and M primers amplified only methylated DNA in the region of gene promoter. W primers amplified unmodified DNA no matter they were methylated or not. Each chemically modified DNA sample was amplified with primers U, M and W respectively.

The PCR mixture contained 12.5 µl hot start polymerase master mix (PyroStart Fast PCR Master Mix, Fermantas), U or M primers 1 µl, bisulfite-modified DNA 0. 1 µg in a final volume of 25 µl. MSP reactions were subjected to initial incubation at 95^{0}C for 10 minutes, followed by 35 cycles of 95^{0}C for 45 seconds, and 60 ^{0}C for 45 seconds and 72 ^{0}C for 60 seconds. Final extension was done by incubation at 72 ^{0}C for 3 minutes. U, M and W controls provided by the kit served as validation of the reagents and PCR conditions. PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The expected PCR products visualized on the gel should be an 154 bp nucleotide for unmethylated P16 and 145 bp nucleotide for methylated P16. The appearance of band W from the sample indicated an incomplete bisulfite conversion, and was considered a sign of unqualified chemical modification of the sample. In vitro methylated DNA (CpG Genome Universal Methylated DNA; Chemicon International) was used as a positive control for methylated alleles, whereas DNA from normal lymphocytes was used as negative controls.

RNA extraction and quantitative real time polymerase chain reaction (qPCR) . The total RNA from clinic sample was isolated by spin colon technique (RBC Bioscience) following mechanical tissue homogenization. RNA isolations were performed from fresh tissue samples which were immediately analyzed by the pathologist after surgical specimen is removed. Concentrations of total RNA were determined with a spectrophotometer (UV-3600, Shimadzu). Approximately 1 µg of total RNA from each sample and a calibrator RNA of a pool of human RNA (Universal Human Normal Tissue RNA, BioTaq) were reverse-transcribed to a single strand cDNA in a final volume of 20 µL using RevertAid™ H Minus first strand cDNA synthesis kit (Fermantas) with 100 pmol random hexamer primers to the manufacturer's instructions. Negative control for reverse transcription was double destilled water water instead of RNA. The synthesized cDNA was either used immediately for PCR amplification or stored at –20°C for further analysis.

Specific primers and hydrolysis probes for CDH1, P16 and 4 reference genes: ACTB (β-actin), B2M (β-2-microglobulin), RPL13A (ribosomal protein L13a) and PUM1 (homolog of Pumilio, Drosophila) were designed by UPL -*universal probe library database*-, Roche Diagnostics (table 1) and qPCR were set up on a 96 well plate Stratagene Mx3000P (Stratagene, La Jolla CA, USA) instrument. A calibrator RNA was used for optimization of primer and probe combinations and plotting standard curves. Standard curves were plotted at 5 serial dilutions of ten fold (from 1 ng to 0.01ng) and with triple replicates.

Table 1 : List of primers and probes for qPCR

Gene	GenBank/EMBL number	Primer 1 (*forward*)	Primer 2 (*reverse*)	UPL Probe no
CDH1	ENSG00000039068	gccgagagctacacgttca	gaccggtgcaatcttcaaa	80
P16	ENSG00000147889	gtggacctggctgaggag	tctttcaatcggggatgtct	34
RPL13A	NM_012423	gaggcccctaccacttcc	aacaccttgagacggtccag	28
B2M	NM_004048	taggagggctggcaacttag	cttatgcacgcttaactatcttaacaa	32
ACTB	NM_001101	ccaaccgcgagaagatga	ccagaggcgtacagggatag	64
PUM1	NM_001020658	agtgggggactaggcgttag	gttttcatcactgtctgcatcc	22

Table 2: CDH1 and P16 promoter methylation distribution according to clinical and histopathologic variables in 62 invasive breast cancer

Variable	CDH1 methylation			P16 methylation		
	Presence N (%)	Absence N (%)	p value#	Presence N (%)	Absence N (%)	p value#
Ages			0.289			0.254
≤50	12 (41.4%)	17 (58.6%)		5 (17.2 %)	24 (82.8 %)	
50<	9 (27.3%)	24 (72.7 %)		10 (30.3 %)	23 (69.7 %)	
Histologic type			0.046*			0.735
Ductal	8 (32 %)	17 (68 %)		5 (20 %)	20 (80 %)	
Lobular	9 (45 %)	11 (55 %)		6 (30 %)	14 (70 %)	
Mixed	4 (23.5 %)	13 (76.5 %)	0.000**	4 (23.5 %)	13 (76.5)	0.493
Stage						
I	0			3 (20 %)		
II	15 (100 %)			12 (80 %)		
	3 (15 %)	17 (85 %)		3 (15 %)	17 (85 %)	
III	10 (52.6 %)	9 (47.4 %)		6 (31.6 %)	13 (68.4 %)	
IV	8 (100 %)			3 (37.5 %)		
Histologic grade	0			5 (62.5 %)		
I			0.000**			0.272
II	0			2 (15.4 %)		
	13 (100 %)			11 (84.6 %)		
	3 (13 %)	20 (87 %)		5 (21.7 %)	18 (78.3 %)	
III	18 (69.2 %)			8 (30.8 %)		
Lymph node	8 (30.8 %)			18 (69.2 %)		
N0			0.000**			0.390
	2 (7.4 %)	25 (92.6 %)		5 (18.5 %)	22 (81.5 %)	
N≤1	19 (54.3 %)	16 (45.7 %)		10 (28.6 %)	25 (71.4 %)	
ER			0.376			0.108
Positive	13 (29.5 %)	31 (70.5 %)		8 (18.2 %)	36 (81.8 %)	
Negative	8 (44.4 %)	10 (55.6 %)		7 (38.9 %)	11 (61.1 %)	
HER2			0.347			0.799
Positive	14 (29.8 %)	33 (70.2 %)		11 (23.4 %)	36 (76.6 %)	
Negative	7 (46.7 %)	8 (53.3 %)		4 (26.7 %)	11 (73.3 %)	

p-value obtained from Fisher's exact test. *significant at the 0.05 level (2-tailed), **significant at the 0.01 level (2-tailed)

Fluorescence reading was performed at the end of the annealing step. All amplification reactions were performed in triplicate. As negative control, samples with PCR mix and reverse transcription PCR negative control were used. The formula $2^{-\Delta\Delta Ct}$ was used to calculate relative quantitation values from data of an individual sample to normalize with its housekeeping gene for comparing with the normal tissue to show the expression differences in folds.

Statistical analysis. NormFinder, which is an excel based software, was used to decide on the most stable reference gene on tumor, adjacent nonmalignant and true normal tissues among studied reference genes [12]. Other statistical analysis were performed using a software package SPSS (Statistical Package for The Social Sciences v13.0, SPSS Inc). The distribution of data was tested for normality using Kolmogorov-Smirnov test. Statistical analysis was performed using Fisher's exact test for differences between groups and Mann Whitney tests between means. Kendall's tau correlation was used to determine the association between gene expressions and methylation status, histopathological parametres. All reported p values are two-sided and considered statistically significant if $p < 0.05$.

Results

Individuals. Hundred twenty four samples (tumor and adjacent nonmalignant tissues) from 62 breast cancer patients and 4 non-tumor normal breast tissues were included in the

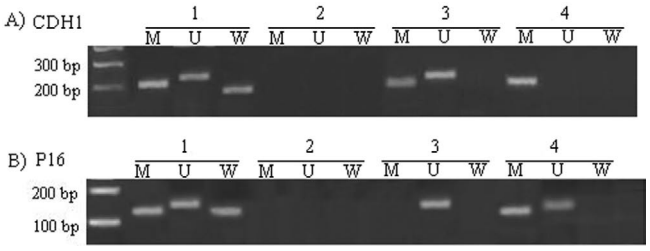

Figure 1: Gel electroporesis view of MSP product of P16 and CDH1 promoters

A) CDH1 promoter region: 1- M, U, W positive control, 2- M, U, W negative control, 3- Sample with both M and U alleles, 4- Sample with presence of only M allele

B) P16 promoter region: 1- M, U, W positive control, 2- M, U, W negative control, 3- Sample with presence only U allele, 4- Sample with both M and U alleles

study. At the time of diagnosis, breast cancer patients ranged in age from 26 to 81 years old (mean 51.2, median 53). At the time of breast reduction surgeries, healthy individuals ranged in age from 30 to 41 years old (mean 36, median 36.5). Table 2 shows their histopathological features.

Methylation analyses. Initially, we analyzed the methylation status in the breast tissues. A representative examples of methylated and unmethylated gene analysis from breast tissues and control was shown in Figure 1.

Methylation incidence of P16 gene and CDH1 gene in tumor tissues were 24.2 % and 33.9 %, respectively. At least one of the genes were methylated in 58.1% of the tumor tissue samples. Table 2 shows the details of associations between CDH1 and P16 gene methylation and clinical and histological parameters in the group of 62 invasive breast cancer. No statistically significant differences in the frequencies of CDH1 and P16 gene promoter methylation were found between the patients at ages ≤ 50 and > 50 years old. CDH1 promotor methylation frequency could significantly explain differences in stage, histologic type, grade and status of lymph nodes (more or less than one positive lymph nodes). No statistically significant differences in the frequencies of P16 gene promoter methylation were found between the patients at histopathological parameters

In adjacent nonmalignant breast tissues, methylation of CDH1 was present in two patients (a ductal type, histologic grade 3, stage 3), when no methylation observed in P16 gene. CDH1 and P16 gene were not methylated in normal breast tissues.

Gene expression analyses. Amplification efficiencies and Rsq values of CDH1, P16 and four candidate references genes which were calculated by the MxPro analysis software of the Stratagene Mx3000P instrument are showmn in table 3. The RSq value (R^2, linear correlation coefficient), an indicator of fit for the standard curve plotted to the standard data points of all genes ranged from 0.996 to 1.000. Based on the slopes of the standard curves, the amplification efficiencies ranged

Table 3: Gene amplification efficiencies and RSq values

Gene	Efficiency %	Slope	RSq
CDH1	100,4	3,313	0.997
P16	100	3,322	1.000
RPL13A	101	3,288	0,998
B2M	86,1	3,717	0,999
ACTB	100,2	3,318	1,000
PUM1	101,4	3,327	0,996

Table 4: Candidate reference gene ranking by NormFinder

Rank	Candidate Genes	Average expression stability values	Standard Error
1	PUM1	0.259	0.104
2	RPL13A	0.525	0.074
3	B2M	0.774	0.076
4	ACTB	1.365	0.102

from 86 % to 101 %. Avarage expression stability values by NomFinder software which are used in deciding the reference gene to be used in relative quantitation calculations are shown in table 4. More stable gene expression is indicated by lower average expression stability values. PUM1 gene is ranked as the most stabile gene among all and preferred for use in normalization.

P16 and CDH1 gene expression levels in tumor and adjacent normal tissues compared to the normal tissue are shown in figure 2. Both P16 and CDH1 had different expression levels in tumor tissues compared to the adjacent normal tissues and in adjacent normal tissues compared to the adjacent normal tissues and in adjacent normal tissues compared to normal tissues ($p<0.05$, $p<0.001$and $p<0.001$, $p< 0.001$, respectively).

Table 5 shows the correlation of P16 and CDH1 gene expression levels with histopathologic features in tumor tissues. No significant correlation was observed between methylation status of P16 gene and expression levels and there were no significant differences in the P16 mRNA levels between P16 methylated and non-methylated groups. P16 expression pattern is found not to be significantly related with any histopathologic features. Six cases out of the 10 which have increased levels of P16 compared to normal tissues were estrogen negative and 4 of them were histologic grade 3.

There was very significant correlation of CDH1 gene methylation with decreased expression of CDH1 mRNA (table 5). The mRNA levels of CDH1 were significantly lower in the CDH1 methylated group compared to the non-methylated cases ($p<0.001$). CDH1 expression was significantly lower in the lobular type compared to the ductal type ($p<0.05$),

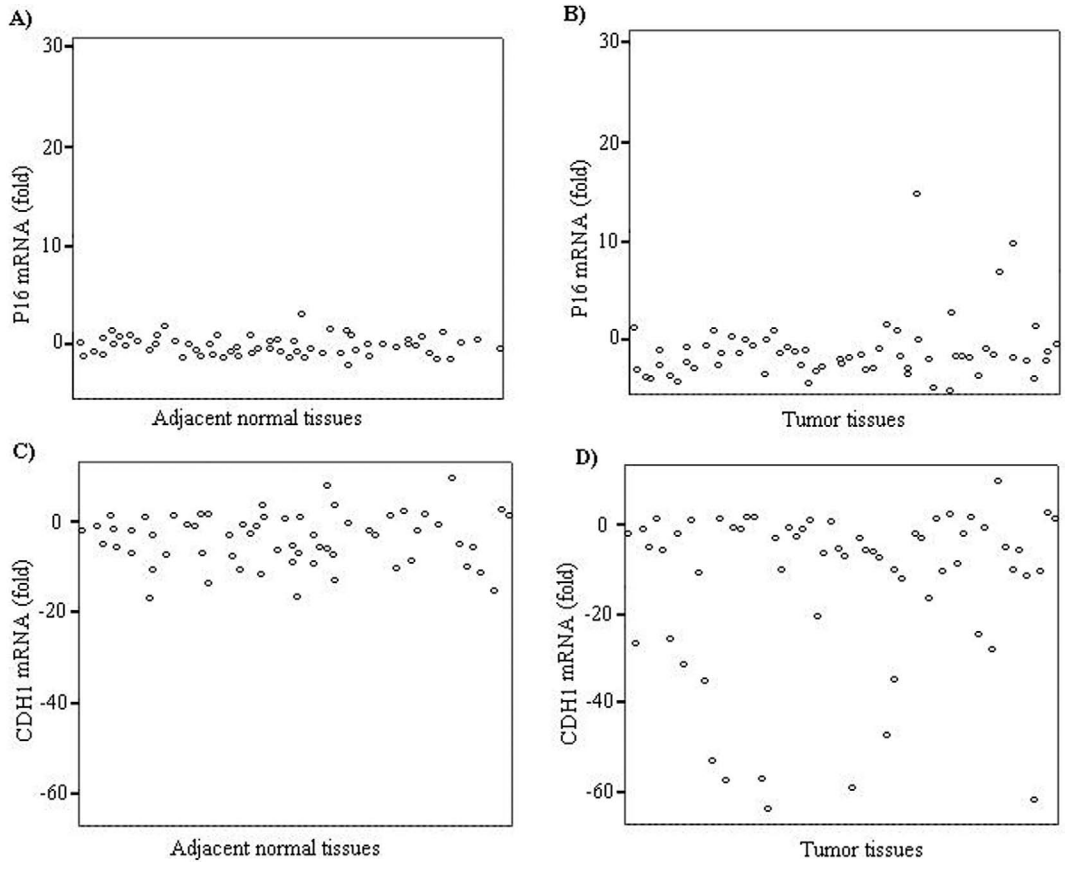

Figure 2: Expression levels of P16 and CDH1genes

A) P16 mRNA levels in adjacent normal tissues compared to the normal non-tumor breast tissue
B) P16 gene expression levels in tumor tissues compared to the normal non-tumor breast tissue
C) CDH1 gene expression levels in adjacent normal tissues compared to the normal non-tumor breast tissue
D) CDH1 gene expression levels in tumor tissues compared to the normal non-tumor breast tissue

but there were no significant difference when compared to the mixed type ($p>0.05$). There were significant correlation between CDH1 expression and stage, histologic type, grade and lymph node involvement (table 5).

Age was found to be not correlated with either P16 or CDH1 expression levels.

Discussion

We studied the promoter methylation status and expression levels of P16 and CDH1 genes in breast cancer tissues and their adjacent normal breast tissues with normal non-tumor breast tissues. We used MSP for analysis of the methylation status of P16 and CDH1 promoter. This method can determine the presence or absence of methylation of a gene locus at a sensitivity level of one methylated allele in thousand unmethylated alleles, appropriate for the detection of neoplastic cells in a background of normal cells [13].

We used qRT-PCR, which is widely and increasingly used for the quantification of mRNA, for analysis of the methylation

Table 5: CDH1 and P16 gene expressions correlations according to clinical and histopathologic variables in 62 invasive breast cancer

Variable	Kendall's tau correlation	CDH1	P16
Ages	Correlation coefficient	0.128	0.148
	Significant	0.264	0.207
Histologic type	Correlation coefficient	0.245	0.104
	Significant	0.037*	0.449
Stage	Correlation coefficient	-0.386	0.003
	Significant	0.000**	0.975
Histologic grade	Correlation coefficient	-0.418	0.035
	Significant	0.000**	0.724
Lymph node	Correlation coefficient	-0.503	0.045
	Significant	0.000	0.670
ER	Correlation coefficient	0.160	0.228
	Significant	0.129	0.068
HER2	Correlation coefficient	0.092	0.081
	Significant	0.384	0.445
CDH1 promoter methylation	Correlation coefficient	0.360	0.001
	Significant	0.001**	0.994
P16 promoter methylation	Correlation coefficient	0.173	0.055
	Significant	0.100	0.605

* significant at the 0.05 level (2-tailed), **significant at the 0.01 level (2-tailed)

status of P16 and CDH1 mRNA. The reliability of qRT-PCR data will be greatly improved by inclusion of a reference gene which passed all steps of the analysis similarly to the gene to be quantified [14]. In this study interestingly, B2M and ACTB that are often referred to as reference genes in gene expression studies are identified to be the most unstable gene among four candidate reference genes with NormFinder (Table 4). Supporting the study of Lyng MB et all., our data displays that PUM1 is the best as an internal reference gene [15]. This demonstrates that a careful screen for reliable reference genes is indispensable for each individual experimental situation.

The frequency of P16 methylation in breast cancer ranged from 14–37.5% [16–18]. In this study, P16 gene promoter was found 24.2% hypermethylated in patients with sporadic breast cancer. The observed differences in the methylation frequencies could be differences in clinical tissue specimens (fresh and formalin fixed paraffin embedded tissues etc) and the role of unknown environmental factors. This study was performed from fresh tissues that had been surgically removed (immediate removal and freezing). The FFPE tissue is still considered as a difficult substrate due to the extensive cross linking of proteins and the degradation and fragmentation of the macromolecules caused by formalin fixation [19]. Though great advances have been made in the development of sensitive techniques for the utilization of formalin fixed material for molecular analysis, the assessment of gene methylation in FFPE tissues is still challenging due to a small volume of tissue and excessive DNA fragmentation. DNA, quality and preservation in the sections from formalin-fixed and paraffin-embedded tissue blocks might not always be optimal.

It is widely argued how P16 promoter methylation affects in primary tumors. Although most of authors hold the traditional viewpoint that detectable P16 methylation necessarily link to the inactivation of P16 protein, or transcriptional silencing of P16 gene [20, 21], coexistence of P16 methylation and P16 expression in tumors has been frequently described [22, 23]. Our findings support coexistence of P16 methylation and P16 expression in tumors. Inconsistencies between P16 promoter methylation status and mRNA expression may result from varying transcript stability, post-transcriptional modifications, or reflect intra-tumor variations [24]. P16 gene hypermethylation may not be the sole or even dominant mechanism for loss of expression of p16. Unlike some previous studies suggest that overexpression of mRNA is a marker of poor prognosis [24], we could not find any correlation between P16 expression levels and any histopathologic parameters. But most of the cases with overexpression of P16 were estrogen receptor negative and histologic grade 3. This inverse relationship between P16 and ER status may support the hypothesis that high P16 levels could reduce the requirement for estrogen for proliferation of breast cancer cellls of Hui et al [24, 25]. However, additional assays in a larger series of patients with longer follow-up times will be necessary, to understand whether P16 promoter methylation and over expression is a prognostic factor in breast cancer.

CDH1 is involved in maintaining cell-to-cell adhesion and is regarded as suppressor of cellular invasion [26]. Furthermore, loss of CDH1 expression in primary tumors has been associated with decreased patient survival [27]. These findings are consistent with our results demonstrating CDH1 methylation and loss of E-cadherin mRNA expression predominates in primary tumors with a more aggressive phenotype (high tumor stage and high histologic grade). We also showed that promoter methylation of CDH1 significantly correlated with the CDH1 expression level, which is previously suggested with a limited number of studies [8].

The most visible feature distinguishing lobular and ductal tumors is the absence of E-cadherin expression in the previous studies [28, 29]. In two different gene expression studies comparing ductal and lobular carcinomas, the unique common discriminator identified was CDH1, which was significantly down-regulated in lobular samples [30, 31]. Loss of E-cadherin mRNA expression for infiltrating lobular tumor types is often a biallelic event resulting from any combination of gene promoter hypermethylation, mutation, or allelic loss, whereas ductal histology often presents with varying levels of expression [32]. In our series, P16 promoter methylation status was not related with histologic subtype, while CDH1 methylation was more common in tumors with infiltrating lobular histology and expression levels were decreased in lobular type.

We cannot detect any methylation of E-cadherin and P16 in our normal non-tumor breast tissue controls. Also, contrary to some of previous studies [33], there had either been no methylation but one in adjacent normal tissues. Considering that the methylation changes begin to appear at early stages, quantitative MSP, which has a sensitivity of 1:10,000 [34] will be a beter technique to be preferred instead of the conventional MSP, which has a sensitivity of 1:1000 [13]. This may particulary be more suitable for the adjacent normal tissues. Adjacent normal tissues showed an expression pattern different from their tumor tissues and also different from the true normal controls, supporting a previous study [35]. The presence of the expression changes in adjacent nonmalignant tissue at the border of resection in larger groups of patients suggests that this could be a marker of disease risk, occult disease, or the tissue's response to an existing tumor.

Analyzing the methylation status and expression levels of many genes in larger homogenous series of patient may be helpful in tumor subtyping and possible molecular prognostic markers in cancer treatment and also add relevant information for clinical management.

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