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Original Research

Methylated p16 gene is associated with negative expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 in breast cancer

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Objective: This study was conducted to determine the relationship between p16 gene methylation and expression of relevant receptors in breast cancer (BC) for subtyping the disease. *Methods*: Methylation-specific PCR (MSP) was carried out to detect the methylation status of p16 gene in 240 tissue samples and 205 serum samples from BC patients treated at our hospital. Immunohistochemistry was used to determine the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Receiver operating characteristics (ROC) curve was analyzed for diagnostic value based on methylation status for triple-negative (TN) BC. *Results*: The overall methylation rates of the p16 gene were 36.7% (88/240) and 35.1% (72/205) in the tissue and serum samples, respectively. In patients with ER, PR and HER2-TNBC, the methylation rate of the p16 gene was significantly higher than that in non-triple negative patients (84.9%, 62/73) vs (25.9%, 35/135, $P < 0.01$). The methylation of p16 gene was negatively associated with the expression of ER, PR and HER2 (r = -0.661, -0.694 and -0.765, respectively, $P < 0.05$), but it was not correlated with the pathological characteristics of BC, such as tumor grade and lymph-node metastasis. Receiver operator characteristic (ROC) curve analysis showed that p16 gene methylation had a significant diagnostic value for TNBC with an AUC of 0.815. Therefore, p16 gene methylation is associated with the subtype of TNBC and can be used as an easy and non-invasive approach to screen patients for TNBC.

Keywords

Breast cancer; Methylation; p16 gene; Estrogen receptor; Progesterone receptor; Human epidermal growth factor receptor; Diagnostic value

1. Introduction

Breast cancer (BC) is made up of 25% of all types of cancer in women and is a leading cause of cancer death. Despite significance advance in diagnosis and treatment of the disease, it remains a global public health issue, resulting in 450,000 deaths annually [1, 2]. Although it is curable in about 70–80% of patients with early-stage, non-metastatic tumor, advanced BC is considered difficult to cure with currently available therapies such as conventional chemotherapy and radiation therapy [3]. During the past decades, five molecular subtypes of BC have been discovered [4]. For BC therapy, molecular subtype plays very important role in defining treatment plans and strategies, determining the therapeutic outcome and prognosis [5, 6]. Triple negative BC (TNBC) is a molecular subtype of BC where the expressions of estrogen receptor (ER), progesterone receptor (PR), and HER2 are negative [7]. It accounts for about 15% to 20% of BC and has poor prognosis [8]. Chemotherapy and radiation are used primarily as systemic therapeutic strategies and FDA-approved targeted therapies are not yet available for TNBC. Since TNBC is highly proliferative and metastatic, without targeted therapies, various therapeutic schemes and the clinical trials currently are underway for TNBC management [3], but effective molecular therapy is still not available for TNBC [9]. Neoadjuvant chemotherapy is shown to benefits TNBC patients with locally advanced disease by downsizing the tumor [10]. It is therefore important to classify BC for their subtype. Currently, BC subtyping is mainly based on immunohistochemistry assay of excised tissues for these genes, in which biopsy or surgical resection is used to generate tumor tissue samples for immunohistochemistry analysis. This is invasive and its accuracy and throughput may be limited due to availability and uneven development of the cancer tissue [11]. One way to resolve this issue is to apply imaging technology for BC subtyping, which is noninvasive and can be used to characterize tumor in details to differentiate the subtypes at the molecular level. It is provided a tool to dynamically evaluate the outcomes during therapeutic process [12, 13].

Using blood sample to detect cancer has been explored for decades. Tumor biomarkers, such as carcinoembryonic antigen (CEA), cancer antigen (CA)15-3 and circulating tumor cell (CTC) count have been developed to clinically diagnose cancer [14, 15]. However, their usefulness is sometime restricted to patients having advanced or metastatic cancer due to low detection limits particularly for BC. In recent years, epigenetic modifications, such as methylation, have been revealed to be involved in the development of BC. Methylation is shown to occur in many BC-related genes [16] and hypermethylation of CpG islands often leads to the silence of a variety of genes, such as *ARHI* [17], *RASSF1A* [18] and the retinoic

acid receptor II gene (*RARβ2*) [19]. High occurrence of *HIN-1* and *RAR β2* methylation has been found in the lymph nodes and lung metastases of BC [15], suggesting that methylation is also associated with clinical presentation and therapeutic outcomes [20].

The tumor suppressor p16 has been observed to alter frequently in many primary tumors and therefore plays important role in carcinogenesis. For example, this gene is silenced through homozygous deletion, methylation of the promoter, and point mutation, resulting in neoplasms [21]. p16 gene acts as a cyclin-dependent kinase inhibitor (CDKI) to suppress the transcription of cell-cycle regulatory proteins and may suppress the migration and invasion of cancer cells to exert its anticancer activity in lobular breast carcinoma [22]. It arrests cells at G1/S phase by suppressing the formation of cyclin D-CDK4/6 complex through binding to CDK [23]. Methylation of CpG islands results in inactivation of the p16 gene and this has been observed frequently found in human cancers such as papillary thyroid carcinoma [24], colorectal cancer [25] and breast cancers [26]. Some reports have also shown that increased p16 expression leads to poor prognosis in breast cancer [27, 28]. Methylated DNA fragment of the p16 promoter region was first detected in plasma samples of BC patients [29]. However, it had been extremely difficult to detect these DNA methylation markers in the blood for routine BC detection and subtyping [30]. With current availability of commercial methylation detection kit and high-throughput DNA assay, it has become feasible to routinely assay the methylation status of DNA extracted from different sources [31, 32] and use methylation status as epigenetic markers to diagnose breast cancer [33].

In the present study, methylation-specific PCR (MSP) was used to determine the methylation status of the p16 gene and analyzed its relationship with ER, PR and HER2 expressions. The findings would provide new way to classify patients based on their BC subtypes for better therapeutic plans and outcomes and offer a high through-put and low-cost screening tools for TNBC.

2. Materials and methods

2.1 Samples

Blood and tumor tissue samples were taken from patients undergoing surgery at our hospital between April 2017 and April 2019. The patients were all female and pathologically proven to have invasive ductal carcinoma (IDC) at TNM I to IV based on Union for International Cancer Control (UICC) TNM Classification of Malignant Tumours. The tissues were excised during surgery and immediately stored at -20°C before analysis. Fasting venous blood was collected in the morning within three days after admission. Demographic and clinical data including age, gender, underlying diseases, treatment methods, medication and laboratory findings were collected. The study protocols were complied with the Declaration of Helsinki and were approved by the institutional ethics committee of Tangshan People's Hospital, Tangshan, China (TPH 211A) and performed in accordance with relevant guidelines and regulations. Written informed consent was obtained from every patient participated in the study.

2.2 MSP

MSP was used to analyze the methylation of the p16 gene as previously described [34]. Briefly, DNA was extracted from the tissue and blood samples using commercial DNA extract kit (Qiagen, Valencia, CA, USA) according to the manufacture's protocols. After the DNA was isolated, the concentrations were quantified using NanoDrop microvolume spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA) for bisulfite conversion. The isolated DNA was diluted to 5 μg/mL. Bisulfite conversion was conducted with the EZ DNA Methylation-Gold kit (Zymoresearch, Irvine, CA, USA). During the bisulfite treatment, unmethylated cytosine in the DNA sequences is transformed to uracil while methylated cytosine is not changed. For MSP, two sets of primers specifically targeting the methylation-prone site of the p16 gene were used. One primer is specific to the methylated sequence that recognizes converted cytosine, whereas the other recognizes unmethylated sequences. The primer sequences are as follows: mp16 forward 5′- TTTT-TAGAGGATTTGAGGGATAGG -3′, mp16 reverse 5′-CTACCTAATTCCAATTCCCCTACA-3′, up16 forward 5′-GTTTTCCCAGTCACGACAGTATTAGGAGGAAGAA AGAGGAG-3′, and u16 reverse 5′- TCCAATTCCCCTA-CAAACTTC -3′. PCR was performed in triplicate at 95 °C for 15 min, followed by 40 cycles of 95 °C for 50 s, 60 °C for 50 s and 72 °C for 50 s, with a final extension at 72 °C for 10 min. The PCR was run in triplicate on C1000 Touch^{TM} thermal cycler (Biorad, Hercules, CA, USA) and amplified DNA was visualized on 2.5% agarose gel after electrophoresis. Universal methylated and unmethylated DNA were obtained from Chemicon International, USA and used as positive and negative controls, respectively.

2.3 Immunohistochemistry assessment

Five μm thick sections were deparaffinized in xylene and rehydrated. Antigen was retrieved by heating in microwave oven in 10 mM citric acid monohydrate for 1 × 5 min at 900 W. Endogenous peroxidase activity was blocked by immersing the sections in 0.5% H_2O_2 for 5 min. The slides were incubated with appropriate dilutions of the primary antibodies overnight in a refrigerator at 4 °C. PBS diluent without antibody was used as negative control. The immunological reaction was visualized using the Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) for ER, the Envision kit (Dako, Copenhagen, Denmark) for HER2 and PR. Diaminobenzidine (DAB) and haematoxylin chromogen (Dako, Glostrup, Denmark) methods were used to develop the immune-reactive bands. The sections were subsequently cleared and mounted using ProLong antifade reagent (Invitrogen). To control variations and allow quantitative comparisons of band intensity, all tissue sections were reacted to antibodies simultaneously. For each sample, cells were counted

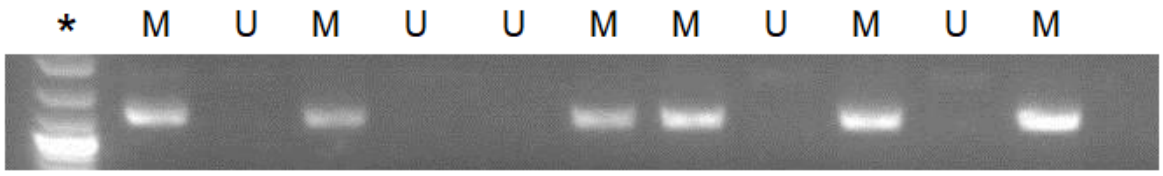

Fig. 1. Representative MSP of *p16* gene. M, U and * denote methylated, unmethylated and DNA molecular weight markers, respectively.

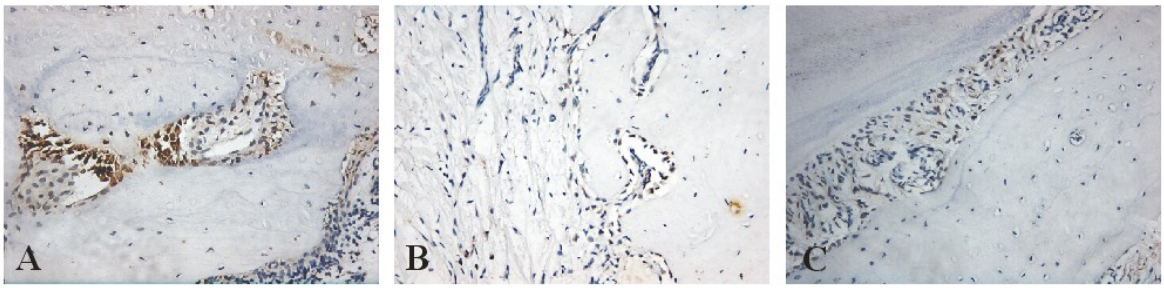

Fig. 2. Representative IHC images of breast tissues showing negative expression of ER (A), PR (B) and HER2 (C). Cancer tissue sections were reacted with antibodies against ER, PR and HER2, and immunoreactivity was visualized using diaminobenzidine and haematoxylin chromogen, showing low intense nuclear staining of neoplastic cells (original magnification 200×).

in five field (×400). For the analyses, the tissue samples were rated as positive for ER, PR and HER2 when ≥ 1% of the tumor cells displayed positive nuclear staining.

2.4 Statistical analysis

The data were analyzed by SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables with normal distribution were presented as mean ± (standard deviation [SD]); non-normal variables were reported as median (interquartile range [IQR]). $\chi 2$ test was used to compare the frequencies. The association of methylation status with the clinical parametres was analyzed by Fisher's exact test. Pearson correlation analysis was performed to analyze the relationship between p16 gene methylation and TNBC. Receiver operator characteristic (ROC) curves were drawn to analyze the predictive values of p16 gene methylation for TNBC. A P value < 0.05 was considered statistically significant.

3. Results

3.1 Patients' clinical data

Patient characteristics are presented in Table 1. A total of 240 subjects were included in this study, all of them were female and a median age was 39.2 (21–69) years. 7.9% of patients had diabetes and 8.8% had hypertension. BC was at TNM stages I to IV (30.4%, 22.5%, 27.9% and 19.1%, respectively). 14.2% of them underwent total resection and the rest had partial resection for BC. Pathological examinations showed that there were lymph-node metastasis, vascular and lymphatic invasion and the majority of the BC were ductal (Table 1). Immunohistochemistry assays showed that 47.7%, 49.6% and 85.8% of the patients were negative for ER, PR or HER-2, respectively, and there were 73 (30.4%) TNBC patients in the cohort (Table 1).

3.2 p16 gene methylation was associated with ER, PR and HER2 expression

We assayed 205 blood samples and 240 tissue samples of the patients for methylation status of the p16 gene isolated using MSP (Fig. 1). The methylation rates were 36.7% (88/240) and 35.1% (72/205) in the tissue and serum samples, respectively. Among the methylated samples, a consistency 92.8% was observed between serum and tissue samples.

We then analyzed the relationship between the immunohistochemistry results (Fig. 2) and MSP results of serum DNA. Among the 73 TNBC, methylation was found in 62 (84.9%, 62/73) patients, which is significantly higher than the rate in NTNBC patients (25.9%, 35/135, $P < 0.01$). Analysis based on Pearson's correlation showed that p16 methylation was negatively associated with expression of ER (r = -0.661), PR (r = -0.694) and HER2 (r = -0.765, $P < 0.05$, Table 2). We also analyzed the correlation between p16 gene methylation and the pathological characteristics of BC and found that the methylation status was not associated with tumor grade or lymph node metastasis (Table 2).

3.3 p16 gene methylation had predictive value for TNBC

We further analyzed the diagnostic value of p16 gene methylation for TNBC using ROC curve. The area under the curve (AUC) of p16 gene methylation to predict TNBC was 0.815 (Fig. 3). At the cutoff point, the diagnostic sensitivity and specificity of the prediction were 85.0% and 82.6%, respectively, and the positive predictive value and negative predictive value were 91.2% and 54.8%.

4. Discussion

Recent studies have revealed that both epigenetic and genetic mechanisms are involved in the BC occurrence and development. Methylation of DNA is one of the most

Table 1. Patient's characteristics.

Patient characteristics		N (%)
Sex		
	Male	0 (0.0)
	Female	240 (100.0%)
Age, years		
	< 30	88 (36.7)
	≥ 30	152 (63.3)
Diabetes		19 (7.9)
Hypertension		21 (8.8)
Tumor grade (TMN)		
	I	73 (30.4)
	II	54 (22.5)
	III	67 (27.9)
	IV	46 (19.1)
Surgery		
	Total resection	34 (14.2)
	Non-total resection	206 (85.8)
Lymph-node metastasis		
	pN0	69 (28.8)
	pN1	90 (37.5)
	pN2	31 (12.9)
	pN3	50 (20.8)
Vascular invasion		
	Positive	21 (8.8)
	Negative	219 (91.2)
Lymphatic invasion		
	Positive	166 (69.2)
	Negative	74 (30.8)
ER status		
	Positive	127 (52.9)
	Negative	113 (47.7)
PR status		
	Positive	121 (50.4)
	Negative	119 (49.6)
HER-2 status		
	Positive	34 (14.2)
	Negative	206 (85.8)
ER/PR/HER-2		
	Positive	167 (69.6)
	Negative	73 (30.4)
Histology		
	Ductal	212 (88.3)
	Lobular	11 (4.6)
	Mixed ductal and lobular	8 (3.3)
	Other	9 (3.8)

prominent mechanisms underlying epigenetic modificaitons [5]. Previously, DNA methylation was mostly detected using DNA extracted from tissues, such as excised tissue from surgery or biopsy. Recently, methylation is also reported in DNA extracted from serum and the results in the tissues are highly consistent with serum DNA [35, 36]. Our study demonstrated that p16 methylation occur consistently both in serum and tissue samples, indicating that serum could be used as the DNA source for characterization of p16 gene

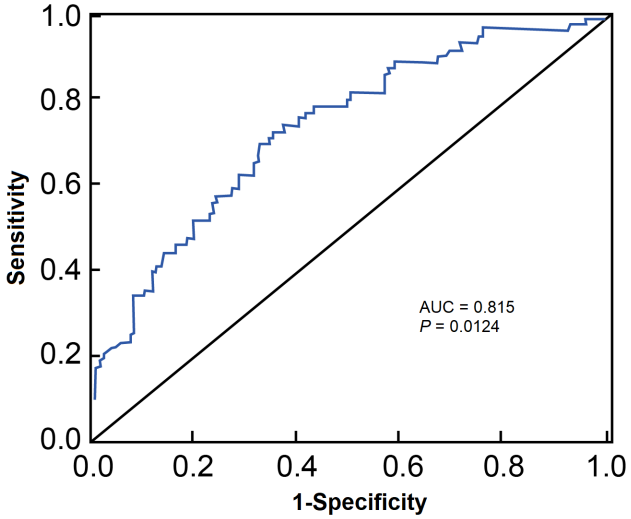

Fig. 3. Receiver operator characteristic (ROC) curves of p16 methylation-based prediction of triple-negative breast cancer. The area under the curve (AUC) of p16 gene methylation to predict TNBC is 0.815, the diagnostic sensitivity and specificity of the prediction are 85.0% and 82.6%, respectively, and the positive predictive value and negative predictive value are 91.2% and 54.8%, respectively.

methylation. This would substantially simplify the assessment since the sampling of serum is basically not invasive and can be performed routinely in any clinical settings. It may also be taken several times as needed over the therapeutic time to monitor the development and progress of BC during the treatment. Previously, serum DNA has been used to detect hepatocellular carcinoma (HCC) in early stage by characterizing their methylation status before diagnosis. Interestingly, methylation was found in serum DNA 1 to 9 years before HCC was clinically diagnosed and different genes were methylated at different frequencies. As a result, epigenetic modifications of *RASSF1A*, *p16*, and *p15* tumor suppressor genes were proposed as serum biomarkers for early detection in populations with high HCC risk [37].

The growth of BC is dependent on estrogens. ER and PR expressions are closely related to the prognosis and outcome of endocrine therapy for BC. In general, ER- and/or PR- positive patients have better therapeutic response than those who are negative for ER- and/or PR. Since TNBC patients response differently to endocrine therapy compared to NTNBC patients, they need to be identified for better treatment and prognosis before treatment plan is defined. HER2 gene has been shown to play important role in the biological behavior and pathogenesis of BC via a variety of intracellular signaling pathways. Serine/threonine protein kinase B (Akt) and nuclear factor-κB (NF-κB) may be activated due to the overexpression of HER2, triggering anti-apoptosis cascades, resulting in the development of resistance to tumour necrosis factor (TNFα) in BC cells and reduced host defense to tumors. Recently, there are some important progresses in treatment of TNBC with endocrine therapy and targeted

Table 2. Correlation between p16 methylation and expression of ER, PR and HER2 and tumor grading in patients with breast cancer.

Variables			Serum DNA of p16 gene		*P*	r
			Methylated	Unmethylated		
Immunohistochemistry						
	ER status	Negative	53	32	0.010	-0.661
		Positive	27	94		
	PR status	Negative	49	38	0.012	-0.694
		Positive	18	100		
	HER-2 status	Negative	123	43	0.010	-0.765
		Positive	14	25		
Tumor grade (TMN)						
	I		12	11	0.210	
	II		35	37	0.326	
	III		32	36	0.510	
	IV		21	21	0.910	
Lymph-node metastasis						
	pN0		44	42	0.410	
	pN1		32	28	0.526	
	pN2		20	19	0.680	
	pN3		11	11	0.540	

therapy against ER and HER2 [29, 30]. Our study showed that p16 gene methylation is negative associated with expression of ER, PR and HER2 and can be used to predict TNBC patients with a reasonable prediction power (AUC = 0.815). This is consistent with previous results that the occurrence of p16 hypermethylated BC cases was remarkably higher in TNBC than in $ER^{+}PR^{+}Her2^{-}$ patients [38]. Since it is based on serum assay, the assay can be applied to screen patients for TNBC either before surgical operation or over treatment, and used in follow-up period to monitor the change and outcome following surgical and chemotherapy. In earlier studies, p16 gene methylation was found to be negatively associated with ER, and could be used as a prognostic indicator for malignancy and high differentiation, particularly in postmenopausal women [31]. Furthermore, the association of p16 gene methylation with the expression of ER, PR and HER2 plays role in regulating the occurrence and progression of BC. For example, in BC patients, p16 methylation in serum DNA is shown to negatively related BC grading [32]. However, we did not observe that p16 gene methylation has correlation with tumor grading and lymph node metastasis, suggesting that methylation in BC might have occurred before clinical diagnosis in this patient cohort. In an earlier study, methylation of tumor-suppressor genes such as RIL and CDH13, but not p16 were found strongly correlated to negative ER, PR, and HR expression [26]. On other hand, high p16 protein expression was found to be related to ER-positive, PR-negative, and HER2-negative tumors, suggesting that p16 protein expression has prognostic value in measuring treatment response [39].

There are limitations in this study. It was a single center study, the sample size was relatively small, blood samples were not analyzed for all patients and the patients were not followed up for changes in methylation status. It is therefore necessary to further validate our results with large study.

5. Conclusions

Our work shows that the methylation of p16 gene can be detected in the circulating blood as well as in tissue sample. It is associated with expression of ER, PR and HER2 and can be used to screen patients for TN subtype of BC. It may be used an easy to operate and low cost tool to diagnosis TNBC patients.

Abbreviations

AUC, the area under the curve; BC, breast cancer; CA15-3, cancer antigen circulating tumor cell; CEA, carcinoembryonic antigen; CTC, circulating tumor cell; ER, estrogen receptor; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor 2; IQR, interquartile range; MSP, methylation-specific PCR; PR, progesterone receptor; ROC, receiver operator characteristic; SD, standard deviation; SP, streptavidin-peroxidase; TNBC, triple negative breast cancer; TNM, tumor, node, metastasis; UICC, Union for International Cancer Control.

Author contributions

SLZ, YQW and JHZ designed the study. SLZ, YQW, JHZ, JWH, JM, ZG and YQW collected the data and performed analysis. SLZ and JJC drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethical committee of this center (TPH 211A) and performed according to relevant guidelines and regulations. Written informed consent was obtained from every participant.

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Conflict of interest

The authors declare no conflict of interest.

Availability of data and material

The datasets used during the current study are available from the corresponding author on reasonable request.

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