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ORIGINAL ARTICLE

Association between promoter hypermethylation of the *DACT2* gene and tumor stages in breast cancer

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Summary

Purpose: *Aberrant methylation of CpG islands in the promoter is a hallmark of cancer, leading to transcriptional silencing of tumor suppressor genes. The aim of this work was to evaluate the promoter methylation status of the DACT2 gene in breast cancer (BC) tissue and to analyze its possible effect on tumor type or grade.*

Methods: *CpG island from the DACT2 promoter in region -240 to -14 from transcriptional start site (TSS) were obtained. Through the use of sodium bisulfite DNA conversion analysis, followed by detection with MSP (methylation specific PCR), we analyzed 79 BC and 15 adjacent healthy samples.*

Results: *The cases analyzed were in stage I (2.5%), II (38%), or III (59.5%). The most frequent tumor type was invasive ductal carcinoma (71.4%). Methylation analysis comparing tumor tissues with adjacent non-cancerous tissues showed statistical significance. Methylation was observed in 32.9% (26/79) of the samples; no methylation was found in adjacent healthy tissue. DACT2 methylation was associated with tumor stage I-II (p=0.03) and stage III (p= 0.004).*

Conclusion: *An association was found of DACT2 promoter methylation with advanced tumor stages. This gene has been suggested as a potential biomarker, however, more investigation is required to validate this function.*

Key words: *breast cancer, CpG island, DACT2, methylation, tumor grade*

Introduction

Cancer is a disease that affects many people worldwide and, regardless of the efforts to prolong survival, improvements in its treatment do not yet achieve a high success rate. This complex disease includes several factors for its development and progression, with aberrant DNA methylation being one of these factors. Epigenetic alterations could inappropriately activate or inhibit different signaling pathways, resulting in the appearance of a tumor. It is well known that epigenetic changes occur in early tumor phases, particularly in the promoter region of genes.

Breast cancer (BC) is the second most common tumor type in women and is the leading cause of cancer-related deaths in less developed countries [1]. In Mexico, BC has also exhibited an increase in health statistics; its estimated incidence is 38.4 per 100,000 women and standardized mortality

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has doubled in the last 20 years [2]. According to the Mexican National Institute of Statistics and Geography (INEGI), the incidence of BC in 2014 was 28.75 new cases per 100,000, with a mortality rate of 14 per 100,000 inhabitants [3]. In different types of cancers at early stages, it is common to observe overall hypomethylation of DNA from tumor cells and hypermethylation of specific promoters, including CpG islands and coasts of CpG islands, with a lower density of CpG, which has led to the suggestion that this deregulation in fact precedes the tumor events occurring in preliminary classic transformers.

The DACT (Disheveled-associated Antagonist of β-CaTenin) family of scaffold proteins has been identified as an important player in tumorigenesis. *DACT2*, an antagonist of TGF-b/Nodal and Wnt/Ca^{2+}-PCP signaling participant, is an important factor in the normal development of vertebrates [4,5].

DACT2 is frequently methylated in lung, hepatic, gastric nasopharyngeal, esophagus, colon, and thyroid cancers [6-10]. In addition, recent studies have demonstrated hypermethylation in the proximal *DACT2* promoter region in BC [11,12], and it has been considered as a possible prognostic biomarker [13]. However, there are few works on the *DACT2* association with tumor grade and its relevance to breast oncogenesis.

Methods

Study subjects

The study included 79 cases of BC and 15 adjacent healthy tissues. Cases with a confirmed diagnosis (without prior history of BC) were recruited in the Mexico City-based Hospital of the IMSS Centro Médico Nacional La Raza. Breast tumor and healthy adjacent tissues were obtained to analyze the methylation status. The study protocol was approved by the Institutional Ethics Committee for Research. Written informed consent was obtained from each patient after being informed on the study aim.

Data collection

Epidemiological and clinical data were collected using structured questionnaires and medical records. Case diagnoses were obtained based on the pathological report. Standard anthropometric (age, height, weight, BMI), gynecological (menarche and menopause), and pathological (type, stage, and tumor size, ER and PR status) data were obtained.

DNA extraction

DNA from tumor and healthy adjacent tissue was obtained with the QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA). DNA concentration was measured by the NanoDrop 8000 Spectrophotometer (Thermo Scientific, MA, USA).

Sodium bisulfite modification and methylation-specific PCR (MSP)

DNA isolated from tumor and healthy adjacent tissues were sodium bisulfite-modified utilizing the EpiTect Bisulfite Kit (Qiagen,Inc.,Valencia,CA,USA) according to the manufacturer's protocol to convert unmethylated C into U. Briefly, 500 ng of DNA were sodium bisulfite-treated, denatured at 95°C for 5 min, and bisulfite-converted at 60°C for 5 hrs. After conversion, samples were desulfonated and purified employing column preparation.

The CpG island from the promoter region was located using the Eukaryotic Promoter Database tool. MSP primer pairs designed to recognize sodium bisulfite-converted DNA were obtained using Methprimer software to detect bisulfite-induced changes affecting Unmethylated (U) and Methylated (M) alleles. Primer sequences to amplify region -240 to -14 based on TSS were as follows : DACT2 (MF) 5'-GGAGGCGTTTAGTTGGTTTC-3'; (MR) 5'-ATCCCGAACTATATCGCGAA-3'; (UF) 5'-AGGTGTTTAGTTGGTTTTGG-3'; (UR) 5'-AATCCCAAACTATATCACAAA-3' PCR for bisulfite-converted DNA was performed utilizing the EpiTect MSP Kit (Qiagen, USA). Twenty ng of DNA treated, 10 μM of each primer and 2X Master Mix MSP in a final reaction volume of 10 μL. Cycle conditions-methylated amplicons were the following: 95°C for 10 min; 40 cycles (95°C for 15 s; 59°C for 30 s, and 72°C for 30 s); 72°C for 7 min. For unmethylated products there were 35 cycles (95°C for 15 s, 55°C for 30 s, 72°C for 30 s); 72°C for 7 min. Each PCR assay included a methylation control, an unmethylated control, and genomic DNA (EpiTect PCR Control DNA Set; Qiagen, USA). The PCR products were analyzed using 3.5% agarose gel electrophoresis. The size of methylated PCR products were 226 bp and 225 pb for unmethylated amplicon.

Statistics

The statistical analyses of the data were carried out employing SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). The association between methylation and covariants was analyzed by one-way Analysis Of VAriance (ANOVA) test. A p value less than 0.05 was considered statistically significant.

Results

The mean patient age was 54.5±11.1 years; clinicopathological characteristics could not be analyzed in all samples because some records were incomplete. Tumor size ranged between 1x1 and 10x10 cm. Receptor status based on immunohistochemical diagnosis was not performed in all samples (Table 1). Tumor type was categorized according to histopathologic diagnosis; tumor stage was categorized according to American Joint Committee on Cancer (AJCC) criteria. The majority of

cases were diagnosed at stage II or III, while the most frequent tumor type was invasive ductal carcinoma (71.4%) (Table 2).

The promoter-region methylation status of the *DACT2* gene was defined and analyzed utilizing MSP in 79 sporadic BC tumors and in 15 adjacent healthy tissues. Methylation was observed in 32.9% (26/79) of BC; no methylation was found in the 15 adjacent healthy tissues. The study included samples from patients with stages I, II, and III. *DACT2* methylation was significantly associated with tumor stage increase (p=0.004), as depicted in Figure 1.

Discussion

DNA methylation plays an important role in gene expression, and it is well known that nearly 80% of the genome is generally methylated in somatic cells. However, epigenetic changes could inappropriately activate or inhibit gene expression, leading to diseases. Changes in epigenetic patterns are observed in very early stages of tumor progression, and it is suggested that this occurs in advance of genetic alterations [14]. Abnormal DNA methylation has been studied in different tumor cells and has been correlated with the ex-

Table 1. Clinicopathological features of DACT2 methylation in breast cancer

		Methylation status		*p value*
Clinicopathological features	*n=79*	*Methylated n (%)*	*Unmethylated n (%)*	
Age, years				0.8
<52	37	13 (50.0)	24 (45.3)	
>52	37	11 (42.3)	26 (49.1)	
Unknown	5	2 (7.7)	3 (5.7)	
Menopause				0.7
Yes	42	12 (46.2)	30 (56.6)	
No	6	2 (7.6)	4 (7.6)	
Unknown	31	12 (46.2)	19 (35.8)	
Tumor size, cm				0.2
≤2	11	4 (15.4)	7 (13.2)	
>2 ≤5	35	9 (34.6)	26 (49.1)	
>5	8	1 (3.8)	7 (13.2)	
Unknown	25	12 (46.2)	13 (24.5)	
ER status				0.2
Positive	15	7 (26.9)	8 (15.1)	
Negative	8	1 (3.9)	7 (13.2)	
Unknown	56	18 (69.2)	38 (71.7)	
PR status				0.4
Positive	13	6 (23.1)	7 (13.2)	
Negative	10	2 (7.7)	8 (15.1)	
Unknown	56	18 (69.2)	38 (71.7)	

Table 2. Methylation status of DACT2 promoter in breast cancer

Samples		*DACT2*		*p value*
	Methylated n (%)	*Partially methylated*	*Unmethylated n (%)*	
Healthy tissue	0 (0.0)	-	15 (100.0)	0.009
Breast cancer	26 (32.9)		53 (67.1)	
ECI	0	1	1	
ECIIA	1	1	8	
ECIIB	3	2	15	
ECIIIA	2	3	8	
ECIIIB	5	4	13	
ECIIIC	0	4	8	

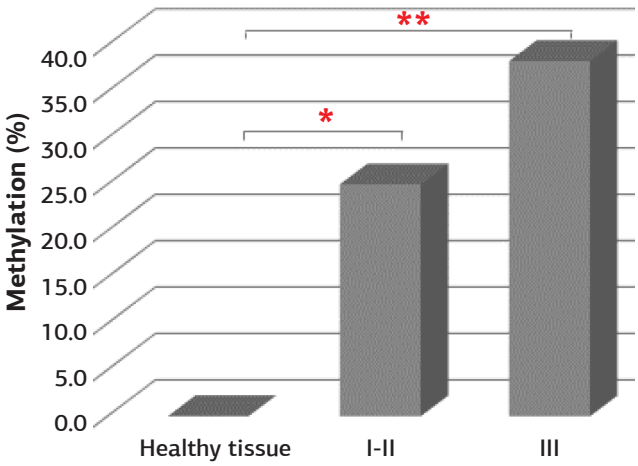

Figure 1. Methylation status of *DACT2* promoter in breast cancer. Comparisons according to breast cancer stages and adjacent non-tumor tissue are shown (*p=0.03; **p=0.004).

pression of some oncogenes or tumor suppressors. Hypermethylation of CpG sites in promoter regions affects gene transcription without gene mutations: in the case of tumor-suppressor genes, silencing by methylation contributes to tumor progression [15]. Thus, it is clear that DNA methylation could be a good biomarker for the early diagnosis of malignancy. Our study revealed that there exists a positive correlation between stage III of BC tumors and the hypermethylation of *DACT2*, demonstrating a significant relationship with poor prognosis. Results reported by Li et al. [12] demonstrate that *DACT2* is frequently methylated in human BC and methylation was associated with tumor size. The importance of *DACT2* in other types of cancer has already been noted, for example, mutations or promoter methylation suppresses the gene's transcription [16]. Hou et al. [13] observed that the greater the amount of *DACT2* protein, the better the survival rate in patients with esophageal squamous cell carcinoma, while *in vitro*, restored *DACT2* expression significantly reduced the growth, migration, and invasion of tumor cells. Analysis of methylation in cell lines, primary tumors, and normal tissues has allowed observing that *DACT2* participates in the regulation of Wnt/β-catenin and TGF-b/Nodal signaling pathways [6,9,12,13]. In all tumor types studied, it was observed a loss of *DACT2* expression that was dependent on promoter methylation. Interestingly, Yu et al. [8] suggest that *DACT2* methylation can be a marker of sensitivity to cisplatin and a marker of insensitivity to paclitaxel, proposing a possible role as biomarker. We found significant differences in *DACT2* methylation in healthy adjacent and tumor tissues. Some reports demonstrated that 50% or more of tumor tissues are methylated but none in normal tissues. In our study, solely 32.9% (26/79) of BC tissues were methylated, but the frequency of methylation was increased at more advanced tumor stages. *DACT2* methylation had been associated with metastasis and it has been suggested for its use as a metastatic biomarker in analyzed tumor types.

In conclusion, we examined the promoter methylation status of the candidate tumor suppressor *DACT2* in BC and we found that *DACT2* promoter was related with advanced tumor stages. Therefore, as has been suggested, promoter methylation of *DACT2* may a potential biomarker of tumor progression.

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Conflict of interests

The authors declare no conflict of interests.

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