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CDH1 methylation and expression of E-cadherin and other markers in breast cancer

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ABSTRACT

Introduction: E-cadherin, encoded by the CDH1 gene, is a glycoprotein involved in cell adhesion, and the methylation of CDH1 can prevent the protein expression favoring tumor invasion. This study investigated the methylation of CDH1 in the DNA extracted from tumor and non-tumor tissues of breast cancer patients. In addition, the expression of E-cadherin, human epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER), progesterone receptor (PR), and the marker of proliferation Ki-67 (Ki-67) was analyzed by immunohistochemistry. **Methods:** Samples of tumor and non-tumor breast tissues were collected from 15 women diagnosed with breast carcinoma at the time of mastectomy to analyze CDH1 methylation. The DNA was extracted, modified by the sodium bisulfite method, and amplified by polymerase chain reaction (PCR). The expression of E-cadherin, HER-2, ER, PR, and Ki-67 was evaluated by immunohistochemistry. **Results:** All the 15 patients had CDH1 methylation in the tumor tissue, and nine had CDH1 methylation in the non-tumor breast tissue. The immunohistochemical analysis showed that one patient had E-cadherin expression, three had HER-2, five had ER, six had PR, and nine had Ki-67. **Conclusions:** Our findings suggest that CDH1 gene methylation prevented E-cadherin expression in breast tumors once only one of the nine patients tested by immunohistochemical analysis showed the protein. The methylation of CDH1 in non-tumor breast tissues observed in nine patients may suggest the presence of infiltrating neoplastic cells or non-neoplastic genetically transformed cells.

KEYWORDS: cadherin-1; methylation; breast neoplasms; immunohistochemistry.

INTRODUCTION

Cadherins, a large superfamily of transmembrane glycoproteins, are integral to cell adhesion and the maintenance of tissue architecture. Among them, E-cadherin, encoded by the *CDH1* gene, is an invasion suppressor, and its dysregulation or mutation can lead to cancer development[1-3]. E-cadherin imbalance is characteristic of several malignancies and is involved in tumor metastasis[2,4]. The protein is particularly significant in the context of invasive lobular carcinoma (ILC), which accounts for 10-15% of all breast cancers[5]. The absence of E-cadherin expression is a characteristic feature of *in situ* and ILCs.

DNA methylation is a biochemical process in which a methyl group (CH_3) is added to the cytosine of a CG dinucleotide in the DNA sequence[2]. This epigenetic phenomenon can alter the gene expression without modifying the base sequence. Aberrant methylation of *CDH1* can inactivate the gene, preventing E-cadherin expression[2]. A study demonstrated the inverse relationship between *CDH1* gene methylation and E-cadherin expression in 50 cases of both ductal-type breast cancer and normal breast samples. The study showed that 94% of ductal-type breast cancers had *CDH1* promoter methylation, and that 95% of full-methylated tumor samples had no E-cadherin expression[6].

In addition to E-cadherin, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2), and the marker of proliferation Ki-67 (Ki-67) are essential markers in breast cancer. ER plays a critical role in the growth and development of breast tumors. More than 70% of breast cancers are ER positive, based on immunohistochemical analysis[7,8]. In these cases, the survival of patients can be improved

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by ER-positive therapy[8]. PR is a prognostic marker in breast cancer, and its high expression is more frequent in tumors with a better prognosis (luminal A) than in tumors with a worse prognosis (luminal B)[9]. HER-2 is a growth-promoting protein, and its excess or amplification of the *HER-2* gene is related to a poor prognosis of breast cancer[10]. Ki-67 is a protein associated with cell proliferation, and a high level of Ki-67 is often indicative of a more rapidly growing breast tumor[11].

This study aimed to analyze the methylation status of the *CDH1* gene in tumor and non-tumor tissues of breast carcinoma patients. Furthermore, the expression of E-cadherin, ER, PR, HER-2, and Ki-67 was examined by immunohistochemistry. We investigated whether *CDH1* methylation inhibited the expression of E-cadherin in the studied patients.

METHODS

Study design and selection of patients

This prospective hospital-based study involved 15 women treated at the *Instituto de Ginecologia* of the *Universidade Federal do Rio de Janeiro*, Brazil. The age of the patients varied between 44 and 78 years (average age: 56.7±9.6 years). All of them were diagnosed with breast carcinoma and underwent mastectomy. Before the surgery, patients were interviewed and invited to participate in the study. Those who agreed to participate were provided with all the necessary information and signed a consent form.

Data collection and ethical aspects

Patient recruitment occurred from October 2018 to July 2021. Demographic and clinical data were gathered from the patients' medical records. The study was approved by the Research Ethics Committee of the *Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro* (Certificate: CAAE #91406118.6.0000.5257, dated September 29, 2018).

Collection of tissue samples

Tumor and non-tumor surrounding tissue fragments of around 1-2 cm in each axis were collected from the breast of patients at the time of mastectomy. The tissue samples were collected at the *Instituto de Ginecologia, Universidade Federal do Rio de Janeiro*.

Extraction of DNA from tumor and non-tumor breast tissues

DNA extraction from tumor fragments and non-tumor breast tissues was performed using the phenol:chloroform method, as previously described by Mccormick et al.[12], using the UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (Invitrogen, USA, Cat. No. 15593-031).

Methylation mechanism

The DNA samples were modified by the sodium bisulfite conversion method and then analyzed by the methylation-specific polymerase chain reaction (MSP) technique. DNA modification was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research, USA, Cat. No. D5005), according to the manufacturer's instructions.

Polymerase chain reaction

After the DNA modification, a fragment of exon 5 of the *P53* gene was amplified by polymerase chain reaction (PCR) to confirm the DNA integrity. The amplification reaction was performed as previously described by Pestaner et al.,[13] generating a 274-base-pair product. In the next step, the *CDH1* gene was amplified by PCR. For the *CDH1* amplification, two pairs of primers were used as follows: *CDH1*-U (unmethylated) forward, 5′-GGTAGGTGAATTTTAGTTAATTAGTGGTA-3′ and *CDH1*-U reverse, 5′-ACCCATAACTAACCAAAAACACCA-3′, producing a fragment of 211 base pairs, and *CDH1*-M (methylated) forward, 5′-GGTGAATTTTAGTTAATTAGCGGTAC-3′ and *CDH1*-M reverse, 5′-CATAACTAACCGAAAACGCCG-3′, producing a fragment of 204 base pairs[14]. The polymerase used was the GoTaq G2 Hot Start Green Master Mix (Promega, USA, Cat. No. M7422). The cycling included an initial denaturation at 96°C for 7 min, followed by 35 cycles of 95°C for 1 min, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min.

Gel electrophoresis and staining

The PCR products were run by electrophoresis in 10% polyacrylamide gel. A negative control and a DNA marker were included in each electrophoretic run. Gels were stained by the silver nitrate method involving DNA fixation with ethanol and acetic acid, impregnation with silver nitrate, and revelation of the DNA bands with sodium hydroxide (NaOH) and formaldehyde[15].

Histopathological and immunohistochemical analysis

The tissue samples were fixed in 10% formalin and embedded in paraffin wax. The tissue blocks were sectioned into 4 μm thickness sections. The hematoxylin-eosin staining was used for the histopathological analysis. The immunohistochemistry was accomplished with monoclonal antibodies for all antigens. The primary antibodies used were rabbit anti-E-cadherin (clone EP700Y, 1:200, Cell Marque), mouse anti-HER-2 (clone CB11, 1:600, Cell Marque), rabbit anti-ER (clone SP1, 1:200, Cell Marque), mouse anti-PR (clone 16, 1:100, Cell Marque), and rabbit anti-Ki-67 (clone SP6, 1:300, Spring). The secondary antibody applied was from the Novolink Polymer Detection System® (Leica Biosystems, UK, product code: RE7280-K), following the manufacturer's instructions.

RESULTS

Table 1 shows the methylation panel of the *CDH1* gene in the tumor and non-tumor breast tissues. All the 15 patients had *CDH1* methylation in the tumor tissue. Nine patients had *CDH1* methylation in the non-tumor breast tissue.

Out of the 15 patients, samples from nine patients were analyzed by histopathology and immunohistochemistry. As indicated in Table 2, E-cadherin expression was detected only in patient five. All the nine patients tested positive for Ki-67. As determined through the immunohistochemical analysis, the classification of tumor subtypes was as follows: luminal A (patients 2, 7, and 8), luminal B (patients 6 and 10), HER-2-positive (patient 5), and triple-negative (patients 3, 4, and 14). The histopathological grades and types of breast carcinomas are described in Table 3.

Table 4 shows the age and TNM stage of the patients. It is noteworthy that all patients with this information available fell within a tumor category of 3 or 4, representing an advanced disease. Photomicrographs of the histological sections of tumors are displayed in Figures 1 and 2.

Table 1. Methylation panel of the cadherin 1 (*CDH1*) gene in tumor and non-tumor breast tissues.

Patient number	Tumor breast tissue	Non-tumor breast tissue
1	M	U
2	M	M
3	M	U
4	M	M
5	M	U
6	M	M
7	M	M
8	M	U
9	M	M
10	M	M
11	M	U
12	M	M
13	M	M
14	M	M
15	M	U

M: *CDH1* methylated. U: *CDH1* non-methylated.

DISCUSSION

Our study investigated the methylation status of the *CDH1* gene in tumor and non-tumor tissues of breast cancer patients. Additionally, we analyzed the expression of E-cadherin, ER, PR, HER-2, and Ki-67 by immunohistochemistry.

The results showed that *CDH1* gene methylation was detected in the tumor of all the 15 patients and in the non-tumor breast tissue of nine patients (Table 1). Otherwise, only patient number five presented E-cadherin protein expression in the immunohistochemical analysis, suggesting that *CDH1* methylation prevented E-cadherin expression in the other patients. This aligns with the findings of Shargh et al.[6], who reported that, in a group of 50 breast cancer patients, 94% had *CDH1* methylation and 95% of full-methylated tumor samples had no E-cadherin expression. In another study, Corso et al. emphasized that the detection of *CDH1* epigenetic alterations in a diagnostic/pre-operative biopsy may be helpful to improve patient management and to infer the prognosis of breast cancer and the pattern of tumor dissemination[16].

Table 2. Invasive ductal and invasive lobular carcinoma: Immunohistochemical analysis reports.

Patient number	Invasive Ductal Breast Carcinoma – Patients 2, 3, 4, 7, 8, 10, and 14 Papillary intraductal carcinoma – Patient 6 ILCs – Patients 5 and 6				
	ER Positive (%)	PR Positive (%)	Ki-67 Positive (%)	HER-2 Positive (%)	E-cadherin Positive (%)
2	100	100	1–5	0	0
3	0	0	80-90	0	0
4	0	0	80-90	0	0
5	Not available	20–30	20–30	Score 3 (>30%)	Positive
6	100	90–95 (infiltrating) 20–30 (intraductal)	30–40	0	0
7	60–70	10–20	5–10	0	0
8	100	100	5–10	0	0
10	100	90–95	50-60	Score 1 (≤10%)	0
14	0	0	80-90	Score 1 (≤10%)	0

ER: estrogen receptor; PR: progesterone receptor; HER-2: human epidermal growth factor receptor-2; Ki-67: marker of proliferation Ki-67; ILCs: invasive lobular carcinomas.

Table 3. Histopathological analysis reports (breast carcinoma types).

Patient number	Ductal infiltrating carcinoma, non special type	Ductal carcinoma *in situ*	Ductal carcinoma *in situ* Grade 1 without comedonecrosis	Ductal carcinoma *in situ* Grade 2 with comedonecrosis	Intraductal papillary carcinoma	ILC
2	Grade 2	Grade 1	P	-	-	-
3	P	-	-	-	-	-
4	Grade 3	-	-	-	-	-
5	-	-	-	-	-	P
6	-	-	-	-	P	P
7	Grade 1	-	-	-	-	-
8	Grade 1	Grades 1 and 2	P	-	-	-
10	Grade 2	Grade 2	-	P	P	-
14	Grade 3	-	-	-	-	-

P: positive for the types of carcinomas of the study patients. Grade 1: well differentiated. Grade 2: moderately differentiated. Grade 3: poorly differentiated. ILC: invasive lobular carcinoma.

Table 4. Age and TNM stage of patients.

Patient number	Age (years)	TNM stage
1	53	T4b N2 Mx
2	73	WD
3	50	WD
4	44	T3 N0 M0
5	49	CT3 CN2 CM0
6	59	T4 N0 Mx
7	44	T4B N1 Mx
8	49	T3 N1 Mx
9	78	WD
10	57	T4 N0 Mx
11	57	T4 N1 Mx
12	56	T4B N0
13	59	T3 N1 M0
14	54	T4b N2 Mx
15	69	T3 N0 M0

TNM acronym refers to TNM Classification of Malignant Tumors, where "T" refers to primary tumors, "N" refers to nearby lymph node involvement, and "M" refers to distant metastasis. WD: TNM stage not described in the records.

Three patients (2, 7, and 8) were diagnosed with luminal A subtype carcinoma, characterized by a strong positivity for ER and PR, a negativity for HER-2, and a weak positivity for Ki-67 (Table 2). As pointed out by De Santo et al.[17], the luminal A subtype is associated with less biologically aggressive neoplasms and is responsive to anti-estrogenic therapy. However, over time, neoplastic cells can develop resistance to this therapy due to mutations in the genes of ERs. This resistance can interfere with the action of anti-estrogen drugs, such as tamoxifen, thereby favoring cancer progression.

Three patients (3, 4, and 14) were diagnosed with the triple-negative subtype, characterized by a high Ki-67+ (80-90%) due to the elevated degree of the proliferation of neoplastic cells. This subtype presents an aggressive clinical behavior, as pointed out by Derakhshan and Reis-Filho[18]. Furthermore, the triple-negative subtype is associated with neoplasms of high combined histopathological grade, which agrees with our findings reported in Table 3 (patients 4 and 14 had Grade 3). This characteristic favors response to neoadjuvant chemotherapy.

Patient number five, diagnosed with ILC, was HER-2+. This patient showed *CDH1* methylation and E-cadherin expression only in the tumor and not in the non-tumor tissue, confirming the heterogeneity of E-cadherin expression in lobular carcinomas[5].

The histopathological diagnosis summarized in Table 3 allowed an initial prognostic assessment. Histopathological analysis is indispensable to direct complementary molecular studies (including immunohistochemistry and methylation analysis). These studies are essential to improve diagnosis and assist in choosing the most appropriate treatments, allowing a better evaluation of the final prognosis in patient survival.

TNM stage information was available for 12 out of the 15 patients (Table 4). All of them had advanced tumors (seven had T4 and five had T3), which is related to late diagnosis, delay in treatment start, and reduced survival, as emphasized by Rivera-Franco and Leon-Rodriguez[19].

To our knowledge, this is the first study that simultaneously explored *CDH1* gene methylation and E-cadherin protein expression in a cohort of Brazilian breast cancer patients.

CONCLUSIONS

Our findings suggest that *CDH1* gene methylation prevented E-cadherin expression in breast tumors once only one of the nine patients tested by immunohistochemical analysis showed the protein. The methylation of *CDH1* in non-tumor breast tissues observed in nine patients may suggest the presence of

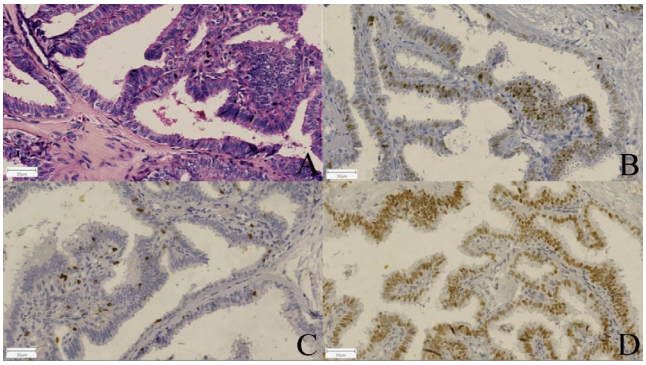

Figure 1. Histological sections of papillary carcinomas. The scales correspond to 50 µm in length. HE: hematoxylin-eosin staining. A: Intraductal papillary carcinoma, HE. B: Intraductal papillary carcinoma with nuclear PR positivity, IH. C: Intraductal papillary carcinoma with nuclear Ki-67 positivity, IH. D: Intraductal papillary carcinoma with nuclear ER positivity, IH.

IH: immunohistochemistry. PR: progesterone receptor. Ki-67: marker of proliferation Ki-67. ER: estrogen receptor.

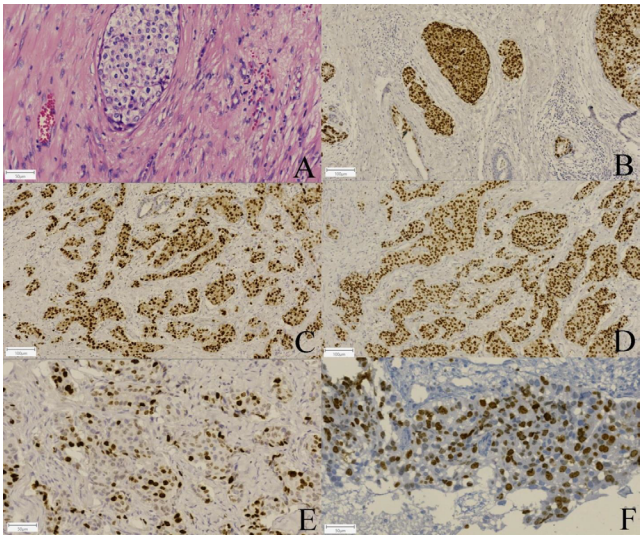

Figure 2. Histological sections of ductal carcinomas. A: in situ ductal carcinoma, HE. B: in situ ductal carcinoma with nuclear PR positivity, IH. C: invasive ductal carcinoma with nuclear PR positivity, IH. D: invasive ductal carcinoma with nuclear ER positivity, IH. E: invasive ductal carcinoma with nuclear Ki-67 positivity, IH. F: invasive ductal carcinoma with nuclear Ki-67 positivity, IH. In A, E, and F, the scales correspond to 50 µm in length. In B, C, and D, the scales correspond to 100 µm in length.

HE: hematoxylin-eosin staining, IH: immunohistochemistry. PR: progesterone receptor. Ki-67: marker of proliferation Ki-67. ER: estrogen receptor.

infiltrating neoplastic cells or non-neoplastic genetically transformed cells. New studies are needed to analyze the methylation of other genes that encode markers for breast cancer, such as ER, PR, HER-2, and Ki-67. Furthermore, these studies should investigate the relationship between gene methylation and the respective marker expression.

AUTHORS' CONTRIBUTION

LFQ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. MSMS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. FCR: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. SBLR: Formal analysis, Investigation, Methodology, Writing – review & editing. HSPS: Funding acquisition, Resources, Writing – review & editing. MGCC: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

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