

# E-cadherin Gene Promoter Hyper-methylation in Blood Samples from Breast Cancer Cases

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## Abstract

In carcinomas, dissemination of cancer cells via blood or lymph circulation constitutes an early event. E-cadherin is a transmembrane calcium dependent adhesion protein. Cellular de- differentiation and plasticity, underlying metastasis, is attributed to the loss of function of E- cadherin (cdh1) gene. The loss of gene expression may arise from promoter hypermethylation, which has been reported in multiple cancers. In the present pilot project, sixty (60) blood samples were collected from the breast cancer patients at a tertiary care hospital in Karachi, Pakistan. DNA was isolated from the cells circulating in the peripheral blood of the participants. Promoter hypermethylation was investigated through sodium-bisulfite treatment of DNA followed by methylation-specific PCR. In 53.3% of the patients, E-cadherin gene promoter hypermethylation was observed. Promoter hypermethylation of E-cadherin has been reported in DNA isolated from the tissue specimen. However, to the best of our knowledge this is the first report of E-cadherin promoter hypermethylation in cells isolated from the peripheral blood of breast cancer patients from a geographically specific population. The results have important implications in tumour staging and selection of treatment regimens.

**Key words:** bladder cancer, clinicopathology, early incidence, surgical oncology.

## Introduction

E-cadherin is a trans-membrane calcium-dependent adhesion glycoprotein. The protein expression is mainly restricted to epithelial cells (Bruner and Derksen, 2018). It is a critical protein at adherens junction, where it connects the epithelial cells to each other (Halbleib and Nelson, 2006). E-cadherin is a tumour suppressor (Berx, 1995; Guilford 1998; Perl 1998; and Derksen 2006). In cancers, the cadherins are frequently non-functional through gene inactivation and/or functional inhibition. Consequently, these play significant role in tumourigenesis and progression (Wong, 2017). E-cadherin is considered to be clinically causal in carcinomas, particularly breast and gastric cancers (Derksen, 2006).

The encoding gene for E-cadherin (CDH1) is mapped to chromosome 16q22.1. A recent CRISPR/Cas9 experiment demonstrated tumour development and progression of breast carcinoma sub-type when dual inactivation of CDH1 and PTEN genes was carried out, (Annunziato, 2016). Clinically, inactivating germ-line and somatic mutations as well as CDH1 promoter hypermethylation have been shown in breast cancers (Berx, 1995, 1996; Masciari, 2007; Strathdee, 2002). The epigenetic silencing of CDH1 has also been reported in some human breast cancer cell lines (Yang, 2001; Szyf, 2004). However, in case of primary breast cancers, information

regarding aberrant DNA methylation is limited. The current data shows frequency variation ranging from 21% to 80% (Cardeira, 2006; Liu, 2016), depending upon tumour sub- type and stage. In the present study, we report the promoter hypermethylation analysis for epigenetic silencing of CDH1 gene in the circulating cells, which were collected from peripheral blood samples of breast cancer patients.

## Methodology

### Patients:

A total of 67, with 60 eligible, blood samples were collected from the breast cancer patients at the Atomic Energy Medical Centre (AEMC), Jinnah Postgraduate Medical Centre (JPMC), Karachi, Pakistan were collected between July 2016 and July 2018. The blood samples were collected in ACD coated vacutainers (BD Vacutainer® BD Franklin Lakes NJ USA) and either processed immediately or stored at 4°C. The study was approved by the ethical review committees (ERCs) of the participating institutions: the independent ERC, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi, Pakistan [ICCBS/IEC-016-BS/HT-2016/Protocol/1.0], and the Atomic Energy Medical Centre

(AEMC), Jinnah Postgraduate Medical Centre (JPMC), Karachi, Pakistan [Admin-3(257)/2016]. The inclusion criteria for patients were diagnosis of primary breast cancer.

### Tumour Information

The information regarding cancer including histological classification, tumour size, tumour grade, tumour stage, lymph node metastasis, and status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) were obtained from the patients' medical records, where available. The patients were interviewed for age, family history of cancer, gynae and obs history, and ethnicity. The patients' information was documented in the form of a questionnaire.

### Bi-sulfite Treatment of DNA Samples

DNA samples were treated with sodium bisulfite following standard protocol (Herman et al., 1996) with slight adjustments. 1µg of DNA sample (total volume adjusted to 45µl with autoclaved deionized water) was taken in 1.5ml microcentrifuged tube. For alkaline denaturation, 5.7µl of 3M NaOH was added to each DNA samples. Samples were then incubated for 17 minutes in shaking waterbath at 37°C, 50RPM. After incubation, unmethylated cytosine were converted to uracil by adding 530µl of freshly prepared mixture of

16mM hydroquinone and 4M liquid sodium bisulfite solution (pH adjusted to 5 with 1M NaOH solution) to each DNA samples. A drop of mineral oil was added to each sample. Samples were incubated for 16-18 hours, in shaking waterbath. Second day, DNA samples were purified using DNA clean-up kit (Bio Basic© 2017 Bio Basic Inc., UK) following the instructions. To each purified DNA, 5.7µl of 3M NaOH was added, followed by incubation at 37°C for 17 minutes. After incubation, DNA was precipitated with 10M ammonium acetate (17µl) and ice-cold absolute ethanol (500µl). Samples were kept at -20°C for precipitation.

The next day, samples were centrifuged at maximum speed (21,000rcf) for 21 minutes at 4°C. The supernatant was discarded and pellets were washed twice with 70% ethanol (500µl), followed by centrifugation at maximum speed for 21 minutes. Supernatant was discarded and tubes were air dried at room temperature. DNA samples were then resuspended in 35µl of autoclaved deionized water. The samples were incubated in shaking waterbath at 37°C for 10 minutes for resuspension. DNA samples were then kept at 4°C till PCR.

### Methylation Specific Polymerase Chain Reaction (MS-PCR)

Primer sequences for methylated and unmethylated PCR are listed in Table 1.

S.no	Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'	Annealing Temperature (°C)	Product size (bp)	Reference
1.	E-cadherin Un Methylated	TAATTTTAGGT TAGAGGGTTA TTGT	CACAACCAAT CAACAACAC A	54	97	Herman, Graff,
2.	E-cadherin Methylated	TTAGGTTAGA GGGTTATCGC GT	TAAGTAAAAA TTCACCTACC GAC	53.5	116	Myöhänen, Nelkin and Baylin (1996)

PCR reactions for methylated and unmethylated genes were carried out separately. For unmethylated PCR, reaction was carried out in a total volume of 25µl containing 1X PCR, 4.5mM MgCl<sub>2</sub>, 1.5mM dNTPs, 1.9µM/µl of primers, 3% DMSO, and 120ng of DNA. Hot-start PCR was carried out. The samples were placed at 95°C for 5 minutes before the addition of 1.5U/µl Taq polymerase (Thermofisher scientific, Inc., USA). Amplification was carried out using Kyratex SC300 super cycler. PCR conditions were: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds. Final extension was carried out at 72°C for 10 minutes (Herman et al., 1996). For methylated

PCR, PCR mix of 25µl comprised 1X PCR buffer, 6.7mM MgCl<sub>2</sub>, 1.5mM dNTPs, 1.85µM/µl of each primer, 10mM β-mercaptoethanol, 2U/µl Taq polymerase and 120ng of DNA. Cycling conditions were similar to unmethylated PCR. Annealing temperature was 53.5°C

### Results

The clinico-pathological features of the patients' samples included in the study are listed in Table 2.

Parameter	N or value %
1 Age (n*=63)	
1a ≤40	26 (41.3%)
1b >40	37 (58.7%)
2 Tumor Site (n*=63)	
2a Right	27 (42.2%)
2b Left	35 (54.7%)
2c Bilateral	01 (1.60%)
3 Molecular Sub-type (n*=35)	
3a Luminal A	17 (48.6%)
3b Luminal B	05 (14.3%)
3c Her 2/neu	04 (11.4%)
3d Basal like	09 (25.7%)
4 Histological Sub-type (n*=63)	
4a IDC	54 (85.7%)
4b DCIS	01 (1.6%)

4e	IDC + DCIS	05 (7.9%)
4f	Others	03 (4.7%)
5	<b>Receptor Status (n*=49 for ER &amp; PR; n*=37 for Her2-neu)</b>	
5a	ER positive	31 (63.2%)
5b	ER negative	18 (36.7%)
5d	PR positive	30 (61.2%)
5e	PR negative	19 (38.8%)
5g	Her2-neu positive	10 (27%)
5h	Her2-neu negative	27 (73%)
6	<b>Tumor Size (n*=58)</b>	
6a	T1(<2cm)	09 (15.5%)
6b	T2(2-5cm)	37 (63.8%)
6c	T3(>5cm)	12 (20.7%)
7	<b>Stage (n*=43)</b>	
7a	Low (I-II)	21 (48.8%)
7b	High (III-IV)	22 (51.2%)

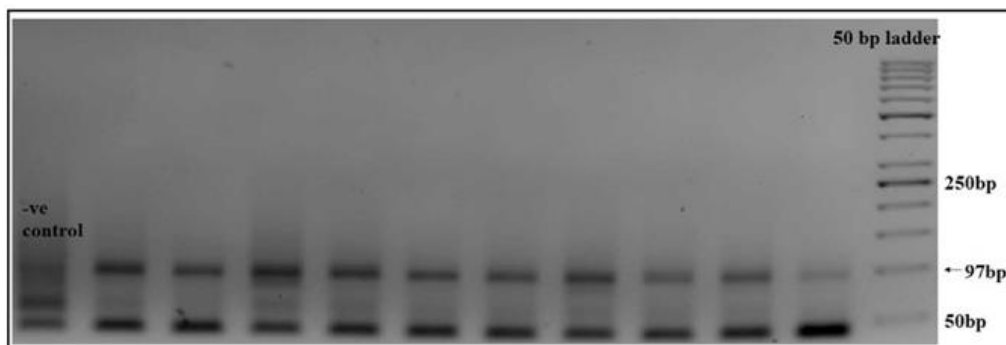
**Table-2** Clinicopathological characteristics of breast cancers cohort

“n\*”: number of samples with available data Representative gels for CDH1 methylation and un-methylation are shown in figures 1 and 2, respectively in the studied cohort of 60 breast cancer patients, E-cadherin

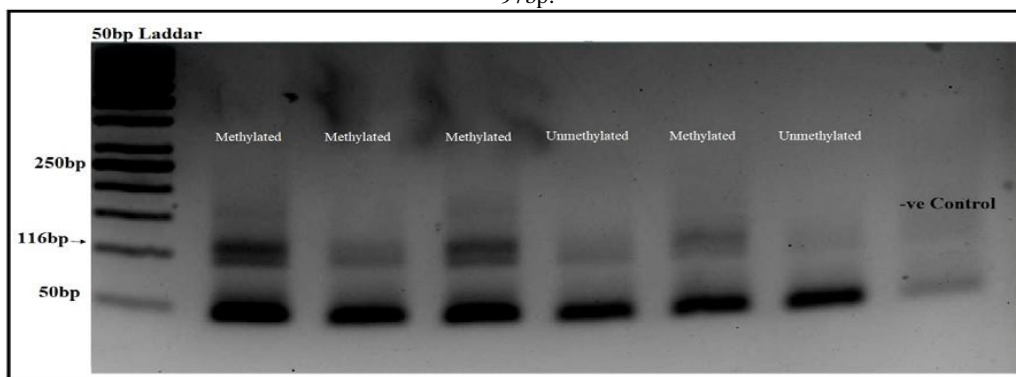
hypermethylation was detected in 53.3% (32/60) of the cases. (Table 3) 49% (18/36) of the cases are above 40 years while 58% (14/24) of the cases are below 40 years.

Methylation status	N=60*	Percentage (%)
Un Methylated	28	46.60%
Methylated	32	53.3%
*Re		

**Table 3: Methylation status of selected breast cancer patient**



**Figure 1:** Agarose gel electrophoresis of MS-PCR product of UnMethylated E-cadherin promoter region, stained with ethidium bromide. Product size is 97bp.



**Figure 2:** Agarose gel electrophoresis of MS-PCR product of methylated E-cadherin promoter region, stained with ethidium bromide. Product size is 116bp.

## Discussion

Epigenetics' is combination of 'epigenesis' and 'genomic' which means 'on top or in addition to genetics. The term was coined six decades ago based on mechanisms of cell fate commitment and lineage specification (Holliday, 1990; Waddington, 1959). Epigenetic changes can reversibly inactivate tumor suppressor gene in a heritable manner (Llinas-Arias and Esteller, 2017). These gene(s) are either packed into heterochromatin or hypermethylation of CpG island occurs. Such changes result in gene silencing that may attribute to tumor progression (Alberts, 2017). E-cadherin being cell adhesion molecule, plays significant role in cancer metastasis. Reduced expression of E-cadherin is one of the molecular events in cancer progression (Nass et al., 2000). In the studied cohort, hypermethylation is correlated with breast cancers progression and metastasis. In the studied cohort comprising both low and advanced stage breast cancer patients, 53.3% of the cases showed promoter hypermethylation of E-cadherin in DNA from circulating cells. To the best of our knowledge this is the first report of E-cadherin promoter hypermethylation in cells isolated from the peripheral blood of breast cancer patients.

Two studies from Asia have analyzed CDH1 promoter hyper-methylation in tissue samples from breast cancer patients. A study based on Kashmiri population has reported hypermethylation in 57.8% of the cases (Asiاف et al., 2014), whereas, another study from Iran reported hypermethylation in 94% of selected ductal-type breast cancer (Shargh et al., 2014). In about 51% of IDC cases, E-cadherin hypermethylation was observed while in different studies, hypermethylation was observed in 30% of IDC cases (Nass et al., 2000).

## Conclusion

The pilot study concludes that E-cadherin hypermethylation is involved in breast cancer progression in our population. Further investigation should be conducted to evaluate the role of E-cadherin in cancer metastasis and response to treatment. This work was presented in a preliminary form in an MPhil thesis of one of the co-authors (SZZ)

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