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Original Article

Oncology Section

Differential Expression of E-cadherin Gene by Real Time RT-PCR and Exon 1 Sequence Analysis in Invasive Breast Carcinoma

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ABSTRACT

Introduction: Genomic alterations in key genes such as tumour suppressor genes have been reported to contribute to human cancers like breast cancer. Loss of E-cadherin (CDH1) mediated adhesion characterises the transition from benign lesions to invasive, metastatic cancer. Genetic changes occurring in the CDH1 gene has not yet been completely studied despite the remarkable biological function of the signal peptide of CDH1. Many of these genomic alterations have altered messenger Ribonucleic Acid (mRNA) and protein expression that play a role in the pathophysiology of cancers and warrant further studies.

Aim: To identify mutations in CDH1 gene (Exon 1) in invasive breast carcinoma and evaluate CDH1 expression by real time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

Materials and Methods: The present study was a retrospective cross-sectional study in which tissue samples were collected from Madras Medical College and VS Hospitals, Chennai, Tamil Nadu, India between March 2010 to August 2011 and molecular biology work and analysis was carried out at Sri Ramasamy Memorial Institute of Science and Technology-Department of Biotechnology (SRM-DBT) Platform for Advanced Life technologies, SRM Institute of Science and Technology (SRMIST) between June 2017 to September 2017. The Exon 1 region of the human E-cadherin gene of normal and breast cancer (Infiltrating Ductal Carcinoma) patients were sequenced by Sanger method. The sequences were compared using the National Centre for Biotechnology Information-Basic Local Alignment Search Tool (NCBI-BLAST) utility. The change in the sequence was identified between the normal and tumour samples. The fold change of E-cadherin gene expression in tumour was calculated by comparing with control non neoplastic breast tissue. The fold change in the relative expression level between tumour and normal sample was determined using real time RT-PCR.

Results: In the present study upon comparing the Exon 1 sequences of normal gene with that of tumour, two deletion mutation and one C→T transition was observed. In present investigation also 0.08-fold down regulation of CDH1 mRNA was observed in the tumour tissue when compared with the normal tissue.

Conclusion: The present study has attempted to study the alterations at genome level (CDH1 gene, Exon 1, encoding for the biologically active signal peptide region), transcriptional and translational level with respect to CDH1. The effect of the mutations detected including two loci with deletion mutations and one single nucleotide change could affect the structural conformation of the protein and functional impact including aberrant expression. Molecular docking studies and in-vitro studies with cell lines and animal studies could be done to confirm these findings.

Keywords: Expression level, Fold change, Reverse Transcriptase Polymerase Chain Reaction, Sequence change, Signal peptide

INTRODUCTION

Invasive carcinoma of breast has two primary histological types, infiltrating ductal carcinoma and infiltrating lobular carcinoma, among which the lobular type is less frequent. Tumour invasion with subsequent regional (axillary lymphnodes) and distant metastasis is the major cause of morbidity and mortality in patients with breast cancer. Patients having distant metastasis have a poor prognosis and is often associated with mortality. E-cadherin is a transmembrane glycoprotein that mediates a cell–cell adhesion in the epithelial tissues [1]. It is one of the key molecules involved in the metastatic potential of tumours and also plays the role of tumour suppressor gene, down regulation of the protein has been implicated in invasion and tumour progression [2,3]. Normal ductal epithelial cells in the mammary gland strongly express E-cadherin protein in the cytoplasmic membrane [4,5]. Lobular carcinoma of breast are associated with reduced/loss of E-cadherin expression resulting in loss of cohesion of tumour cells and showing single cell infiltration pattern on histology.

Aberrant expression of E-cadherin and Estrogen Receptor (ER) expression is not yet fully established, though many studies on this have been done in the past [6]. Expression of E-cadherin in an aberrant manner in infiltrating ductal carcinoma breast has also been noted in cases with metastatic tumour deposits in distant sites [7,8].

E-cadherin knockout models have been associated with non viability and abnormal epithelial morphogenesis [8]. Loss of E-cadherin expression as a cell adhesion molecule has shown to cause dyscohesiveness of tumour cells and aid in invasion and metastasis in many human cancers [9,10]. In various cell lines, a reciprocal relationship has been shown between levels of E-cadherin expression and invasiveness [11].

Many mutations have been identified in E-cadherin gene in breast cancer that can affect protein function including promoter methylation events and also truncating mutations in signal peptide region [11-15]. Aberrant expression of CDH1 mRNA has also been reported in advanced tumours [16-19]. Genetic changes occurring in the signal peptide domain of E-cadherin gene have not been completely understood though the biological function of the region has been widely acknowledged in previous studies [20-22]. Differential expression of E-cadherin protein has also been detected in malignant breast cancer tissue [23-29] but the overall mechanisms involved in tumour progression is still not completely understood.

Hence the present study was undertaken to study the biologically significant signal peptide region of E-cadherin (encoded by CDH1) at the Deoxyribonucleic Acid (DNA) (genome) level, at the transcriptional and translational levels evels and compared with breast cancer tissue and normal control samples.

The aim of the study was to identify mutations of the Exon 1 region encoding the signal peptide domain of the human E-cadherin (CDH1) gene in tissue from infiltrating ductal carcinoma of breast. Quantitative analysis of CDH1 mRNA in normal and malignant breast cancer tissue by real time RT-PCR. Also evaluation of E-cadherin protein expression in normal and malignant tumour tissue by Western blot analysis.

MATERIALS AND METHODS

The present study was a retrospective cross-sectional study in which tissue samples were collected from Madras Medical College and VS Hospitals, Chennai, Tamil Nadu, India, between March 2010 to August 2011. All cases collected during the above study period were enrolled in the study. Informed consent was obtained from all participants. The samples were collected from Madras Medical college, Chennai, Tamil Nadu, India, after obtaining the Ethical Committee Clearance for the study (No: 13112010). Thirty cases were collected during the study period according to the inclusion and exclusion criteria.

Inclusion criteria: (i) Cases diagnosed as infiltrating ductal carcinoma of breast, (ii) Cases with adequate tissue for molecular biology analysis (iii) Fresh tumour tissue which was later frozen and stored.

Exclusion criteria:(i) Cases diagnosed as lobular breast carcinoma as they do not have E-cadherin expression by definition, (ii) Formalin fixed tissue samples.

Molecular biology work including DNA sequencing and RT-PCR and bioinformatics analysis was carried out at the SRM-DBT Platform for Advanced Life Technologies, SRM Institute of Science and Technology (SRMIST), Kattankulathur, Tamil Nadu, India, between June 2017 to September 2017.

The patients included in the study were aged between 30-60 years. The tissue samples were collected from the patients immediately after mastectomy procedure and stored in phosphate buffered saline in a deep freezer (-20°C). A portion of the tissue was processed and studied for pathological changes.

Controls: Tissue representing normal non neoplastic breast parenchyma, 6 cm away from tumour tissue isolated from the same patient served as controls. A total of 23 control samples were used in the study. The tissues collected were kept in appropriately labelled containers and was subjected to routine histopathological studies.

DNA extraction from tissue: DNA was extracted from all tissue samples using QIAGENDNeasy Blood and Tissue kits. The isolated DNA was assessed for purity and quantity by Nanodrop 1000 Spectrophotometer and appropriately stored at -20°C.

PCR amplification of the E-cadherin gene: The PCR amplification was carried out as per the primer sequences [Table/Fig-1] and protocol of Berx G et al., [11]. The PCR reaction mixture consisted of 7 µL of genomic DNA, 2+2 µL of primers of concentration 20 picomoles, 25 µL of PCR master mix and 14 µL of distilled water. PCR was performed for 35 cycles consisting of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds on a thermal cycler [Table/Fig-2]. The amplified PCR products were then subjected to agarose gel electrophoresis. The bands were identified and photographed.

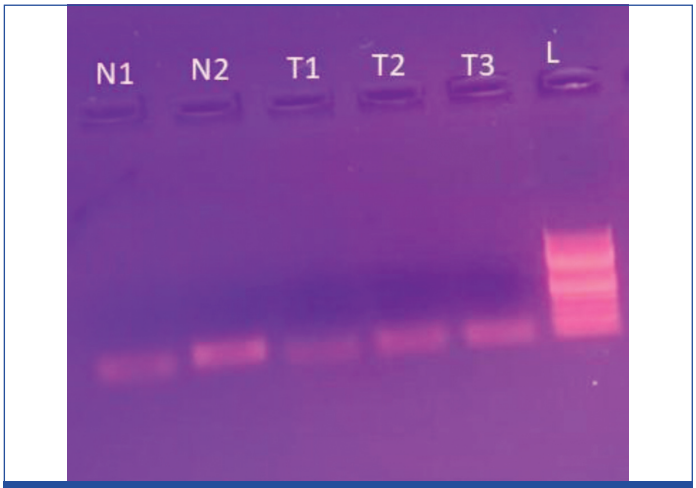
Exon No.	Sequence of S primer (5'-3')	Sequence of AS primer (5'-3')	Amplicon length (bp)
1	(-53) TACGGGGGGCGGTG CTCCGG	(+59) CTGGGGCGCGGAG CTTGCGG	282

[Table/Fig-1]: PCR primers' Sequences of E-cadherin gene- Exon 1 amplification.

Temperature and time	PCR				Melt Curve		
	Step 1	Step 2	Step 3	Step 4	Step 1	Step 2	Step 3
Temperature (°C)	95	94	60	72	95	60	95
Time	10 mins	30 secs	30 secs	45 secs	15 secs	1 min	0.01 secs
		35 cycles					

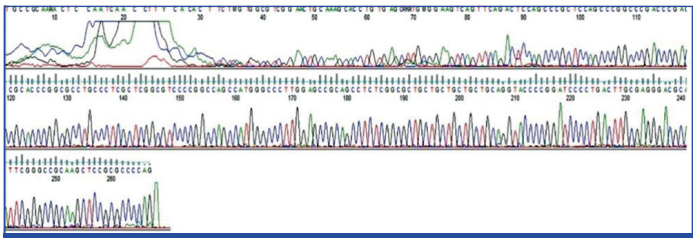
[Table/Fig-2]: PCR cycle conditions of E-cadherin gene- Exon 1 amplification.

PCR Product analysis by agarose gel electrophoresis: The PCR amplified product (282 bp) was analysed on 1.5% agarose gel with intercalating ethidium bromide [Table/Fig-3]. The gel was visualised under Ultravoilet (UV) transilluminator and the product was compared with molecular weight marker.

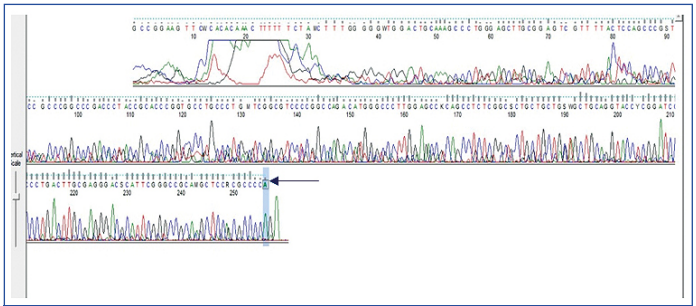


[Table/Fig-3]: Agarose gel electrophoresis. E-Cadherin- Exon1 amplified PCR product from normal and tumour DNA.
A single PCR product identified (282 bp) in lanes N1 to T3 (left to right); Lanes N1, N2: Normal control tissue; Lanes T1, T2, T3: Tumour tissue; Lane L – Molecular weight marker (ladder)

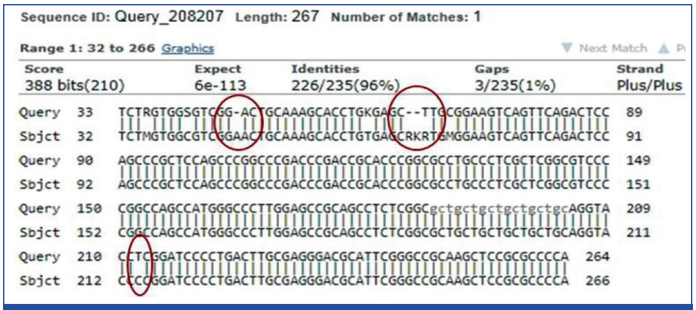
DNA Sequencing (Sanger method): Exon 1- CDH1 gene and variant analysis by bioinformatics: The amplified PCR products were then purified, isolated and sequenced by Sanger DNA sequencing method on DNA Seq Studio (Applied Biosystems) using universal primers. The sequence of normal and tumour DNA was compared [Table/Fig-4,5] using NCBI nucleotide Basic Local Alignment Search Tool (BLAST) search using the webpage: https://blast.ncbi.nlm.nih.gov/Blast.cgi [Table/Fig-6].



[Table/Fig-4]: DNA sequencing (Sanger method)- E cadherin Exon 1- Normal Control DNA.
Chromatogram showing nucleotide (A,T ,G, C) sequence within exon 1 region, CDH1 gene



[Table/Fig-5]: DNA sequencing (Sanger method)- E cadherin Exon 1- Tumour DNA (Invasive Breast Carcinoma).
Highlighted area above shows site of nucleotide sequence variation in tumour DNA compared to Normal Control DNA noted in [Table/Fig-4].



[Table/Fig-6]: BLAST Analysis- Exon 1: Tumour vs Normal DNA.
Normal sample - labelled as "Sbjct"; Cancer sample- labelled as "Query"; Mutations in cancer sample (highlighted in circle)

E-cadherin Transcript Analysis (Real time RT -PCR): Cytoplasmic RNA was isolated from tissue using QIAGEN RNeasy kit and reverse-transcribed using the primers:

Forward, 5' CAG CAC GTA CAC AGC CCT AA 3' and Reverse, 5' ACC TGA GGC TTT GGA TTC CT 3' for CDH1 [11]. Concentration and purity of total RNA was assessed using a Nanodrop 1000 Spectrophotometer. Total RNA from each sample was reverse-transcribed into single strand cDNA. Real time RT PCR was performed using Applied Biosystems Quant Studio 5 instrument. Expression levels from control non neoplastic breast tissue was used as baseline level and compared with expression levels from malignant breast cancer tissue. Relative expression of CDH1 mRNA was measured (ddct- *delta-delta-Ct value*) and fold change was calculated.

Immunoblotting: Whole cell lysates from both tumour and normal tissues were prepared using lysis buffer {13 PBS, 1% (NP-40) Nonyl phenoxy polyethoxyl ethanol-40, 0.5% sodium deoxycholate, 0.1% SDS} with protease inhibitors and protein concentrations were determined. Between 10 mg and 100 mg of protein were separated by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE), electro-transferred on to nitrocellulose membrane and incubated with E-cadherin Monoclonal Antibody (HECD-1) (Catalog Number 13-17, Thermo Fischer Scientific). Immunoreactive bands were visualised, quantified, compared and analysed. Chemicals and solvents used for the analyses were of analytical grade.

STATISTICAL ANALYSIS

Statistical analysis was done by Statistical Package for the Social Sciences (SPSS) version 22.0. A two-tailed Student's t-test was done to compare values between controls and tumour samples.

RESULTS

PCR Amplification of Exon 1, CDH1 gene: A single discrete band (282 bp size) was observed [Table/Fig-3] in tumour and normal control tissue corresponding to the targeted region in Exon 1 of CDH1 gene. CDH1 gene was amplified in all 23 control DNA samples (non neoplastic breast tissue) and 30 breast cancer samples.

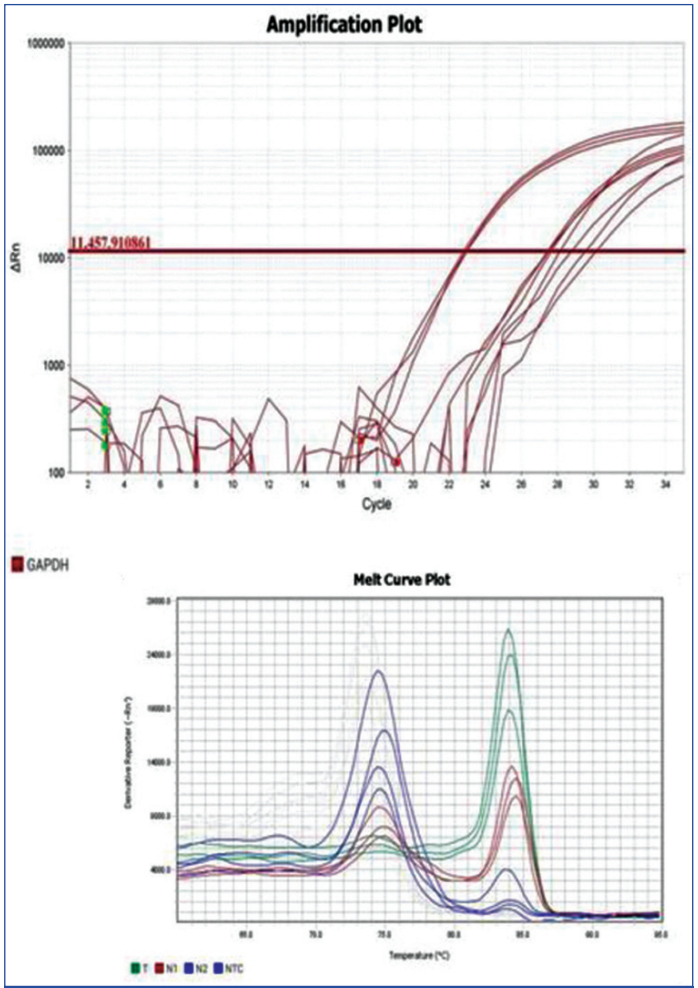
DNA Sequence analysis (Sanger method): The chromatogram output from Sanger sequencing of the amplified PCR products from control and tumour samples cane be visualised in [Table/Fig-4,5] respectively.

Exon 1, CDH1 gene Mutation profile- BLAST analysis: The exon 1 region of CDH1 gene isolated from normal and cancer tissue DNA was amplified, sequenced and compared using Genbank Accession Number MK 503194. The sequence of normal control was named "subject" and cancer tissue sequence named as "query". Bioinformatics analysis [Table/Fig-6] showed nucleotide position 46 with a single deletion mutation and positions 67 and 68 both with deletion mutations and position 214 with one single nucleotide change (C→ T) [Table/Fig-7].

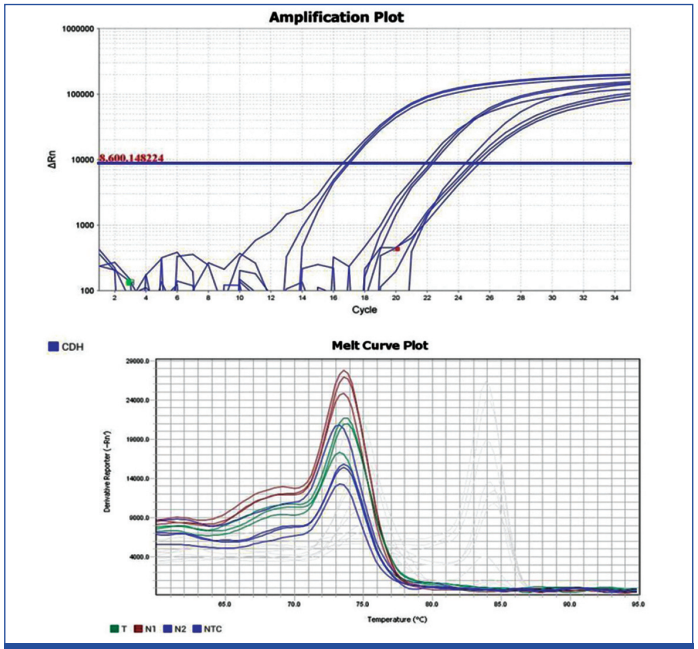
CDH1 mRNA Transcript Analysis- Real time RT-PCR: Real time RT-PCR was performed using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as housekeeping gene [Table/Fig-8] and CDH1 mRNA as test [Table/Fig-9] and amplification plots and melting curve plots.

Nucleotide position	Normal	Tumour
46	A	Deletion
67,68	R (A/G) K (G/T)	Deletion
214	C	T (single nucleotide change)

[Table/Fig-7]: Summary of DNA Sequence variations in CDH1 gene, Exon 1 (Tumour Vs Normal Control).



[Table/Fig-8]: Real time RT-PCR (q PCR): GAPDH (House-keeping gene)- Amplification plots and Melt-curve plot.
T- tumour tissue, N1, N2- normal control, NTC- no template control

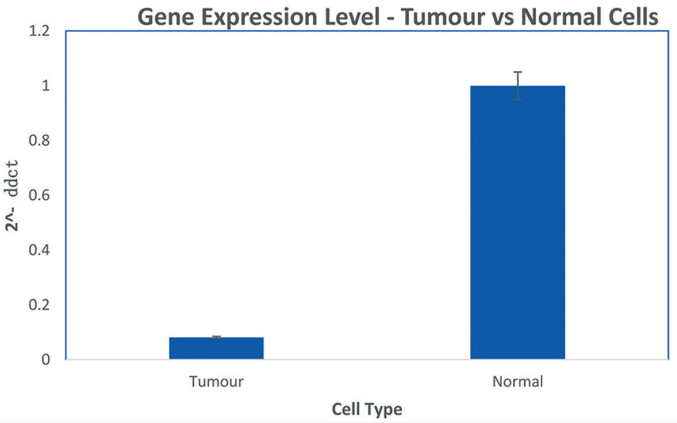


[Table/Fig-9]: Real time RT-PCR (q PCR) of E- Cadherin (CDH1) mRNA transcript-- Amplification plots and Melt -curve plot.
T- tumour tissue, N1, N2- normal control, NTC- no template control

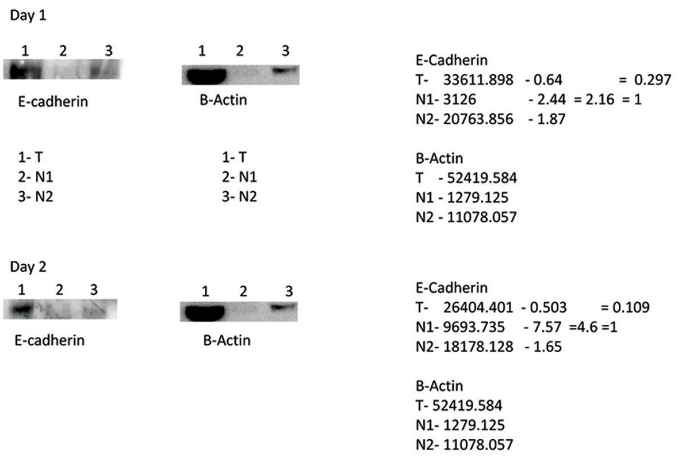
E-cadherin protein expression confirmation by immunoblotting showed a decreased expression of CDH1 in breast cancer samples compared to normal samples [Table/Fig-10,11].

Cell type	2-ddct*
Tumour	0.08099
Normal	1

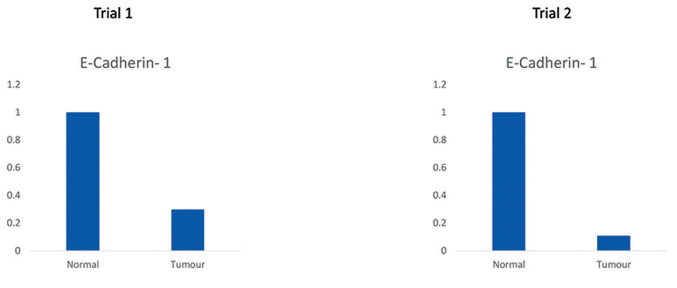
[Table/Fig-10a]: Gene expression profile- CDH1 (Real time RT PCR) -Tumour vs Normal control.
*delta-delta-Ct value for relative change in gene expression



[Table/Fig-10b]: Variation in gene expression profile of CDH1 (Real time RT PCR)- tumour vs control DNA- Graphical representation.
*delta-delta-Ct value for relative change in gene expression



[Table/Fig-11a]: Immunoblotting: E-cadherin protein expression- tumour vs normal.
T- tumour tissue, N1, N2- Normal control



[Table/Fig-11b]: Immunoblotting: E-Cadherin protein expression variation- tumour vs normal- Graphical Representation.

DISCUSSION

E-cadherin, a key cell adhesion molecule with a likely tumour suppressor function and plays an important role in maintaining the integrity of intercellular junctions [1,2]. E-cadherin is expressed normally in breast epithelium and in addition to the role of cell adhesion, it forms a key mechanism in Epithelial-Mesenchymal Transition (EMT) [4-6]. Loss of E-cadherin expression is typically associated with lobular carcinoma of breast but downregulation is also associated with infiltrating ductal carcinoma of breast in tumour progression, invasion, metastasis and poor prognosis [6-8]. At the molecular level, it is encoded by the CDH1 gene which has an important signal peptide region which is an important docking site and taking part in recognition of translation codes of various proteins [10-15]. Germ line mutations in CDH1 gene in familial gastric cancers were reported by Richards FM et al., [16] and Brooks-Wilson AR et al., identified germ line CDH1 mutations in 42 families of hereditary gastric cancer and proposed new genetic screening criteria [17]. Both the mutations were predicted to yield a truncated E-cadherin protein in the signal peptide domain. A splice acceptor site mutation, an A-to-G transition at position -2 from nucleotide 49 at the start of Exon 2 of the CDH1 gene and also a germline G-to-A transition at nucleotide 59 in exon 2 was identified by Richards FM et al., [16]. This mutation was found to result in a truncated E-cadherin gene product corresponding to the signal peptide domain [18-20].

The present study has focused on Exon 1 of CDH1 gene which has the signal peptide region that plays an important role in its normal as well as aberrant expression. CDH1 exon 1-2 region bears a 5' high-density CpG island, while other exons lack such features. The results show two loci with a total of three deletions in Exon 1 of CDH1 gene have been observed in this study [Table/Fig-6]. Position 46 with a single deletion mutation and positions 67 and 68 both with deletion mutations and position 214 with one single nucleotide change (C→T) [Table/Fig-7]. Such deletions may have functional impact on E-cadherin protein function. Previous studies reported that mutation p.L13_L15del cells yield low levels of E-cadherin, decreased cell adhesion and enhanced cell invasion. Functional alteration of the signal peptide domain structure has shown inhibition of binding to other components in the cell and in translation of important proteins interacting with E-cadherin [14-16].

E-cadherin protein expression is reduced or absent in invasive ductal carcinoma [4-6,16-18]. Diminished expression has been related to the acquisition of invasiveness within experimental tumours and many advanced cancers including breast cancers. In the present study, real time RT-PCR analysis of CDH1 mRNA has shown a 0.08-fold downregulation in the tumour. E-cadherin aberrant expression at this transcriptional level is in conformation with previous studies [17-19] on breast cancer. This observation of downregulation also conformed to previous studies [23-26] on E-cadherin protein expression detection by Western blotting technique. An earlier study by Yang L et al., [23] suggested that low expression or deletion of E-cadherin was positively associated with infiltration and metastasis in breast ductal carcinoma.

Limitation(s)

The study has a limitation related to smaller sample size of breast cancer tissue samples. Though procurement of unfixed tumour tissue at the time of mastectomy surgical procedure could be difficult, extension of the study protocol with a larger sample size might show more tumour heterogeneity and more genetic alterations with increased frequency.

CONCLUSION(S)

The genomic alterations in the biologically active and significant signal peptide region of E-cadherin protein, encoded by CDH1 at Exon 1 region has not been completely studied. Earlier studies have suggested methylation of CDH1 promoter to be the reason for CDH1 downregulation. The present study has attempted to study the alterations at genome level (CDH1 gene, Exon 1, encoding for signal peptide region), transcriptional and translational level with respect to E-cadherin. The effect of the mutations detected including two loci with deletion mutations and one single nucleotide change could affect the structural conformation of the protein and functional impact including aberrant expression. Confirmation of the above findings with association of mutations detected at the

genome level and the relative downregulation of CDH1 mRNA as well as E-cadherin protein level in tumour tissue needs to be done by molecular docking studies and in-vitro functional assays using cell-lines and/or animal studies.

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