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*International Journal of Current Research*
*Vol. 5, Issue, 10, pp.2890-2894, October, 2013*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

**ISSN: 0975-833X**

**RESEARCH ARTICLE**

# E-CADHERIN EXON 4-5, EXON 7, EXON 8, EXON 9 AND EXON 16 MUTATIONS IN SPORADIC INFILTRATING DUCTAL CARCINOMA OF THE BREAST

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**ARTICLE INFO**

***Article History:***
Received 17th July, 2013
Received in revised form
16th August, 2013
Accepted 20th September 2013
Published online 10th October 2013

***Key words:***
Breast cancer,
Ductal carcinoma,
E cadherin,
CDH1 mutations,
Carcinoma,
Metastatic protein

**ABSTRACT**

The cell- cell adhesion molecule E-cadherin is a potent invasion suppressor molecule. In human cancers, partial or complete loss of E-cadherin expression correlates with malignancy .Inactivating mutations have been identified for the E-cadherin gene (CDH1) in diffuse gastric cancers and lobular cancer of the breast. Hence in the present study we tried to study the occurrence of tumour specific mutations in exon 4-5, exon7, exon 8, exon 9 and exon 16 of E-cadherin gene in sporadic ductal carcinoma of the Breast. About 50 breast cancer patients were involved in the study. In exon 4-5 we observed 3 mutations ,one deletion mutation and 2 insertion mutation; in exon 7 we observed two deletion mutation; In exon 8 and exon 9 one deletion mutation was observed. In exon 16 one insertion mutation was observed. These mutations are suggestive of loss of growth control and tumour suppressive role of E-cadherin.



## INTRODUCTION

Breast cancer is cancer originating from breast tissue, most commonly from the inner lining of the milk ducts or the lobules that supply the ducts with milk (Sariego, 2010). In order to understand the mechanism of metastasis, it is important to know how cancer cells detach from the primary tumors. Cadherins are major cell- cell adhesion molecules. Perturbation of cadherin function causes disaggregation of tumour cells and thus may promote the invasion and metastasis of such cells (Berx *et al*., 1995). The calcium dependent interaction among E-cadherin molecules is critical for the maintenance of adherent junctions in areas of epithelial cell-cell contact. Loss of E-cadherin mediated adhesion characterizes the transition from benign lesions to invasive, metastatic cancer (Berx *et al*., 1995). The human epithelial E-cadherin geneCDH1 maps to chromosome 16q22.1 (Clenton *et al*., 1994). Cell- cell adhesion determines cell polarity and participates in cell differentiation, establishment and maintenance of tissue homeostasis. During oncogenesis this organized adhesion is disturbed by genetic changes. This results in changes in signaling, loss of contact inhibition and altered cell migration and stromal interaction (Van Roy *et al*., 2008). The human E-cadherin gene comprises 16 exons. Exon 4-5 is the extracellular domain of the protein. Exon 7,8,9 is identified as muation cluster region in diffuse gastric cancer (Berx *et al*., 1998).

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Exon 8 and 9 encode for the calcium binding sites of the E -cadherin protein. Exon 14-16 encode for the cytoplasmic domain of the protein. Exon 16 is the terminal end of the gene encoding the cytoplasmic tail necessary for binding β catenin or plakoglobin.The cytoplasmic tail of E-cadherin is linked via catenins to the actin cyto skeleton (Cowin, 1994). The exon 4-13 is involved in a molecular zipper mediating cell-cell adhesion (Shapiro *et al*., 1995). These observations urged us to analyze these exons of E-Cadherin gene in ductal carcinoma of the breast. We believe that the genomic mutation screen presented here will be a valuable molecular tool for understanding the role of E-cadherin in tumourogenesis.

## MATERIALS AND METHODS

### Patients and tissue samples

50 Breast cancer patients were involved in this study. The patients were aged between 30-60 years. Informed consent was obtained from all participants. The samples were collected from Madras Medical college, Chennai, Tamil Nadu ,India ,after obtaining the ethical clearance for the same (No: 13112010). The tissue samples were collected from the patients soon after their mastectomy and stored in phosphate buffered saline in a deep freezer. Portion of the tissue was sliced and studied for pathological changes. The samples confirmed malignant were selected for the study. Normal tissue isolated from the same patient served as controls.

### Genomic DNA isolation

DNA was extracted from all tissue samples using the HipureA ™ mammalian genomic DNA isolation /purification spin Kit, Himedia. The isolated DNA was labeled appropriately and stored at -20$^{o}$C.

### PCR amplification of the E-Cadherin gene

The PCR amplification was carried out as per the protocol of Berx *et al*., 1995. PCR was performed for 35 cycles consisting of 94 $^{o}$C for 30 s, 55-70 $^{o}$C for 30s and 72 $^{o}$C for 45 s on a thermal cycler. The PCR reaction mixture consisted of 7µl of genomic DNA, 2+2 µl of primers of concentration 20 picomoles, 25 µl of PCR master mix and 14 µl of distilled water. The amplified PCR products were then subjected to agarose gel electrophoresis. The bands were identified and photographed.

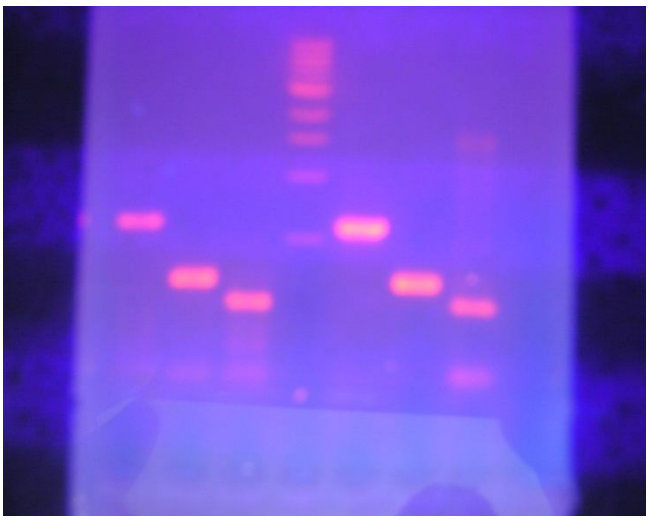

**Fig. 1. PCR amplification of the exon 4-intron-exon5, exon 7 and exon 9 of E-cadherin gene**

Lane 1- Exon4-intron-exon5 (normal); Lane2- Exon7 (normal); Lane3- Exon9 (normal); Lane4-Ladder; Lane5- Exon4-intron-exon5 (Tumour); Lane6- Exon7 (Tumour); Lane 7-Exon9 (Tumour)

### Sequencing of the PCR products

The amplified PCR products were then purified, isolated and sequenced. The cycle sequencing reaction was performed using Big Dye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (from Applied Biosystems, P/N: 4337457).

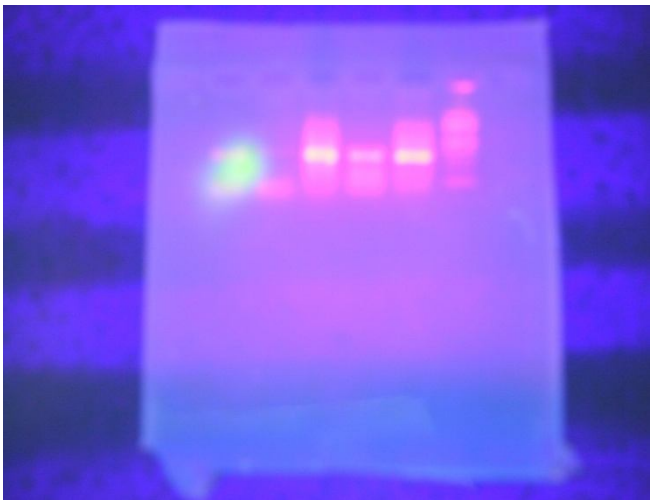

**Fig. 2. PCR amplification of the exon16**

Lane 1-3: Exon 16 (normal); Lane4, 5: Exon 16 (tumour); Lane 6: Ladder

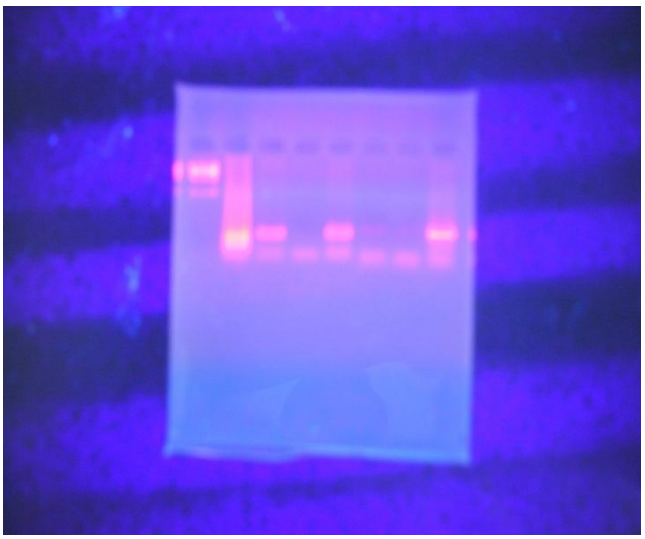

**Fig3: PCR amplification of the exon8**

Lane 1-4-Exon 8 (Tumour); Lane 5-7-exon8 (normal)

The sequencing reaction - mix was prepared by adding 1ul of Big Dye v3.1, 2ul of 5x sequencing buffer and 1ul of 50% DMSO. To 4ul of Sequencing reaction –mix was added 4 Pico moles of primer (2ul) and sufficient amount of plasmid. The constituted reaction was denatured at 95°C for 5 minutes. Cycling began with denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension for 4 minutes at 60°C and cycle repeated for a total 30 cycles in a MWG thermocycler. The reaction was then purified on sepheadex plate (Edge Biosystems) by centrifugation to remove unbound labelled and unlabelled nucelotides and salts. The purified reaction was loaded on to the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 hours.

### Mutational screening

The sequence of normal and tumour DNA was compared using NCBI nucleotide blast search. blast.ncbi.nlm.nih.gov/

## RESULTS

Comparison of the amplified aberrant tumour DNAs with the amplified normal DNA isolated from the normal breast tissue revealed various mutations. (Table1). The sequences were submitted to Gen Bank and an accession number was provided for the same (accession number JX 519564).

| Exon | Mutation observed | Nucleotide number |
|---|---|---|
| Exon 4-intron-exon5 | Deletion of T | 183 |
| | Insertion of G | 196 |
| | Insertion of G | 573 |
| Exon 7 | Deletion of G | 41 |
| | Deletion of A | 48 |
| Exon 8 | Deletion of G | 52 |
| Exon 9 | Deletion of A | 199 |
| Exon 16 | Insertion of A | 4 |

In exon 4-intron-exon5 we observed 3 mutations one deletion mutation (deletion of T) and two insertion mutatation (insertion of G).

In exon 7 on comparing the tumor and normal sequences we observed 2deletion mutations, deletion of G and deletion of A.

In exon 8 we observed one deletion mutation of G

Exon 9 also showed one deletion mutation of A.

Exon 16, the terminal region of the gene showed an insertion mutation of A.

```
NUCLEOTIDE SEQUENCE BLAST
fig :1
Blast results tumour Vs Normal Exon 4-5.

>lcl|57965 OciSeq_4-3_45F_037.ab1
Length=423

 Score =  730 bits (395),  Expect = 0.0
 Identities = 403/406 (99%), Gaps = 3/406 (1%)
 Strand=Plus/Plus

Query  177  GAGGGA-TTTGGCAGAGAAGTACCAAGGAGAGAAAGGGAAAAGACCCAGTGTTGGGATCC  235
            ||||||  |||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  20   GAGGGATTTTGGCAGAGAA-TACCAAGGAGAGAAAGGGAAAAGACCCAGTGTTGGGATCC  78

Query  236  TTCTTTACTAATTCTTTTTCTTTCATTTTGTCTTCAGATCAAATCCAACAAAGACAAAGA  295
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   TTCTTTACTAATTCTTTTTCTTTCATTTTGTCTTCAGATCAAATCCAACAAAGACAAAGA  138

Query  296  AGGCAAGGTTTTCTACAGCATCACTGGCCAAGGAGCTGACACACCCCCTGTTGGTGTCTT  355
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  AGGCAAGGTTTTCTACAGCATCACTGGCCAAGGAGCTGACACACCCCCTGTTGGTGTCTT  198

Query  356  TATTATTGAAAGAGAAACAGGATGGCTGAAGGTGACAGAGCCTCTGGATAGAGAACGCAT  415
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  TATTATTGAAAGAGAAACAGGATGGCTGAAGGTGACAGAGCCTCTGGATAGAGAACGCAT  258

Query  416  TGCCACATACACTGTAAGTATCTCTTAGAAGCTTGTTGACACCGGGGTAACATCCACCCA  475
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  TGCCACATACACTGTAAGTATCTCTTAGAAGCTTGTTGACACCGGGGTAACATCCACCCA  318

Query  476  GGATTTTTTGGTCAACCCATGCTGGATCCGCAGATCAGAGGCTCTGAACACATGAGGAGC  535
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  GGATTTTTTGGTCAACCCATGCTGGATCCGCAGATCAGAGGCTCTGAACACATGAGGAGC  378

Query  536  TTAACTTGACACCTCTTGACCTGTTGCTAAGGAGAAGGTGATGGGA  581
            |||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  379  TTAACTTGACACCTCTTGACCTGTTGCTAAGGAGAAG-TGATGGGA  423
```

**DATA 1. Nucleotide Blast result of Exon 4-5 Tumor Vs normal**

```
Fig :2
Exon 7 tumour Vs Normal

>lcl|22539 OciSeq_7-5_7F_043.ab1
Length=132

 Score =  209 bits (113),  Expect = 1e-59
 Identities = 118/120 (98%), Gaps = 2/120 (2%)
 Strand=Plus/Plus

Query  36   GGTCA-GGGGTC-AGGAGGATCCAGAGGGTGTGGAGGACAAATGTGTATTAGCTCAATCC  93
            ||||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13   GGTCAGGGGGTCAAGGAGGATCCAGAGGGTGTGGAGGACAAATGTGTATTAGCTCAATCC  72

Query  94   CGTGGACAAAGCAAAATCCTGCTAGGCCAGTTGTCAGTTAATACAGTGATGGTCTAAGCA  153
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73   CGTGGACAAAGCAAAATCCTGCTAGGCCAGTTGTCAGTTAATACAGTGATGGTCTAAGCA  132
```

**DATA 2. Nucleotide Blast result of Exon 7 Tumor Vs normal**

```
Fig:3
>lcl|37223 OciSeq_C_E-COd8F_001.ab1
Length=142
exon 8 TUMOUR vS NORMAL BLAST RESULTS

 Score =  211 bits (114),  Expect = 4e-60
 Identities = 117/118 (99%), Gaps = 1/118 (1%)
 Strand=Plus/Plus

Query  49   AGT-CACTGACACCAACGATAATCCTCCGATCTTCAATCCCACCACGGTAATTCTATAAC  107
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25   AGTGCACTGACACCAACGATAATCCTCCGATCTTCAATCCCACCACGGTAATTCTATAAC  84

Query  108  TCCTTAGAGGGTTTCCAAAGAAAGGTCTTTTGTTGTTCATGAACTAAGGGTACCACTA  165
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   TCCTTAGAGGGTTTCCAAAGAAAGGTCTTTTGTTGTTCATGAACTAAGGGTACCACTA  142
```

**DATA 3. Nucleotide Blast result of Exon 8 Tumor Vs normal**

```
Fig:4
>lcl|17293 OciSeq_G_E-COd9F_005.ab1
Length=315
tumour vS nORMAL EXON 9

 Score =  298 bits (161),  Expect = 1e-85
 Identities = 164/165 (99%), Gaps = 1/165 (1%)
 Strand=Plus/Plus

Query  39   TGATGCTGATGCCCCCAATACCCCAGCGTGGGAGGCTGTATACACCATATTGAATGATGA  98
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGATGCTGATGCCCCCAATACCCCAGCGTGGGAGGCTGTATACACCATATTGAATGATGA  60

Query  99   TGGTGGACAATTTGTCGTCACCACAAATCCAGTGAACAACGATGGCATTTTGAAAACAGC  158
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TGGTGGACAATTTGTCGTCACCACAAATCCAGTGAACAACGATGGCATTTTGAAAACAGC  120

Query  159  AAAGGTTTGTATGGTACCTGGCAAGATGCAGAAACTGGCAA-CCA  202
            |||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  121  AAAGGTTTGTATGGTACCTGGCAAGATGCAGAAACTGGCAAACCA  165
```

**DATA 4. Nucleotide Blast result of Exon 9 Tumor Vs normal**

```
Fig :5
>lcl|37591 089-III330_N4_F-D11.ab1
Length=254
exon 16 tumour vs Normal

 Score =  433 bits (234),  Expect = 2e-126
 Identities = 237/238 (99%), Gaps = 1/238 (0%)
 Strand=Plus/Plus

Query  1    CTGAAAGCGGCTGATACTGACCCCACAGCCCCGCCTTATGATTCTCTGCTCGTGTTTGAC  60
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18   CTG-AAGCGGCTGATACTGACCCCACAGCCCCGCCTTATGATTCTCTGCTCGTGTTTGAC  76

Query  61   TATGAAGGAAGCGGTTCCGAAGCTGCTAGTCTGAGCTCCCTGAACTCCTCAGAGTCAGAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   TATGAAGGAAGCGGTTCCGAAGCTGCTAGTCTGAGCTCCCTGAACTCCTCAGAGTCAGAC  136

Query  121  AAAGACCAGGACTATGACTACTTGAACGAATGGGGCAATCGCTTCAAGAAGCTGGCTGAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  AAAGACCAGGACTATGACTACTTGAACGAATGGGGCAATCGCTTCAAGAAGCTGGCTGAC  196

Query  181  ATGTACGGAGGCGGCGAGGACGACTAGGGGACTCGAGAGAGGCGGGCCCCAGACCCAT  238
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  ATGTACGGAGGCGGCGAGGACGACTAGGGGACTCGAGAGAGGCGGGCCCCAGACCCAT  254
```

**DATA 5. Nucleotide Blast result of Exon 16 Tumor Vs normal**

## DISCUSSION

In humans partial or complete loss of E-cadherin expression correlates with malignancy. E-Cadherin plays an essential role in the formation and maintance of normal architecture and function of epithelial tissues (Takeichi, 1995, Bracke *et al*, 1996). This 120 KD trans membrane glyco protein localized in lateral cell-cell contacts and enriched in the Zonula adherence junctions, mediates intercellular adhesion through homophilic interactions (Takeichi, 1991).

**Exon 4-5 mutations**

The exon 4-13 is involved in a molecular zipper mediating cell-cell adhesion (Shapiro *et al.,* 1995) and hence the detected mutations in this exonic region may have significant role in determining the metastatic potential of the tumor.

**Exon 7 mutations**

Kanai *et al.*, 1994 reported in their study that with lobular breast tumors, 2 (10%) of the 20 cases examined, a sequence abnormality was detected in E–cadherin exon 7, i.e. a point mutation of codon 315 (AAT to AGT) which resulted in a single amino acid substitution (Asparagine to Serine). They reported that this mutation may abolish the E–cadherin–mediated cell–cell adhesion and be at least partly responsible for the weak intercellular adhesiveness and scattered histological pattern of the tumor. *In vitro* created missense mutations in the $Ca^{2+}$binding sites, encoded by exon 7 of the mouse E-cadherin gene, abolishes the adhesiveness of the protein (Ozawa *et al.,*1990). Guilford *et al.,* 1998 reported that in gastric cancers sequencing of the E-cadherin gene revealed a G to T nucleotide substitution in the donor splice consensus sequence of exon 7, leading to a truncated gene product.

**Exon 8 mutations**

Earlier studies of E-cadherin transcripts in 63 gastric carcinomas by RT-PCR and direct cDNA sequencing revealed six exon8 skippings, seven exon 9 skippings and four in frame deletions .Exon 8 and exon 9 could be considered as a mutation hot spot in E-cadherin gene. Exon skipping of exon 8 or 9 destroys essential calcium binding sites of the E-cadherin gene (Berx *et al*, 1998).

**Exon 9 mutations**

Deplazes *et al.,* 2009 demonstrated that E-cadherin harboring an in-frame deletion of exon 8 had reduced ability to activate Rac1 and to inhibit Rho.The lack of Rac1 activation influenced the downstream signaling of Rac1, as shown by a decrease in the binding of the Rac1 effector protein to rac1-GTP.Reduced membranous localization of p120 –catenin in mutant E-cadherin expressing cells was associated with the lack of negative regulation of Rho by mutant E- cadherin. The enhanced motility and invasion associated with mutant E-cadherin was sensitive to the inhibition of Rac1 and Rho. Therefore it was concluded that the mutation of E-cadherin had a reciprocal influence on Rac1 and Rho activation and that Rac1 and Rho are involved in the establishment of the migratory and invasive phenotype of tumor cells harboring an E-cadherin mutation.

**Exon 16 mutations**

The C-terminal cytoplasmic domain of ~150 residues is highly conserved in sequence, and has been shown experimentally to regulate the cell-cell binding function of the extracellular domain of E-cadherin, possibly through interaction with the cytoskeleton. The juxtamembrane region of the cadherin cytoplasmic tail has been identified as a functionally active region supporting cadherin clustering and adhesive strength. Exon 14-16 encode for the cytoplasmic domain of the protein. Exon 16 is the terminal end of the gene encoding the cytoplasmic tail necessary for binding β catenin or plakoglobin.The cytoplasmic tail of E-cadherin is linked via catenins to the actin cyto skeleton (Cowin, 1994). The cytoplasmic domain of E-cadherin may modulate the Wnt signalling pathway by inhibiting the availability of free cytoplasmic β-catenin. In response to wnt signaling, cytoplasmic beta-catenin is stabilized, accumulates in the cytoplasm and enters the nucleus, where it finds a partner, a member of the DNA binding protein family LEF/TCF (Tcell factor-lymphoid enhancer factor). Together they activate new gene expression programs. One of the target genes for β-catenin/TCF encodes c-MYC protein (Guilford *et al.,* 1999). This clearly explains the constitutive activation of the wnt pathway which can lead to cancer. In general studies have reported that all these mutations are predicted to generate a secreted E-cadherin fragment instead of a transmembrane protein with cell-cell adhesion activity. Soluble E- cadherin fragments have been identified in the serum and urine of cancer patients (Katayama *et al.,* 1994) and in the medium of the human breast cancer cells MC7 ( Damsky *et al.,* 1983; Wheelock *et al.,* 1987).

It is expected that these mutations will have severe effects on the normal functions of the E-cadherin protein. Aberrant expression of E-cadherin has been associated with the development of metastases in patients with breast cancer. Even though the expression of E-cadherin has been studied in primary breast tumors, little is known about its expression at the distant metastatic sites (Paul J Kowalski *et al.,* 2003). In breast cancer, inactivating point mutations in the E-cadherin gene are frequently found in invasive lobular carcinoma (ILC) but never in invasive ductal carcinoma (IDC). Lobular carcinoma *in situ* (LCIS)

adjacent to ILC has previously been shown to lack E-cadherin expression, but whether LCIS without adjacent invasive carcinoma also lacks E-cadherin expression and whether the gene mutations present in ILC are already present in LCIS is not known. The method of blocking E-cadherin down regulation in tumors is one of the important future approaches in gene therapy. To target this molecule is the logical path to prevent metastazing potential of almost any epithelial tumor. E-cadherin down regulation is caused by many different mechanisms, ranging from mutations and gross deletions to repression of gene transcription, as well as signal transduction stimulation of E-cadherin adhesion complex formation. Loss of E-cadherin expression or E-cadherin mutations appears to be a major determinative step in the metastatic progression. Therefore identification of E-cadherin mutations and deletions in human cancers may improve prognosis in these cancers, and further helps us to understand how cells escape from their normal location and spread through the body. Earlier studies with 98 sporadic breast cancers reported E-cadherin mutations solely in the histological subtype of lobular breast cancers.For ductal carcinomas of the breast so far no E-cadherin mutations were reported (Berx *et al*., 1995; Kashiwaba *et al*., 1995). However in our study we observed E-cadherin mutations in the ductal carcinoma of the breast .Mutations were observed in exon 4-intron-exon5, exon 7, exon 8, exon 9 and exon16. The detected mutations in this exonic region may have significant role in determining the metastatic potential of the tumor.

**Acknowledgements**

The authors thank Asia's first women neurosurgeon Dr.T.S.Kanaka for her constant support and guidance.

**Conflict of Interest**: None

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