Mutational spectrum of Gelsolin and its down regulation is associated with breast cancer

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Abstract. Cytoskeletal rearrangement occurs in variety of cellular processes and involves a wide spectrum of proteins. Gelsolin super family proteins control actin organization by severing and capping filament ends and nucleating actin assembly. Gelsolin is the founding member of this family and plays important role in pathogenesis of human neoplasia.

This study aimed to investigate the germline mutations and expressional profile of Gelsolin in human breast cancer tissues. For germ line screening PCR-SSCP technique was used while expression was analyzed through quantitative real time PCR. Different types of mutations were observed in Gelsolin coding regions on exons 4, 10, 11, 14 and 15. These mutations include 3 missense nonsynonymous substitution mutations, 2 deletions, 1 insertion and 1 synonymous substitution mutation. Gelsolin transcript level was found significantly lower in breast tumor tissues compared to control samples ($p = 0.03$). Low level of Gelsolin was found in metastatic patients ($p = 0.002$) and patients who died from breast cancer ($P = 0.03$) compared to disease free patients at final follow up. This study shows that level of Gelsolin is down regulated in breast cancer tissues and is linked with metastasis development and death in patients. It is concluded that genetic changes in coding regions of Gelsolin can potentially contribute to genetic instability. These genetic variations and expressional correlation with patient survival may prove to be of significant importance.

Keywords: Gelsolin, germline mutations, PCR-SSCP, expressional analysis, quantitative real time PCR, breast cancer

1. Introduction

Gelsolin protein super family is a conserved family of proteins present in mammalian and non-mammalian organisms [22]. This super family consists of seven different proteins that contain homologous repeats of gelsolin-like (G) domain. Gelsolin, a protein of 82–84 kDa, is the founding member of this family encoded by a gene on chromosome 9. It exists as a cytoplasmic as well as a plasma isoform and can bind, sever and cap actin filaments [21]. It is expressed in a wide variety of cell types and contains six gelsolin-like (G) domains. It was first described as a protein able to bind and sever actin filaments, and to control polymerization of barbed ends. This protein also initiates formation of actin filaments by binding two monomeric actin molecules. Gelsolin activity is regulated by Ca^{+2}, intracellular pH, phosphoinositides and tyrosine phosphorylation [12,14,27,38,50]. It is a downstream effector of Rac for motility in dermal fibroblasts and regulates phosphoinositide signaling pathways and ion channel function *in vivo*. It also acts as a regulator and effector of apoptosis [38]. Gelsolin is one of the most important actin structure regulating proteins. Its expression in almost all eukaryotic cells shows its fundamental importance in maintaining an organized actin cytoskeleton [8,9].

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HeLa cells that normally do not express gelsolin are rendered more susceptible to apoptosis by over expressing gelsolin [51]. Point mutations in mouse gelsolin grant it tumor-suppressor activity against H-ras oncogenes transformed NIH 3t3 cells. Gelsolin has a histidine instead of a proline residue at position 321 and suppresses the tumorigenicity of EJ-NIW3T3 (ras oncogene-transformed NW3T3 cells) cells [16,56]. Moreover, transfection of the gelsolin gene in a human bladder cancer cell line strongly reduces colony-forming ability and tumorigenicity *in vivo* [42]. It has been reported that gelsolin promoter activity is much higher in normal mammary cells (184A1N4) than in breast cancer cells (T47D and MCF-7) [44]. Gelsolin as a cytoskeleton-regulated factor may control cell mobility and inhibit tumor growth and metastasis [38]. Expression of gelsolin is noted to be reduced in human cancer tissues, particularly in human breast cancer tissues and non-small cell lung carcinomas [31, 15,60]. Over-expression of gelsolin has been shown to inhibit metastasis *in vivo* by its binding with actin molecules [16].

Variations in gelsolin expression affect major cytoskeletal changes during differentiation and carcinogenesis. Diminished expression of gelsolin was observed in multiple transformed cells [16,42]. It is reported that gelsolin expression is significantly low in murine fibroblasts transformed by *H*-ras oncogene. [16]. In human fibroblasts and epithelial cells transformed with SV40 virus, gelsolin is one of the most striking down regulated markers of the transformed state [34]. Low or undetectable expression of gelsolin was observed in seven of the eight gastric carcinoma cell lines [58]. There have been similar reports of gelsolin expression in human breast cancers and colon cancers [6,53]. In a study 6 bladder cancer cell lines and 14 of the 18 bladder cancer tissues showed reduced or undetectable gelsolin expression compared with normal control bladder mucosa [45]. Gelsolin is able to bind PIP2 and can inhibit PIP2 hydrolysis by PLCγ *in vitro* [62,48], it was speculated that the tumor suppressive function of gelsolin is also provoked by the inhibition of signal transduction through phosphoinositides [45].

Partial or total loss of Gelsolin expression is commonly observed in breast cancer patients with an increase in PIP2 levels [13,40]. Similar to these observations Sagawa et al. [47], suggested that Gelsolin suppresses the activation of Protein kinase C (PKCs) thus inhibiting cell proliferation and tumor growth [47].

Several reports indicate that gelsolin acts as a tumor suppressor [3,18,19,29,37,45,47,55]. There are other studies who reported Gelsolin as a tumor activator [1, 5,23,35,36,39,57]. It is perceived that role of Gelsolin as a tumor suppressor depends upon the type of cancer [25]. Abbeele et al. [4] showed that gelsolin act as a tumor activator as it's down regulation significantly reduces the invasive and motile properties of cells, as well as cell aggregation *in vitro* [4].

It has been observed that increased gelsolin expression may play a critical role in converting a superficial tumor to an invasive tumor [30]. Shieh et al. [11] observed a biphasic gelsolin expression pattern with limited gelsolin staining in oral pre-cancerous lesions and increased gelsolin staining in primary and metastatic lesions [11]. A decreased trend in Gelsolin expression was observed in malignant progression, from normal epithelium to DCIS to invasive breast cancer [19]. Down-regulation of gelsolin might have a role in progression of inverted papilloma [26].

Many studies have been reported in context to Gelsolin role in cancer but the presence of any germ line mutations on this gene in breast cancer patients has not been evaluated before. This work was planned to screen gelsolin for germ line mutations in sporadic breast cancer cases of Pakistani population. Breast cancer is one of the major death causes amongst women in world as well as in Pakistan [46,59]. Expression profile of gelsolin was also examined in breast cancer patients and compared with clinical outcomes of patients in the cohort.

2. Materials and methods

2.1. Identification of patients and samples collection

Present case-control study consisted of pathologically confirmed breast cancer cases. Two study groups were used in this study. The cohort#1 was used for screening germline mutations. It consists of 350 blood samples along with age and gender matched, healthy and disease free normal individuals as controls. While cohort#2 was used for evaluating Gelsolin expression at mRNA level and comprises 60 tissue samples. Tumor matched normal adjacent tissues were used as control. Presence of tumor cells in the collected tissues was verified by examination of frozen sections following H and E staining by a consultant pathologist. Details of the patients in study cohort#1 and 2 are given in Tables 1 and 2 respectively. Samples were recruited from Nuclear medicine, Oncology and Radiotherapy Institute (NORI), Pakistan Institute of Medical Sci-

Table 1
Characteristics of the study population (cohort#1)

	Associated factors	Number of patients (P value)
1	Age	
a	$\leqslant 40$ years	123 (< 0.0001)
b	Above	227
2	Age at menarche	
a	< 13 years	149 (< 0.0001)
b	$\geqslant 13$ years	186
c	$\geqslant 17$ years	15
3	Age at first birth	
a	< 25 years	223 (< 0.0003)
b	$\geqslant 25$ years	127
4	Menopausal status	
a	Pre menopausal	146 (< 0.03)
b	Post menopausal	204
5	Age at menopause	
a	< 48 years	126 (< 0.02)
b	$\geqslant 48$ years	78

ences (PIMS) Islamabad and Military Hospital (MH) Rawalpindi, Pakistan. These samples were collected with a prior approval from Ethical Committees of both CIIT and hospitals. All study subjects participated on a volunteer basis with informed consent.

2.2. DNA isolation and quantification

DNA was isolated from leukocytes (blood), using organic protocol with phenol–chloroform extraction as previously described [24,52]. Electrophoresis was performed on isolated DNA in 1% ethidium-bromide stained agarose gel and photographed (BioDocAnalyze Biometra). 5 ng/μl dilutions were made for isolated DNA sample and stored at 4°C until further usage.

2.3. RNA extraction and reverse transcription-polymerase chain reaction

RNA was extracted using Trizol reagent in accordance with the provided protocol by manufacturer (Invitrogen, Scotland, UK) from tissue samples. Sample RNA was quantified using a spectrophotometer (WPA UV 1101, Biotech Photometer, Cambridge, UK) and standardized to a concentration of 500 ng per reaction. This RNA was used as a template to reverse transcribe cDNA using the Super Script First-Strand Synthesis System (Invitrogen, USA)

2.4. PCR amplifications, SSCP analysis and sequencing

PCR amplifications were performed in 10 μl PCR mixture containing 2 μl of each primer (10 mM), 5 μl

master mix, 1 μl PCR water and 2 μl (5 ng/μl) extracted DNA. Reaction mixture was placed in 9700 thermal cycler/ Veriti of ABI systems. PCR profile consisted of an initial melting step of 94°C for 5 min, 35 cycles of 94°C for 40sec, annealing at 55°C for 40 seconds and 72°C for 1min and a final extension step of 72°C for 10 min and hold at 4°C. All patients and control DNA was amplified for gelsolin gene with exon specific primers.

PCR product was analyzed by single stranded conformational polymorphism (SSCP) as previously described [24,52] and results were examined with gel documentation system (BioDocAnalyze Biometra) after ethidium bromide staining and photographed for further analysis. Amplified products showing abnormal SSCP patterns were prepared as per instructions and shipped to MCLab (USA) for DNA sequencing. The sequenced results were made forward complementary before analysis using BioEdit v 7.0.5 software and analyzed.

2.5. Mutational analysis

Sequencing results were analyzed via Alamut bio software version 2.0 for detection of mutation location as well as its effect on genomic, cDNA and protein level. Missense mutations were also predicted by Align GVGD and SIFT [49] using this software.

2.6. Quantitative-Polymerase Chain Reaction (Q-PCR)

Real-time quantitative PCR was used to assess gelsolin transcript levels as previously reported [2]. Results are given as number of transcripts/μl based on an internal standard and the results were further normalized using the expression of GAPDH in these samples. Q-PCR technique used the Amplifluor system (Intergen Inc., New York, US), Q-PCR Master Mix (ABgene, Surrey, UK) and a universal probe (Uniprimer^{TM}, Intergen) to record the fluorescence emitted by the z-sequences. Conditions for Q-PCR were: an initial 15 minute 95°C period followed by 60 cycles of 95°C for 15 seconds, 55°C for 60 seconds and 72°C for 20 seconds.

2.7. Statistical analysis

Data were collected, compared and analyzed using a two-sample, two-tailed t-test. Data values given represent the mean value $\pm$ SEM, and values of $p \leqslant 0.05$ were considered to be statistically significant. Confidence Interval was calculated using the null hypothesis approach.

Table 2
Clinical and pathological information of the study cohort#2

Grouping	Number				
Age					
> 50	23				
< 50	37				
Grades	Grade1	Grade2	Grade3		
	17	22	21		
Histology	Ductal	Lobular	Others		
	47	8	5		
Clinical outcome	Disease free	With metastasis	With local reoccurrence	Died of breast cancer	Died of unrelated disease
	36	**8**	**3**	**8**	**5**

Table 3
Mutations observed in the Gelsolin gene in Breast Cancer Patients

No of cases	Frequency of variation	OR (95% CI)	Exon	cDNA level	Genomic level	Protein level	Effect
12	0.08	0.09 (0.0053–1.5348)	4	NM_001127662.1: c.11A>G	Chr9(GRCh37): g.124064260A>G	p.Glu4Gly	Missense substitution
27	0.186	0.22 (0.0139–3.7747)	11	NM_001127662.1: c.985C>T c.987_988del	Chr9(GRCh37): g.124080952C>T g.124080954_124080955del	p.Leu329Phe p.Pro330X	Missense substitution& Deletion
18	0.124	0.14 (0.008–2.3665)	15	NM_001127662.1: c.1637A>T	Chr9(GRCh37): g.124089635A>T	p.Lys546 Ile	Missense substitution
36	0.248	0.33 (0.0201–5.4173)	10	NM_001127662.1: c.897_898insGCAGGC	Chr9(GRCh37):g.124080694_ 124080695insGCAGGC	p.Ala299_Asn300 insAlaGly	Insertion, Frame shift mutation
20	0.137	0.16 (0.0096–2.623)	14	NM_001127662.1: c.1436del	Chr9(GRCh37): g.124088809del	p.Lys479 ArgfsX7	deletion, Frame shift mutation
32	0.220	0.28 (0.0172–4.6548)	10	NM_001127662.1: c.906G>A	Chr9(GRCh37): g.124080703G>A	p. =	Synonymous substitution

3. Results

3.1. Characteristics of the study population (cohort#1)

General characteristic features associated with breast cancer were studied in patients (Table 1). These features include patients age, age at menarche, age at first full term pregnancy and age at menopause. Mean age of patients were noted to be 47 years. Mean age at menarche was 13 years. Age at first full term pregnancy in 39% patients was > 25 years while 61% had < 25 years. While mean Age at menopause was 45 years.

3.2. Mutational results

An extensive screening of Gelsolin gene was conducted for mutations and variations among 350 samples and 350 cancers free control samples in study cohort#1.

Different types of mutations were observed which include 3 nonsynonymous substitutions, 1 synonymous substitution, 3 frame shift mutations (comprising of 2 deletions and 1 insertion) (Table 3). Mutations described are of 7 different types and were observed in a total of 145 samples.

3.3. Non-synonymous substitutions

3 nonsynonymous substitution mutations at exon 4, 11 and 15 were observed. Transition A to G in exon 4 "11A>G" cause missense substitution mutation and change Glutamine at position 4 to Glycine in 12 samples (Fig. 1A). Transition C to T, cause missense substitution mutation at exon11 at position 985 by altering Leucine at position 329 to Phenylalanine in 27 samples (Fig. 1B). Transversion from A to T in exon 15 "1637A>T" resulted in missense substitution by changing Lysine at amino acid position 546 to Isoleusine in 18 samples (Fig. 1C).

3.4. Frame shift mutations

One insertion and two deletion mutations were observed in this study. Insertion of 6 bps "GCAGGC" in exon 10 at position 897_898 caused the insertion of 2 residue (s) Alanine and Glycine in 36 samples (Fig. 1D). While deletion of 2bp "TC" and 1bp "A" observed at exon 11, position 987_988 in 27 samples (Fig. 1B) and at exon 14, position 1436 in 20 samples (Fig. 1E) respectively.

Table 4
Distribution of Gelsolin mutation types among age group and menopausal status in Breast cancer patients

| Types of mutation	Pre menopausal N (%)	Post menopausal N (%)	OR (95% CI)	Age < 40 N %	Age > 40 N %	OR (95% CI)
11A>G	5 (41.6%)	7 (58.33%)	1.1224 (0.3465–3.6355)	3 (25%)	9 (75%)	0.5488 (0.1451–2.0757)
985C>T 987_988 TC/–	9 (33.3%)	18 (66.6%)	0.7606 (0.3279–1.7643)	4 (14.48%)	23 (85.18%)	0.2644 (0.0887–0.788)
1637A>T	7 (38.8%)	11 (61.1%)	0.9943 (0.3726–2.6537)	4 (22.2%)	14 (77.77%)	0.4606 (0.1474–1.4394)
897_898——/GCAGGC	12 (33.33%)	24 (66.6%)	0.7527 (0.3584–1.5807)	16 (44.4%)	20 (55.5%)	1.414 (0.6962–2.8721)
1436 A/-	11 (55%)	9 (45%)	2.0193 (0.8062–5.058)	13 (65%)	7 (35%)	3.443 (1.3258–8.9413)
906G>A	13 (56.52%)	19 (82.60%)	1.0795 (0.5082–2.2928)	15 (46.87%)	17 (53.12%)	1.572 (0.7483–3.3024)
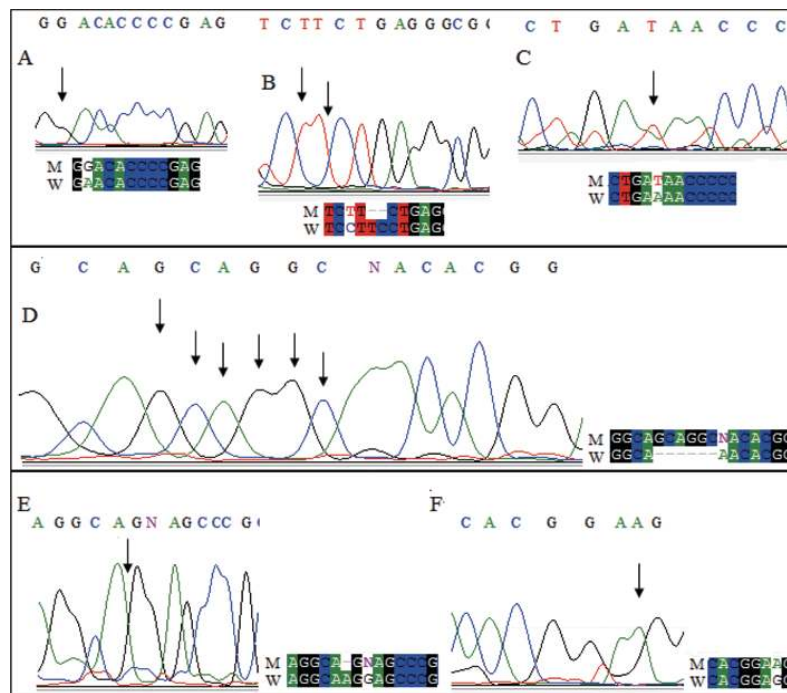
Fig. 1. Mutations observed in coding sequence of Gelsolin (study cohort#1). A **c.11A>G**, B **c.985C>T** and C **c.1637A>T**: nonsynonymous missense mutations on exon 4, 11 and 15 respectively. D **c.897_898insGCAGGC**, B **c.987_988del** and E **c.1436del**: Frameshift mutations at exon 10, 11 and 14 respectively. F **c.906G>A**: Synonymous substitution at exon 10. Arrows show observed change, M is for mutated sequence while W is wild sequence. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-120952)

3.5. Synonymous substitution

Transition from G to A in exon 10, "906G>A" resulted in synonymous substitution in 32 samples (Fig. 1F) as codon GAG changed to GAA and both encode for Glutamic acid.

When analyzed according to menopausal status, most common mutation in premenopausal women was frame shift mutation that was 906G>A at exon 10. Frame shift mutation 897_898——/GCAGGC in at exon 10 was most observed among postmenopausal patients. This variation is also more frequent among patients in age group < 40. While 987_988 C>T, 987_988 –/TC at exon 11 was wide spread among patients > 40 years of age. Distribution of all these mutations according to age and menopausal status is summarized in Table 4.

3.6. Expression of Gelsolin in human breast tissues

This study further quantified the levels of Gelsolin transcript in breast tumor tissues (cohort#2). Levels of Gelsolin transcripts were quantified in breast tumor tissues and were normalized by GAPDH. Gelsolin level in breast cancer tissues was lower compared with normal tissues and the difference was statistically significant ($p = 0.03$) (Fig. 2A).

When Gelsolin transcript level was observed in accordance to grades, a stepwise decrease in level of Gelsolin from grade1 to grade 3 tumors was noted. How-
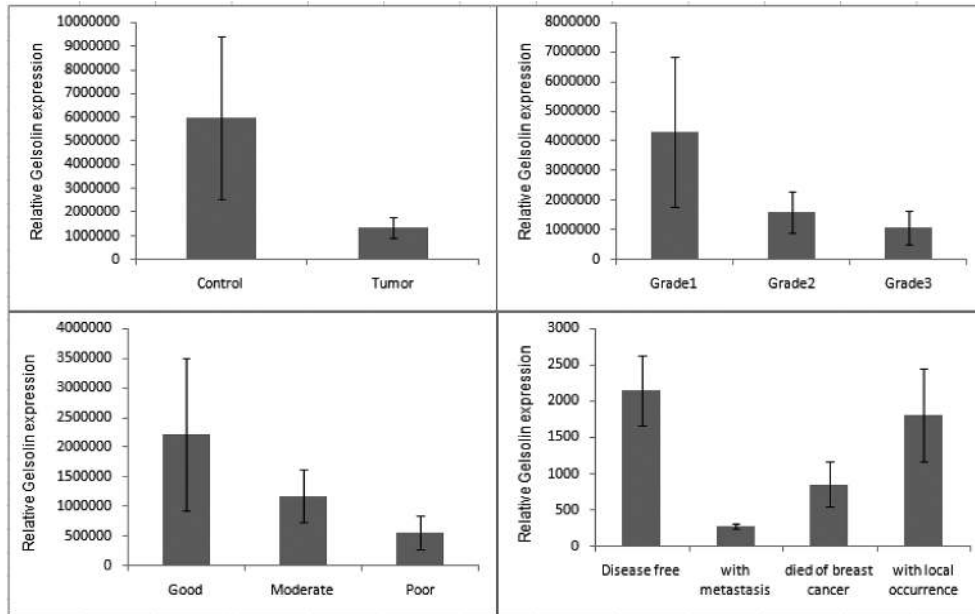
Fig. 2. Quantitative analysis of Gelsolin transcript levels in the study cohort#2 in control and tumor tissues ($p = 0.03$) (A), Relative expression of Gelsolin in breast cancer tissues in connection with grades (B), predicted prognosis was based on the NPI value of each patient and that good, moderate and poor prognosis had NPI value either < 3.4, $3.4–5.4$ or > 5.4. (C). Patients who develop metastasis ($p = 0.002$) and who died of breast cancer ($p = 0.03$) had significantly low level of Gelsolin than those who were disease free at final clinical outcome (D).

ever, the difference observed was not significant ($p = 0.31$ and $p = 0.21$, grade 1 vs. grade 2 and grade 1 vs. grade 3 respectively) (Fig. 2B). Nottingham prognostic index (NPI) was calculated to determine prognosis following surgery for breast cancer. Patients were divided in to three groups, good, moderate and poor prognosis based on the NPI value < 3.4, $3.4–5.4$ or > 5.4. Its value is calculated using three pathological criteria that are size of the lesion, number of involved lymph nodes and grade of the tumor. Although there is a gradual decrease in Gelsolin transcript level in tumors with NPI value < 3.4 (good prognosis) to > 5.4 (poor prognosis) but this difference is not statistically significant ($p = 0.43$ and $p = 0.26$, good vs moderate and good vs poor prognosis respectively) (Fig. 2C).

Based on the clinical outcome at the final follow up, patients were divided into following categories; those who remained disease free, with metastatic diseases, with local recurrence, and those who died of breast cancer (patients died of causes other than breast cancer were excluded from the current analysis). As can be seen from Fig. 2D, patients who remained disease free has the highest levels of Gelsolin amongst all the patients. It is interesting to note that patients who developed metastasis and who died of breast cancer exhibited significantly lower levels compared with those

who remained disease free ($p = 0.002$ and $p = 0.03$, respectively).

4. Discussion

Multistage model of carcinogenesis proposes the growth of consecutive genetic abnormalities in oncogenes and tumor suppressor genes during tumor progression [45]. One of the most fundamental characteristics of malignant and transformed cells is the aberrant organization of the cytoskeleton [10].

Gelsolin, one of the major actin-binding proteins, is widely expressed by normal cells [38] and may be down-regulated with transformation of multiple cell types, including breast epithelium [31,40]. It has therefore been considered a candidate tumor suppressor gene [6,34,47]. Although gelsolin-null mice survive, they have multiple defects in cell morphology and motility [61].

Purpose of this study was to screen Gelsolin for germ line mutations in sporadic breast cancer cases and to verify the expressional profile of Gelsolin in breast cancer patients, of Pakistani population and to compare it with the clinical data. Different risk factors were also analyzed in association with breast cancer in patients but no role of any of these factors was observed in

studied population which is in accordance with other studies [54].

Gelsolin consists of six domains S1-S6 [6,28,33,41]. Different types of mutations were observed in these domains in this study at germline level. Missense mutation observed at exon 4 resulted in change in amino acid Glutamine to Glycine at position 4. When analyzed by Alamut biosoftware version 2.0, this change was found to be present in highly conserved nucleotide (score: 0.99 [0–1]) and in moderately conserved amino acid (considering 15 species). This change was tolerated as was analyzed by SIFT (score was 0.23). Same is the case with substitution A>T at exon 15 which was observed in S5 domain, C-terminal of gelsolin. Mutation in this domain can impair nucleation of actin filaments [10]. Its SIFT score is 0.07 and this variation in Gelsolin is also tolerated. Missense mutation resulted from substitution at exon 11 that is C>T changes amino acid Leucine in to phenylalanine in putative type 2, Ca^{+2} binding site in S2 domain. Changes in Ca^{+2} binding site might hinder Ca^{+2} binding thus, Gelsolin functions [41]. This change is deleterious according to SIFT score (0.02). SIFT score less than 0.05 is considered deleterious [49].

It was observed that two additional amino acids, Alanine and Glycine were added at exon 10 in S3 domain of Gelsolin, in consequence of insertion of 6 base pairs. This S3 domain of Gelsolin is important in severing of actin filaments and is functionally relevant for regulation of Gelsolin activities [10,56]. Two deletion mutations that were observed at exon 11 and exon 14 interrupt reading frame prematurely. In exon 11 deletion of two nucleotides i.e. c.987_988del, replaced codon Pro330 by a STOP codon in ion binding site which lies in S3 domain and is important in binding of Ca^{+2} and polyphosphoinositide [10]. Mutation in this site could impair Gelsolin functions. Deletion at exon 14, c.1436del creates a frame shift starting at codon Lys479. Resultant new reading frame ends in a STOP codon, 6 positions downstream and mRNA produced might be targeted for nonsense mediated decay (NMD). This mutation is also important in context that it is in actin binding interface in S4 domain of Gelsolin [10, 41].

As all these mutations were observed in important domains of Gelsolin so they may be important in context of their consequence and could contribute to growth and behavioral defects. Data generated in this study shows that all mutations described may play a significant role in presenting Gelsolin as a potential risk factor for breast cancer.

Variations in Gelsolin expression may play an important role in the development of breast cancer and serve as a biomarker of prognostic diagnostic importance [45]. In this study expression of Gelsolin was observed in 60 tumor samples along with their controls by Q-PCR. Down regulation of Gelsolin was confirmed by quantitative PCR analysis. It was noted that Gelsolin transcript levels were significantly lower in tumor tissues compared to controls. There is consistent decrease in gelsolin mRNA levels associated with higher tumor grade or poor prognosis. However, these differences do not appear to be statistically significant. Small sample size might be the reason for non- significant results.

The most important observation here is the link between level of Gelsolin expression and the clinical outcome. A highly significant link was observed between low levels of expression and a poor clinical outcome and metastatic development and overall survival. It is perceived that role of Gelsolin as a tumor suppressor depends upon the type of cancer [25]. It might be the reason that no difference was observed in Gelsolin levels in local occurrence and in disease free individuals.

This data indicate that Gelsolin may act as a potential prognostic indicator and the molecule may act as a protective factor in patients with breast cancer. In many cancers, down regulation of Gelsolin has been identified as a factor mediating aberrant cytoskeletal organization. Reduced levels of Gelsolin were observed in bladder cancer [45], breast cancer [19], non-small cell lung cancer (NSCLC) [15], gastric cancer [29], osteosarcoma [55] and in ovarian cancer [3].

Present study shows that Gelsolin is of crucial importance and is inversely correlated with metastatic development of breast cancer. Mutations described here suggest that there is a possible contribution of these variants in genomic instability of Gelsolin which can lead towards metastatic potential of breast cancer.

Results from this work suggest that an association may exist between mutational spectrum of Gelsolin, its down regulation and breast cancer metastasis. Concluded from literature survey it might be the first study reporting germline mutations in Gelsolin in breast cancer patients. Further, this is a first report of Gelsolin screening in breast cancer patients from this particular study cohort.

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Conflict of interest

Authors declare that they have no conflict of interest.

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