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Polymorphic Analysis of Connexin 43 in Early as well as Advanced Stages of Breast Cancer in Kashmiri Population

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Authors' contributions

This work was carried out in collaboration between all authors. Author SGA designed, formulated and performed the lab work and wrote the manuscript. Authors RD and SR helped in Lab work. Author KIA over all supervised the work. All authors read and approved the final manuscript.

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ABSTRACT

Background: Down-regulation of connexin-43 gap-junction protein is involved in primary tumor formation as well as metastasis in breast cancer patients and restoration of gap-junction intercellular communication by up-regulation of connexins has been shown to restore normal phenotypes. However the molecular mechanisms behind these processes remain unknown and a better understanding of the key events is necessary to design anti-cancer treatment models against breast cancer.
Methods and Materials: In this study, Connexin 43 was analyzed for polymorphic changes using PCR method to establish its role (if any) in breast cancer in our cohort of population.
Results: After sequence analysis, none of the screened samples revealed any kind of variations in early or advanced stage of the disease.
Conclusion: Our studies imply that mutational deactivation does not play any role in the down regulation of Connexin 43 expression in breast cancer patients. Instead, some other regulatory mechanism like hypermethylation or mutation of the promoter region of the gene may be involved.

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1. INTRODUCTION

Connexin 43 (Cx43) encoded by the Gap Junction protein alpha 1 gene (*GJA1*), is the predominantly expressed gap junction protein in normal breast tissue and plays an important role in normal mammogenesis, lactogenesis and involution [1,2]. Various studies have shown high expression of Cx43 during mammary gland development and differentiation process of various mammary cells [1,2]. Depending upon the role of different genes during mammary gland development, many parallels have been drawn between protein function during development and deregulation in breast cancer. Cx43 has long been considered a tumor suppressor based on the studies showing down-regulation of Cx43 gap-junction protein in human breast cancer tissues or a relocalization of the connexin to intracellular compartments, resulting in a predicted loss of GJIC compared to matched normal or benign breast tissue [3,4]. Decreased expression of Cx43 gap junctions has also been linked to the control of processes associated with breast cancer at various stages of progression, and restoration of gap-junction intercellular communication by up-regulation of Cx43 has been shown to restore normal phenotypes [5,6]. Cx43 expression also contributes to decreased metastasis *in vivo* as various studies have shown that its expression in cancer cells leads to decreased expression of proteins involved in increased motility, invasion and metastases [7, 8]. Down-regulation of endogenous Cx43 expression by small interfering RNA has shown to promote a more aggressive phenotype in human breast cancer cell lines [9]. *In vivo* and in vitro studies have shown increased metastatic capacity in tumors cells with lesser number of gap junction plaques and weak GJIC capacity [10,11]. Re-expression of Cx43 in tumor cells is shown to reduce growth of tumors in nude mice and led to fewer metastases to the lungs [12,13]. Moreover, breast tumor metastasis to lung also increased in mice expressing mutant form of Cx43 [8].

However the molecular mechanisms behind the down-regulation of Cx43 and contribution to development of primary tumor and its metastasis in breast remain elusive. A better understanding of the key events that lead to the down-regulation of Cx43 in breast cancer is necessary to gain information relevant to the designing of anti-cancer treatment models against breast cancer. Multiple mechanisms appear to be responsible for the down regulation of Cx43 in breast neoplastic tissue, and one of the potent mechanisms can be mutations in gap-junctions genes. In this study, we propose to study the sequence variations of Cx43 in early as well as advanced stages of breast cancer patients of this region, and workout association of such variations (if any) with the disease phenotype.

2. MATERIALS AND METHODS

Patients presenting for treatment of Breast cancer for the first time at the Sheri-I-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir were recruited for the study with prior informed consent. Patients underwent fine needle aspiration cytology (FNAC) and histopathological examination to establish the clinical profile. Surgically resected breast tissue (which included tumor tissue and adjacent normal tissue) was collected from 100 breast cancer cases. All samples were snap-frozen at –70°C until analysis. A questionnaire was used to collect the information on Clinico-epidemiological characteristics such as age, family history of disease, body mass, menopause status, site of tumor, marital status, provisional diagnosis, lymph node/s involved and clinical tumor stage of patients.

PCR primers were designed with mismatches to the pseudogene sequence to specifically amplify GJA1 and exclude any contribution from processed pseudogene (GJAIP) that has been identified in humans. The desired amplicons were obtained using Nested PCR method. In the first round, 1331bp product which contains the entire coding region plus a small portion of an intron at the 5' UTR was amplified using primers "F 5'CGTGAAACCGTTGGTAGTA; R 3' GCACTTTCTACAGCACCTT".In the second round, the generated amplicons were used as template to specifically amplify the N-terminal, mid-region and C-terminal region of Connexin 43 using primersF5'CGTGAAACCGTTGGTAGTA3',R3'CCCCTCGCATTTTCACCTTACC5';F 5'TTTGAGGTGGCCTTCTTGCTGA3', R3'TAGGCGAGAGGGGAGCGGT5' &F 5'TGGGTACAAGCTGGTTACTG3', R 3'GCACTTTCTACAGCACCTT5'.

Purified PCR products were further subjected to denaturation and renaturation proceduresfor

generation of potential heteroduplexes and analyzed by Conformation Sensitive Gel Electrophoresis (CSGE). Tumor Samples which demonstrated unusual mobility during this assay were finally sequenced along with the adjacent controls to check for the presence of any sequence variations. Sequence results obtained in Fasta and PDF formats were analyzed using Clustal X version 2 software, and Chromas Pro version 1.49 beta 2 software was used for the detailed inspection of individual chromatograms [14].

3. RESULTS

The clinico-epidemiological features of all the patients are presented in Table 1. Prior to DNA sequencing, samples were screened for the presence of mutations by CSGE and only those 58 samples were sent out for commercial sequencing that showed differential migration on heteroduplex assay by CSGE (Fig. 1). Heteroduplex analysis was done to minimize the sequencing load although the results are not 100% but still huge sequencing burden was reduce to a great extent.

Table 1. Clinico-epidemiological features of breast cancer patients

Features	
Age	
≤ 45 years	40
≥ 45 years	60
Residence	
Rural	80
Urban	20
Menopause Status	
Pre-menopausal	40
Post-menopausal	60
Breast Involved	
Right	32
Left	68
Provisional Diagnosis	
IDC	100
IBC	0
Paget's Disease	
Lymph Nodes Involved	
Yes	72
No	28
Tumor Details	
Clinical Tumor Stage	
II (a,b)	40
III (a,b)	36
IV	24

The sequencing analysis did not reveal any kind of variation in Connexin 43 in any of the patients whether in early or advanced stage of the disease.

4. DISCUSSION

Connexins are transmembrane proteins characterized by their ability to form intercellular gap junctions that facilitates the intercellular communication between neighboring cells by allowing transfer of ions and small molecules [15]. As communication between the cells form an important part of various cellular processes like cell growth, differentiation and tissue homeostasis, the disrupted connexin expression plays a key role in the development and progression of various cancers including breast [16,1]. One of the most highly expressed connexins, Connexin 43 (Cx43) plays a very important role in breast development and has been reported to be aberrantly expressed in breast cancer [11].

As the available evidence suggests that loss of Cx43- directed GJIC promotes malignant transformation of breast/mammary epithelial cells. it can be expected that mutational deactivation of these genes during carcinogenesis can constitute strong evidence in favor of their tumor-suppressive capacity [17,4]. In contrast, our results show that Cx43 gene mutations are rare in breast cancer making it apparent that Cx43 mutational deactivation is a rather rare event in breast tumor formation. Henceforth our study implies that Cx43 down regulation in breast tumors is not due to any mutations. Instead some other regulatory mechanisms may play an important role in the down regulation of this gene.

Although some studies have also reported mutations in Cx43 in advanced stage of the cancer; on the contrary, we also did not report any kind of variation in the patients who were in the advanced stage of the disease. Dubina *et al.* [18] have reported frame-shift mutations in the carboxyl-terminal region of Cx43 in three out of six exophytic colon tumors which were morphologically classified as well or moderately differentiated. In two exophitic adenocarcinomas, a tumor specific single nucleotide deletion in the second position of codon 311 resulted in shift of reading frame from Ala to Val and substituted the next 35 residues followed by a premature stop codon, which shortened the carboxy lterminal tail of Cx43 by 36 residues was reported. Insertion of a single nucleotide at the second position of codon 358 was detected in another

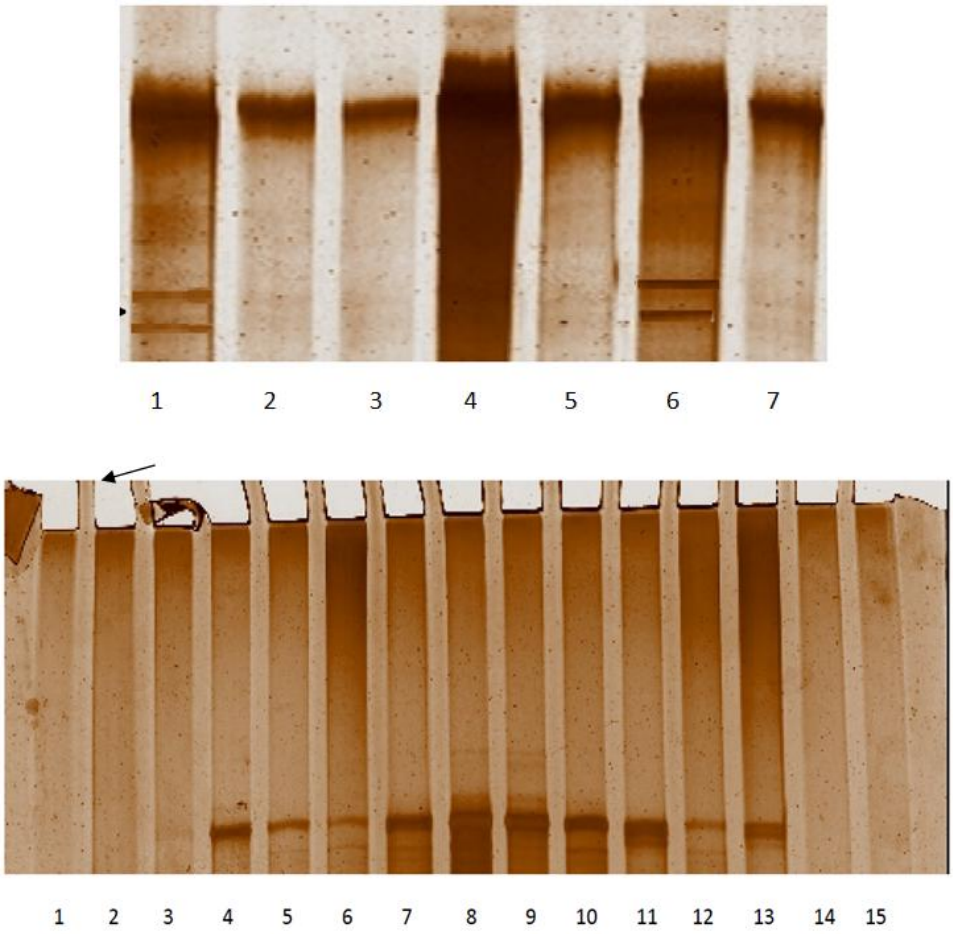

Fig. 1. Heteroduplex analysis of different amplicons by CSGE. Different heteroduplex patterns were obtained which were suggestive of the presence of variation. (1a). Well no 1 & 6 (1b) Well no 1, 2, 3, 4, 14,15 show the heteroduplex pattern of samples that doesn't not show any sequence variation and (1a) Well no 2, 3, 4, 5, 7 (1b) Well no 5, 6, 7, 8, 9, 10, 11, 12, 13 show heterodulex pattern of samples that showed sequence variations

adenocarcinoma also resulting in the shift of reading frame changing Ile to Asn and altering the next 18 residues, followed by an early stop codon, the protein becoming five residues shorter [18].

Some human hereditary diseases are also reported to have certain mutational alterations of individual connexins arising in patients with mutations in the gene encoding connexins. Mutations in Cx32 gene are associated with X-Linked Charcot-Marie-Tooth disease, an inherited peripheral neuropathy. (Bergoffen et al., 1993; Nelis et al., 1999; Krutovskikh and Yamasaki, 2000). Around 100 unique mutations in connexin 26 gene are associated with recessive deafness and those of Cx30 [19] and Cx31 with skin disorders [20]. Similarly autosomal mutations in Cx43 gene in the carboxyl-terminal tail of the protein are associated with complex human heart malformations [21,22]. However, the lack of link of these diseases with cancer incidence suggests major difference in the connexin expression pattern in the two pathological conditions.

5. CONCLUSION

Thus our studies strongly implicate that mutational deactivation of this gene does not play any role in primary tumor formation or its metastasis in the breast. Although the gene encoding Cx43 is not found mutated while the protein expression level is frequently altered, which presumably could be due to the mutation

of an upstream regulator or other factors, thus Cx43 can be described as class II tumor suppressor. However it is debatable how far downstream Cx43 is from the primary assault and whether its regulation is a key player in breast carcinoma or simply a distant secondary effect.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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