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RESEARCH ARTICLE

INVOLVEMENT OF MITOGEN-ACTIVATED PROTEIN KINASE KINASE 6 IN BREAST TUMORIGENESIS.

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Abstract

MKK6 expression analysis has been reported in various primary tumors, like esophagus, stomach, colon and prostate cancers but its significance in primary breast tumor patients remains unclear. We investigated the expression of MKK6 in cancer tissues through immunoblotting and immunofluorescence techniques in 25 resected cases of human breast carcinomas. Increased MKK6 protein expression was observed in 56% (14 ⁄ 25) of breast cancer cases. These results indicate that the upregulated expression of MKK6 in cancer tissues has significant role in tumor progression and the clinical prognosis of patients with primary breast carcinoma.

Introduction:-

Breast cancer is one of the most common cancers with more than 1.3 million cases and 0.45 million deaths each year worldwide [1]. It is the number one cancer amongst females accounting for 5.59% of total cancers in females of Kashmir valley only [2]. Breast cancer development involves progression through chain of intermediate processes, which starts with ductal hyper-proliferation, followed by subsequent progression to carcinoma in situ, invasive carcinoma, and finally into metastatic disease [3]. Studies have shown that metastasis is a series of diverse events, generally known as a metastatic cascade. These steps include fleeing of malignant cells from the site of primary tumor, propagation and shifting to a discontinuous secondary site(s), and lastly, the survival and growth into clinically detectable metastases, a process termed metastatic colonization [4]. In order to significantly augment our understanding of the metastatic process and to identify the specific targets for cancer therapy we need to single out the proteins and signaling pathways necessary for regulation of metastasis. In various cancers many proteins show differential expression and regulation [5-7]. One such protein is mitogen-activated protein kinase 6 (which from now onwards will be referred to as MKK6). MKK6 belongs to the mitogen-activated protein (MAP) kinase super family and has a role in both apoptosis [8,9] as well as proliferation [10]. MKK6 has been implicated in regulating tumorigenesis. MKK6 has been shown to play contradictory roles in suppressing metastatic colonization in human ovarian carcinoma [11] on other hand it has been shown to be up-regulated in prostate cancers [12]. As of now, very little is known about the regulation of expression of MKK6 in the cell [13]. MKK6 activates downstream all the isoforms of p38 MAPK [14]. P38α has been implicated in regulating cancer progression, by regulating invasion, inflammation and angiogenesis which are key oncogenic steps in tumorogenesis[15-17].

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In our previous study we have reported a dramatic up-regulation of the MKK6 in esophageal, stomach and colon cancers [7], and as already mentioned an upregulation has also been reported in prostatic carcinomas [5]. The expression pattern of MKK6 has never been systematically evaluated in breast cancer and the specific role of MKK6 in breast cancer progression is incompletely understood. Thus we focused this study on checking the expression pattern of MKK6 protein in human breast cancerous tissues that might help in providing an attractive target for future biomarkers against the said disease.

Materials and Methods:-

Human tissue procurement:

Twenty five patients (1 male, 24 females) examined and treated at Shri Maharaja Hari Singh Hospital (Srinagar City, India) for breast carcinomas were evaluated for this study. The study was approved by the Ethics Committee for Clinical Research of the hospital. Primary tumors and adjacent non-neoplastic breast tissue were obtained at the time of surgery, evaluated by a pathologist, and used for the studies.

Chemicals &Antibodies:

Chemicals used for carrying out protein extraction and western blotting were of analytical grade and were purchased from SIGMA-ALDRICH (USA). From Genei laboratories (India), we purchased Bradford micro-protein estimation kit. PVDF membrane was purchased from WHATMANN (Germany). All electrophoresis reagents were obtained from SIGMA-ALDRICH (USA), Thermo Scientific (USA), Qualigens (India) and Spectrochem (India), ECL solutions for protein detection were obtained from Thermo Scientific (USA). Rabbit Polyclonal MKK6 antibody was purchased from Cell Signaling Technology Inc., phospho-MKK6 was purchased from ABCAM, anti-rabbit HRP-conjugated Secondary Antibody and Anti-Rabbit igg-FITC antibody was purchased from SIGMA-ALDRICH (USA). Anti-Vinculin antibody was purchased from MILLIPORE.

Protein extraction and estimation:

Protein extraction and estimation were carried out as done previously [18].

Immunoblotting:

35 μg of protein extract, preheated at 100°C for 3 min in reducing sample buffer containing 50 mm TrisCl (ph 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100 mm β-mercaptoethanol, were run on 10% SDS-polyacrylamide gel and proteins were transferred onto PVDF membrane (Whatmann). Membranes were then blocked with 5% BSA in TBS followed by an overnight incubation with primary antibodies (1:1000 dilution of anti-MKK6 in 5% BSA in TBS, 1:1000 dilution of anti-VinculinAb in 5% skimmed milk in TBS) at 4°C. After washing with TBS containing 0.1% Tween-20, the membranes was incubated with horseradish peroxidase-conjugated secondary antibody (1:7000) dilution in 5% skimmed milk in TBS) for 2 hours at room temperature. Protein bands were detected with chemiluminescence as done previously [18]. Three independent experiments were done to confirm all the results.

Immunofluorescence:

The experiments were carried out as done previously [18] and the photographs of the sections were taken by a camera mounted on a Leica fluorescence microscope.

Results:-

The expression of MKK6 (molecular weight 38kda) was examined in 25 breast carcinomas and the adjacent normal breast tissues by Western blotting and Immuno-fluorescence using anti-rabbit MKK6 polyclonal antibodies. Out of 25 patients, 14 patients (56%) showed upregulation of MKK6 protein. The age of the patients ranged from 20-70 years. The majority of the cases were in the age group of 40-60 years [Table - 1]. In cancerous breast tissue the expression of MKK6 was found to be considerably up-regulated as compared to the normal breast tissue (Figure 1A). In all the experiments, blots were stripped and treated with anti-Vinculin antibody used as loading control which showed equal expression (lower panel in figure1a). Immunostaining was also carried out alternatively in human tissues and the expression of MKK6 was found to be significantly higher in terms of recorded fluorescence signal as compared to the surrounding normal breast tissue (Figure 1B). Western blot analysis using phospho-MKK6 antibody was also carried out to check for the activation of MKK6 (phosphorylated form) and the obtained results indicated an increase in the level of activated MKK6 in cancerous tissue as compared to normal breast tissue (Figure 1C).

Discussion:-

In the present study, we carried out Immunoblotting and Immunofluorescence of human breast tissue specimens to detect the protein expression of MKK6 and we have shown that clinical specimens of tumor tissues had increased expression of MKK6, but not in adjacent normal breast tissues. To our knowledge, this is the first study to detect protein expression of MKK6 protein in breast carcinoma. We also report that MKK6 expression might be a useful prognostic marker for the breast carcinoma patient survival. Using Western Blot analysis and Immunofluorescence techniques increased MKK6 protein expression was observed in 56% (14⁄25) of breast cancer cases. The reported over expression may be due to an increased transcription of the related genes or due to posttranscriptional modifications. In conclusion, our results suggest that increased MKK6 expression might play an important role in progression of breast tumorigenesis and may have diagnostic and therapeutic consequences.

Conflict of interest: The authors declare no conflict of interest.

Ethical clearance: The study was approved by the Human Ethics Review Committee of SMHS Hospital and proper consent was taken from the patients.

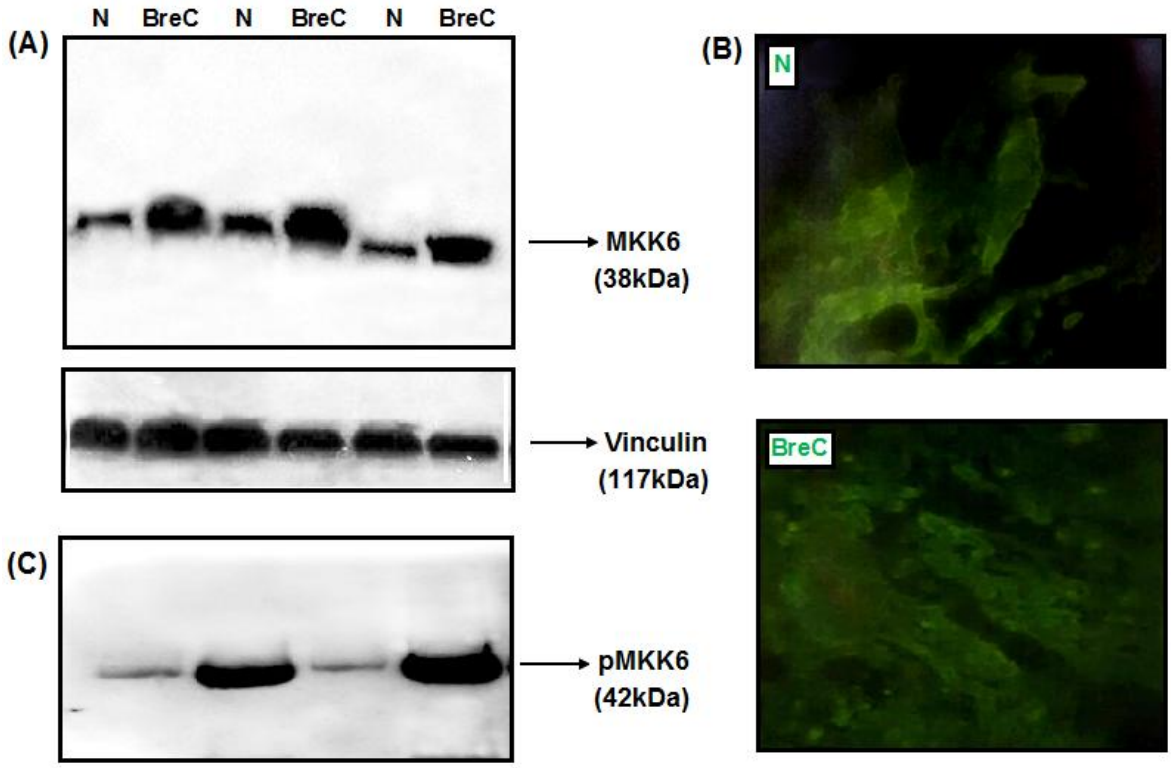

Figure 1:-Up-regulation in the expression of MKK6 protein **A:** Representative Western blot showing the increase in the expression of MKK6 in Breast tissues (Cancerous Vs. Normal) **B:** Immunofluorescence staining showing up-regulated expression of MKK6 protein in Breast tissues (Cancerous Vs. Normal) **C:** Representative Western blot showing the activation of MKK6 in Breast tissues (Cancerous Vs. Normal) *The analysis was carried out on different individuals each time.

Table 1:-

Age Group	**No. Of Cancer Patients**	**Healthy Controls**	**Upregulated MKK6 in Cancer Patients**	**Upregulated MKK6 in Healthy Controls#**		**Strength of association**		
					P	***Odds ratio***	***Sensitivi***	***Specifici***

					value	***(95% CI)***	***ty (95% CI)***	***ty (95% CI)***
20-30	1	1	Nil	0	1	1 (0.011 to 92.52)	0 (0.0 to 0.98)	1 (0.02 to 1.0)
30-40	2	1	1	0	1	3 (0.060 to 151.3)	0.5 (0.01 to 0.99)	1 (0.020 to 1.0)
40-50	7	3	3	0	0.475	5.44 (0.20 to 144.2)	0.42 (0.09 to 0.81)	1 (0.29 to 1.0)
50-60	12	5	8	0	0.0294 *	20.78 (0.92 to 467.3)	0.66 (0.35 to 0.90)	1 (0.48 to 1.0)
60-70	3	2	2	0	0.4	8.33 (0.22 to 320.7)	0.67 (0.09 to 0.99)	1 (0.16 to 1.0)
Total	**25**	**12**	**14**	**0**	**0.0009 *****	**31.52 (1.68 to 591.2)**	**0.56 (0.35 to 0.75)**	**1 (0.73 to 1.0)**

**p < 0.5 (significant); **p<0.01 (most significant); ***p<0.001 (highly significant). Data were analyzed using Fisher's exact test across each age –group.*
 # Vs. Adjacent normal of Cancer Tissue

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