

Mitogen Activated Protein Kinase Kinase-4 Upregulation is a Frequent Event in Human Stomach and Colon Cancers

Research Article

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Abstract

Mitogen-activated protein kinase kinase 4 is a dual specificity protein kinase that is encoded in humans by the MAP2K4 gene. The protein belongs to the Ser/Thr protein kinase family and has two isoforms, isoform-1(44KD) and isoform-2 (41 KD). The role of MKK4 in tumors is controversial, with initial evidences suggesting as tumor suppressor role but recent studies support its pro-oncogenic role in many tissues. MKK4 has been reported as pro-oncogenic in gastric cancers but it has not been studied at protein expression level. We attempted to investigate the expression analysis of MKK4 protein infifty samples of three different types of cancers from both the sexes (28 males, 22 females) which include 28 stomach cancer samples, 18 colon cancer samples, and 12 rectal cancer sample sand compared them with their respective normals. We found MKK4 protein expression to be up-regulated in stomach and colon cancers however analysis of rectal tumors shows no expression differences. Results observed were consistent for the chosen solid tumors when compared to their respective normals. We invoke higher expression of MKK4 in cancer might have a role to play in tumorigenesis and could possibly be used as a novel diagnostic or prognostic marker in these cancers.

Keywords: MKK4; Cancer; Signal transduction; Stomach cancers; Colon cancers

Introduction

Gastrointestinal tract cancers are very common in Kashmiri population [1] with esophageal cancers in lead followed by stomach cancers. Also cancers of lower GIT i.e., colon and rectum have high frequency in Kashmiri population [1,2]. Cancer is a very complex disease in which signal transduction pathways is deregulated. Many proteins show differential expression in gastric and other types of cancers [3-5]. Mitogen-activated protein kinase kinases (MAPKKs) have a potential to play an important role in this disease. MAPKKs are a family of tyrosine/threonine dual specificity protein kinases,

upstream of the Mitogen-activated protein kinases (MAPKs). There are four main families of MAPKs, including extra- cellular signal-regulated protein kinases (ERKs), p38-MAP Kinases, c-Jun N-terminal kinases (JNKs) and ERK5, also referred as BMK1[6,7]. MAPKKs are activated by upstream MAPKKKs that respond to a number of signaling molecules finally lead to MAPK phosphorylation and activation which initiate different cellular responses as cell proliferation, differentiation, survival, death, and transformation via phosphorylation of a number of cytoplasmic and nuclear regulatory proteins [8-10].

The MKK4 (also called JNKK1, MEK4, MAP2K4 and SEK1); a dual-specificity protein kinase is a member of the MAPK family. The protein is activated by many upstream kinases and active MKK4 mutually act with MKK7 to phosphorylate and thereby activate c-Jun NH2-terminal kinase (JNK) and p38 kinases by dual phosphorylation in the Thr-Pro-Tyr and Thr-Gly-Tyr motifs within the activation loop respectively [11-14]. The activated forms of JNKs and p38 kinases eventually initiate a wide array of biological processes, such as cell proliferation, differentiation and apoptosis [14,15]. There are two isoforms of this protein. Isoform-1 contains 399 amino acids (MW 44,288 Da) and Isoform-2 contains 410 amino acids (MW 45,584Da). The sequence of isoform-2 differs from sequence the isoform-1 as follows: 39-39: G → GFQINFCEKAQS [16]. Initially MKK4 was identified as tumor suppressor protein in different cancer cell lines derived from tumors of breast, colon, lungs, pancreas, prostate, ovary and testis [17,18]. Normal ovarian surface epitheliums express high level of MKK4 as compared to ovarian cancer omental metastases. Reduction in the level of expression of MKK4 was observed in prostate and ovarian metastatic tissues as compared to normal prostate and ovarian epithelial cells, thereby signifying that MKK4 has a metastatic suppressive role [19,21]. These findings were supported by clinical finding that MKK4 null states will encounter a growth advantages as compared to MKK-4 positive cell lines in pancreatic cancer thus lack of expression of MKK4 correlated with shorter survival time and MKK4 positive carcinomas having half the risk of death than Mkk4-negative carcinomas [22]. Mice injected with the MKK4^{-/-} pancreatic cancer cell line PL5 had very few lung metastases compared to those injected with the MKK4 positive or MKK4^{+/-} cells [23]. However in some cases MKK4 also functions as pro-oncogenic protein. It has been identified pro-oncogenic in breast and pancreatic cancer cell lines Ectopic expression of MKK4 in MDAMB-231 cell line by adenoviral delivery in MKK4-negative cancer lines stimulated the cell proliferation and invasion, where as knock down of MKK4 expression by small interference RNA in an MKK4-positive result in suppression of tumor growth [24]. It was also discovered that skin specific MKK4 deficient mice are resistance to carcinogen induced tumorigenesis. The mechanism by which MKK4 promotes cell proliferation and the formation of tumors is by increasing EGFR expression via the c-jun N-terminal protein kinase (JNK)/c-jun signaling pathway [25]. It was found that in MKK4 leads to over expression of JNK2 downstream, while the expression of JNK1 decreases indicates that JNK1 acts as a suppressor, while JNK2 is a promoter of skin tumor [26,27]. A pro-oncogenic function of MKK4 has been further supported by the analysis of gastric cancers and laryngeal squamous cell carcinomas. Patients with MKK4 present in tumor tissues had a significantly shorter survival compared to patients with reduced MKK4 expression [28,29].

From previous studies it has been shown that MKK4 might be involved in tumor development and metastasis progression. In quest to that, a clear systematic investigation is needed to know the expression pattern of MKK4 in different types of human cancers prevalent in Kashmir Valley. In this study we have carried out the expression analysis of MKK4 protein in human stomach, colon and rectal cancerous tissues. This is the first demonstration of MKK4

expression in above mentioned types of cancers and this information may be used to target MKK4 protein as a potential therapeutic target.

Materials and Methods

Collection of tumor specimens

Cancer tissue samples from the patients who underwent surgical operation at Department of Surgery at Shri Maharaja Hari Singh Hospital, a total of fifty samples from both the sex (28 males, 22 females) were collected. The samples collected include 28 stomach cancer samples, 18 colon cancer samples, and 12 rectal cancer samples. All study participants gave their written informed consent. The study was also approved by the Human Ethics Review Committee of the Hospital. The adjacent normals taken for the study were obtained by dissecting about 3cm away from the tumorigenic area.

Chemicals and 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 from Genei laboratories (India). 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 Monoclonal MKK4 antibody was purchased from UPSTATE-MILLIPORE, anti-rabbit HRP-conjugated Secondary Antibody and Anti-Rabbit IgG-FITC antibody was purchased from SIGMA-ALDRICH (USA). Anti-Vinculin antibody was purchased from EPITOMICS.

Western blotting

The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked in 5% non-fat milk and incubated overnight with primary antibodies. The membranes were then incubated with primary antibodies (1:1000 dilution of anti-MKK4 in 5% BSA in TBS, 1:5000 dilution of anti-vinculin Ab in 5% skimmed milk in TBS) overnight at 4 °C. After serial washing with TBS containing 0.1% Tween-20, the membranes was incubated with HRP-conjugated secondary antibodies (1:7000 dilution in 5% skimmed milk in TBS) for 2 hours at room temperature. Immunoreactive bands were visualized using ECL system (Cell Biosciences). Blots were stripped and re-probed as required.

Immunofluorescence and HE Staining

Formalin-fixed paraffin-embedded specimens were sectioned in 5µm-thick slices and sections were de-paraffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was carried out by placing specimens in citrate buffer at 98 °C for 5 min. The sections were then incubated with rabbit anti-MKK4 antibody (1:100) diluted in blocking buffer overnight, followed by incubation with secondary

antibody conjugated with FITC. All the steps were followed as done previously [3] and the photographs of the sections were taken by a camera mounted on a Leica fluorescence microscope. Hematoxylin and Eosin staining was used as the counterstain and done as carried out previously [3].

Results

Hematoxylin and eosin staining of tissues

The tissue morphology was studied using Haematoxylin and Eosin stains (Figure 1). HE staining of tissues as expected is supposed to look different in tumor versus normal. This is so because the morphology of cancer cells is different when compared to normal cells. The structured organization as we see in the normal tissue of almost all organs of the body gets disorganized with the passage and/or stage of cancers.

MKK4 is up-regulated in Stomach and down-regulated in Colon cancers

In this study, we investigated the expression analysis of the MKK4 protein in stomach, colon and rectal cancers. We carried out western blot analysis, to check the expression of MKK4 protein in histologically confirmed cancerous tissues of stomach (Figure 1A), colon (Figure 1B) and rectal (Figure 1C) cancers isolated from independent patients. Results indicate a consistent increase in MKK4 protein levels in stomach and colon cancers, when compared to their adjacent normal controls. Rectal cancers did not show any difference in the expression levels of MKK4 protein. In all the experiments, blots were stripped and treated with anti-Vinculin antibody used as loading control which showed equal expression (lower panels in all figures) (Figure 2).

Immunofluorescence studies reveal a higher level of MKK4 presence in Stomach and lower level of protein in Colon cancers

To further validate the results, immunostaining was also carried out alternatively in human tissues and the expression of MKK4 was found to be significantly higher in terms of recorded fluorescence signal as compared to the surrounding normal in stomach and colon cancers (Figure 3).

Discussion

The role of MKK4 in cancers is very much paradoxical and intriguing. It has been shown that skin specific MKK4 deficient mice are resistance to carcinogen induced Tumorigenesis. A pro-oncogenic function of MKK4 has been further supported by the analysis of gastric cancers and laryngeal squamous cell carcinomas. Patients with MKK4 present in tumor tissues had a significantly shorter survival compared to patients with reduced MKK4 expression. It has also been identified as proto-oncogenic in breast and pancreatic cancer cell lines [29]. Our results are in consonance with the earlier studies showing a proto-oncogenic role for MKK4 protein in cells. In the present study, we carried out Immunoblotting and Immunofluorescence of human stomach, colon and rectal tissue samples to detect the protein expression of MKK4. We have shown that clinical specimens of tumor tissues had increased protein expression of MKK4 in stomach and colon adenocarcinomas, but not in adjacent normal tissues, whereas the expression analysis of MKK4 in rectal samples don't show any difference. To our knowledge, this is the first study to detect protein expression of MKK4 protein in stomach, colon and rectal carcinomas. We also report that MKK4 expression might be a useful prognostic marker for these type carcinomas patient survivals. Western blot analysis and Immunofluorescence techniques showed an increased

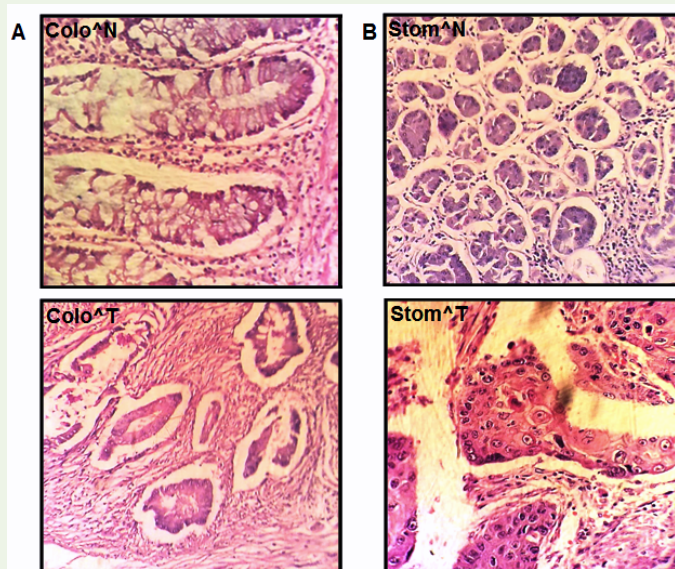


Figure 1: Hematoxylin and Eosin staining of the normal and cancerous tissues of stomach and colon.

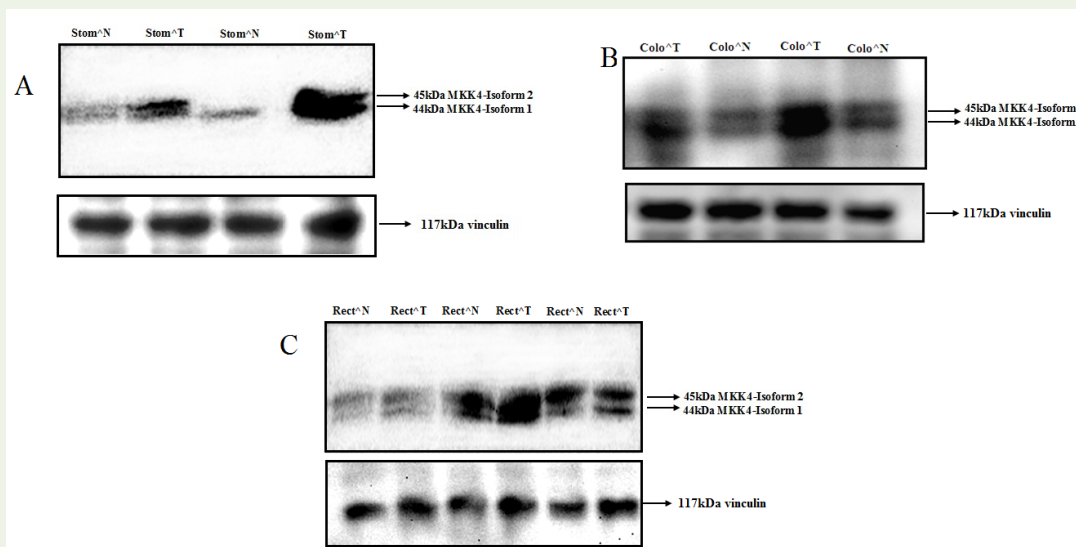


Figure 2: Up-regulation in the expression of MKK4 protein A: Representative Western blot showing the increase in the expression of MKK4 in Stomach tissues (Cancerous Vs. Normal) B: Representative Western blot showing the increase in the expression of MKK4 in colon tissues (Cancerous Vs. Normal) C: Representative Western blot showing the increase in the expression of MKK4 in rectal tissues (Cancerous Vs. Normal).

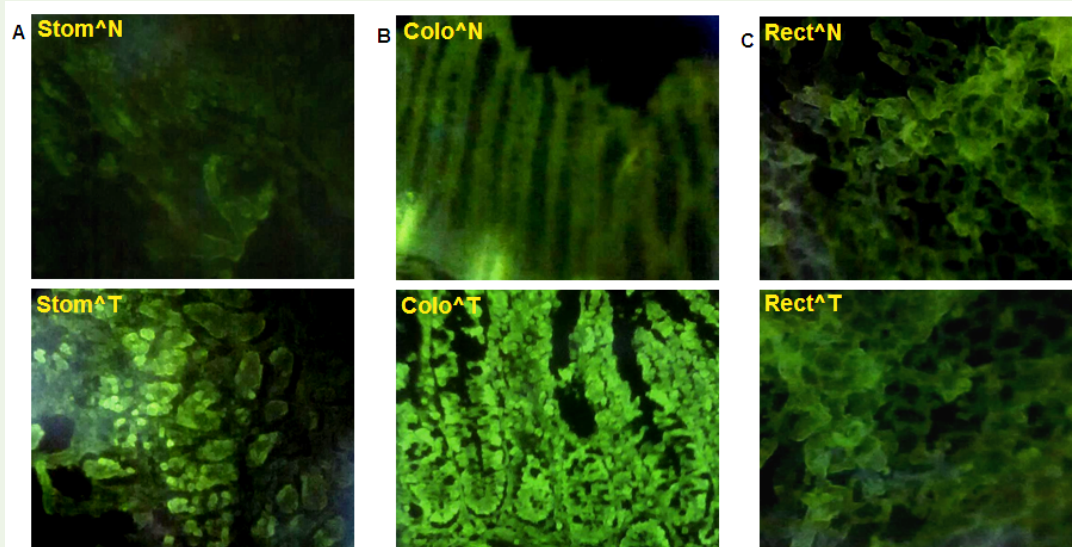


Figure 3: Immunofluorescence staining showing up-regulated expression of MKK4 protein in stomach and colon tissues (Cancerous Vs. Normal).

MKK4 protein expression in stomach and colon cancer cases. The reported over expression may be due to an increased transcription of the related genes or due to post-transcriptional modifications.

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Ethical clearance

The study was approved by the Human Ethics Review Committee of SMHS Hospital after obtaining patient consent and by ethical

committee of University of Kashmir.

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