

E3B1/ABI-1 isoforms are down-regulated in cancers of human gastrointestinal tract

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Abstract. The expression of E3B1/ABI-1 protein and its role in cancer progression and prognosis are largely unknown in the majority of solid tumors. In this study, we examined the expression pattern of E3B1/ABI-1 protein in histologically confirmed cases of esophageal (squamous cell carcinoma and adenocarcinoma), gastro-esophageal junction, colorectal cancers and corresponding normal tissues freshly resected from a cohort of 135 patients, by Western Blotting and Immunofluorescence Staining. The protein is present in its phosphorylated form in cells and tissues. Depending on the extent of phosphorylation it is either present in hyper-phosphorylated (M. Wt. 72 kDa) form or in hypo-phosphorylated form (M. Wt. 68 kDa and 65 kDa). A thorough analysis revealed that expression of E3B1/ABI-1 protein is significantly decreased in esophageal, gastro-esophageal junction and colorectal carcinomas irrespective of age, gender, dietary and smoking habits of the patients. The decrease in expression of E3B1/ABI-1 was consistently observed for all the three isoforms. However, the decrease in the expression of isoforms varied with different forms of cancers. Down-regulation of E3B1/ABI-1 expression in human carcinomas may play a critical role in tumor progression and in determining disease prognosis.

Keywords: Eps8 binding protein, abelson interactor protein, signal transduction, esophageal squamous cell carcinoma, adenocarcinoma, colorectal cancers

1. Introduction

E3B1 (Eps8-SH3 domain binding protein1), which is a human homolog to the mouse Abelson interactor-1 gene (Abi-1) [1], was isolated as an Eps8 binding protein [2]. E3B1/ABI-1 is an adaptor protein that has been found to mediate a number of signal pathways related to cancer cell proliferation, apoptosis, adhesion, and migration in breast cancer, BCR-Abl-induced leukemia, colon cancer, and melanoma, both *in vitro* and *in vivo* [3–8]. E3B1/ABI-1 was found to be part of several macromolecular complexes, including a trimer-

ic signaling complex, where it closely interacts with Eps8 and Sos-1, activating Rac1 [9]. In turn, Rac1 has been shown to be involved in production of Reactive Oxygen Species [10,11] and WAVE2 signaling complex. Activated WAVE proteins lead to enhanced actin nucleation via the Arp2/3 complex [12–14]. As an adaptor protein, E3B1/ABI-1 is involved in actin reorganization and lamellipodia formation which are believed to play critical roles in cell migration during metastasis [15]. E3B1/ABI-1 has been proposed as a potential tumor suppressor [16]. Recent findings have indicated that the gene encoding E3B1 is deleted in prostate cancer, and also loss of its expression accompanies the development and progression of some types of leukemia's [17,18]. Its role in cell mobility and decreased expression in some forms of cancers may be an indicative that E3B1/ABI-1 is involved in one or more of the steps leading to tumor progression or

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Table 1

Cancer type	Males		Females		NO. of samples showing %age decrease of E3B1 EXPRESSION
	n	%	n	%	
(Total n = 135)					
Gender	81	60.00	54	40.00	–
Average years*	59 ± 14.84	–	51.89 ± 14.23	–	–
Smoking status					
Non-smokers	57	70.37	48	88.88	–
Ex-smokers	24	29.62	6	11.11	–
Esophageal squamous Cell	15	18.51	12	22.22	70%
Poorly differentiated	6	7.40	3	5.55	75%
Well differentiated	9	11.11	9	16.66	65%
Esophageal adenocarcinoma	7	8.64	6	11.11	72%
Gastro-esophageal junction	20	24.69	12	22.22	90%
Colon	18	22.22	10	18.51	88%
Rectal	21	25.92	14	25.92	94%

*Values are expressed as Mean ± SD.

metastasis. To understand this role a careful analysis of E3B1/ABI-1 protein expression needs to be carried out in different forms of human cancers. Here, in this study we have carried a systematic protein expression analysis on human esophageal (squamous and adenocarcinoma), gastroesophageal junction and colorectal cancers.

2. Materials and methods

2.1. Patients

A total of 135 tissue specimens were obtained with consent from patients who underwent curative surgical resection from April 2008 to November 2010 at the Department of General Surgery, Shri Maharaja Hari Singh Hospital, Srinagar, (India). Archival materials were reviewed by experienced pathologists (Department of Pathology, Shri Maharaja Hari Singh Hospital and Department of Pathology, Sher-i-Kashmir Institute of Medical Sciences), to select a representative tumor block and surrounding normal mucosal tissue block for each case. The patient group included 81 men and 54 women (Table 1). Clinical diagnosis was confirmed in all cases by histo-pathological examination. The study protocol was approved by the Shri Maharaja Hari Singh Hospital, Research Ethics Committee. The patients were not given any radiotherapy or chemotherapy before surgery. The adjacent normals taken for the study were obtained by macro-dissection, about 3 cm away from the tumorigenic area. Tissues were immediately snap frozen by immersion in liquid nitrogen N₂ (l) and stored at –80 degree until use.

2.2. Chemicals

Bradford microprotein estimation kit was purchased from Genei Laboratories (Bangalore, India). PVDF membrane was purchased from Whatman GmbH, (Dassel Germany). Electrophoresis reagents were obtained from Sigma-Aldrich (St Louis, MO, USA), Qualigens (Mumbai, India) and Spectrochem (Mumbai, India). All the chemicals for carrying out protein extraction and Western blotting were of analytical grade and were acquired from Sigma-Aldrich.

2.3. Protein extraction and estimation

Tissue was disintegrated using 0.5% trypsin-EDTA at 37°C for 5 min, centrifuged at 12 000 rpm for 5 min, rinsed twice with ice-cold PBS, pH 7.4. Whole cell lysates were incubated on ice with NP-40 lysis buffer containing protease and phosphatase inhibitors (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 1% glycerol, 2 mM EDTA, 10 mM NaF, 1 mM PMSE, protease inhibitor cocktail 10 µl per 1 ml of lysis buffer) for 45 minutes and then clarified by centrifugation at 12,000 rpm for 10 minutes at 4°C to obtain the extract. Protein concentration was determined spectrophotometrically (Shimadzu, Kyoto, Japan) at 595 nm with the Bradford assay kit.

2.4. Antibodies

Rabbit polyclonal antibodies against E3B1 and Vinculin, and Alkaline phosphatase conjugated anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Anti-Rabbit IgG Alexa Fluor-488 antibody was gift from Dr. Ellora Sen (NCBR, New Delhi).

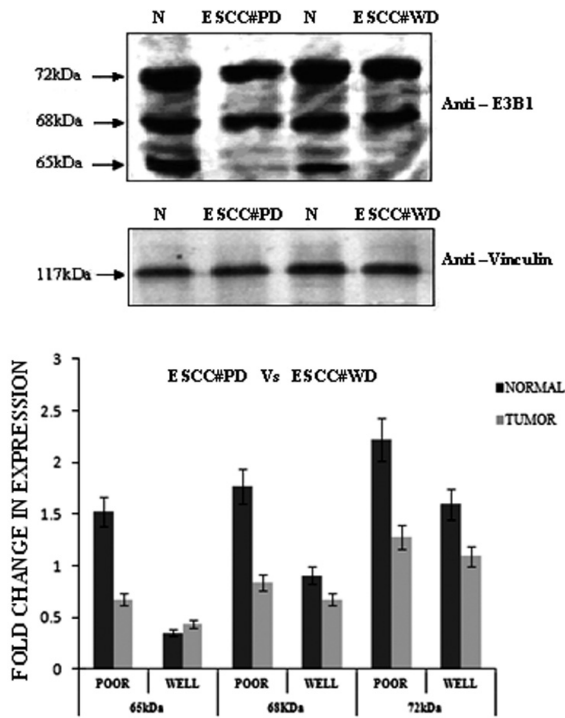


Fig. 1. Immunoblot showing the decreased expression of E3B1 protein and Bar chart comparing the fold change in expression levels in well differentiated (ESCC#WD) and poorly differentiated (ESCC#PD) esophageal squamous cell carcinoma as compared to their adjacent normals (N).

2.5. Western blot analysis

10% SDS-PAGE was used to resolve 25 micrograms of protein extract preheated at 100°C for 3 min in reducing sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100 mM β -mercaptoethanol and the resolved proteins were transferred onto PVDF membrane (Whatmann) blocked with TBS supplemented with 0.1% Tween 20 and 5% (w/v) fat-free powder milk for 1.5 hrs. The membranes were then rocked overnight with primary antibody (1:500 dilution of anti-E3B1 Ab in 5% skimmed milk in TBS, 1:1000 dilution of anti-Vinculin Ab in 5% skimmed milk in TBS) at 4°C. Thereafter, the membranes were washed in TBS supplemented with 0.1% Tween 20 followed by incubation with anti-rabbit IgG (1:3000 dilution in 5% skimmed milk in TBS) conjugated to alkaline phosphatase for 2 hrs. The blots were then washed again and antibody binding signals were detected by treating the membrane with BCIP/NBT solution, incubated and protected from light for 10–15 min until the desired color intensity was ob-

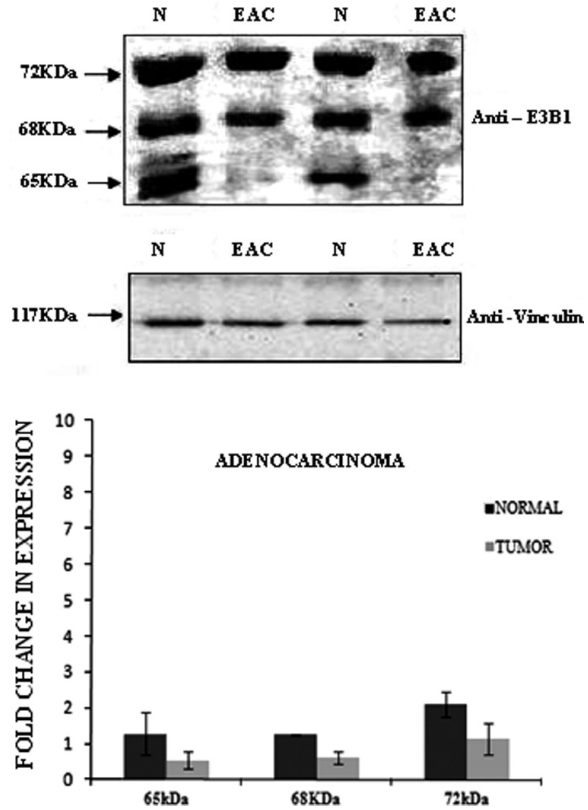


Fig. 2. Immunoblot showing the decreased expression of E3B1 protein and Bar chart comparing the fold change in expression levels in the esophageal adenocarcinoma (EAC) as compared to their adjacent normals (N).

tained. All results were confirmed in at least three independent experiments.

2.6. Immunohistochemistry

Formalin fixed, paraffin-embedded tissues were cut into 5 μ m serial sections on a conventional microtome, overlaid onto glass slides and processed for immunohistochemistry. The sections were de-waxed, washed thoroughly in phosphate-buffered saline (PBS) and microwaved in 0.1 M citrate buffer (pH 6.0) for 5 min (x2), again washed with PBS, and post-fixed with 4% paraformaldehyde and finally washed thrice with PBS. The sections were then blocked with 1% non-fat dried milk in buffer for 2 h at room temperature, and then incubated with primary anti-E3B1 antibody (1:100) diluted in blocking buffer overnight at 4°C. After thoroughly washing in PBS (3 \times 5 min), the sections were then incubated with an Anti-Rabbit IgG Alexa Fluor-488 antibody was diluted (1:200) in blocking buffer. After washing the tissue three times with PBS, the sec-

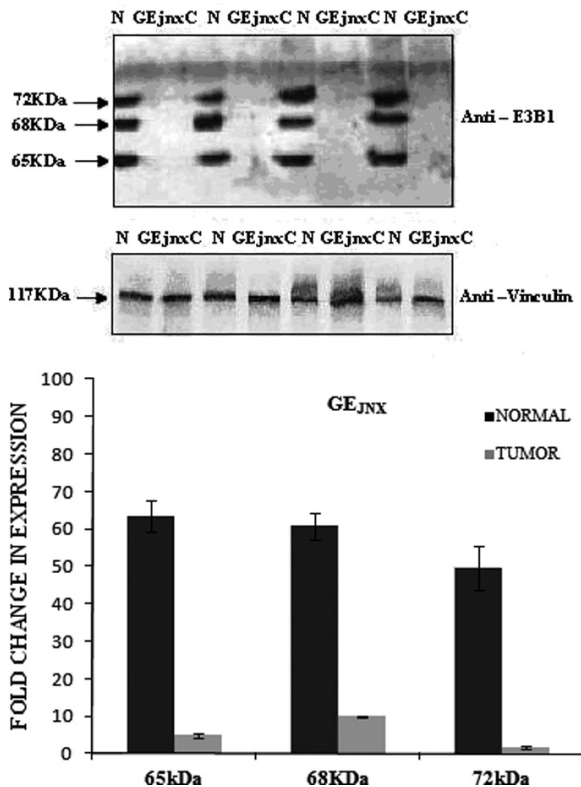


Fig. 3. Immunoblot showing the reduced expression of E3B1 protein and Bar chart comparing the fold change in expression levels in gastro-esophageal junction carcinoma (GEJnC) as compared to their adjacent normals (N).

tions were viewed under fluorescence microscope (Leica). For negative control studies, the primary antibody treatment was omitted (data not shown).

2.7. Hematoxylin and eosin staining

Tissue sections were deparaffinised followed by rinsing in xylene, re-hydration in absolute alcohol and final rinse with graded alcohol. Slides were washed briefly in distilled water and stained in hematoxylin solution for 8 minutes and followed by wash in running tap water for 5 minutes. Slides were differentiated in 1% acid alcohol for 30 seconds and again followed by wash with running tap water for 1 minute. Bluing was carried out in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute and washing was repeated as in the previous step. Slides were rinsed in 95% alcohol (10 dips). Now slides were counterstained in eosin-phloxine solution for 30 seconds to 1 minute and dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each. Slides were finally now washed in xylene and mounted for viewing.

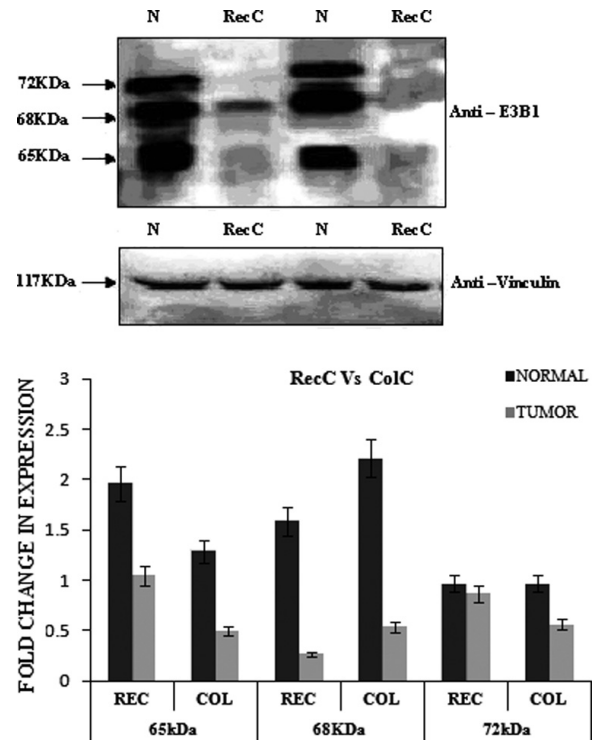


Fig. 4. Immunoblot showing the down-regulated expression of E3B1 in Colon cancer (ColC) and Rectal cancer (RecC) as compared to their adjacent normals (N). *Bar chart comparing the fold change in expression levels of E3B1 protein in Rectal and Colon cancers as compared to their normals.

2.8. Statistics

The results were calculated as mean \pm SD. For statistical analysis of the data, t-test (Microsoft Excel) was used, to evaluate association between clinicopathological variables. Statistical significance was considered when $p < 0.05$.

3. Results

E3B1/ABI-1 a polypeptide of length 480 amino acids is unique in having large number of Ser and Thr residues. This protein is present as a phosphorylated form in cells and tissues. Depending on the extent of phosphorylation it is either present in hyper-phosphorylated (M. Wt. 72 kDa) form or in hypo-phosphorylated form (M. Wt. 68 kDa and 65 kDa). p65 E3B1 is a phosphoserine-containing protein and p68E3B1 and p72E3B1 are hyperserine-phosphorylated forms of p65E3B1 [2]. In this study we have looked at the expression pattern of these iso-

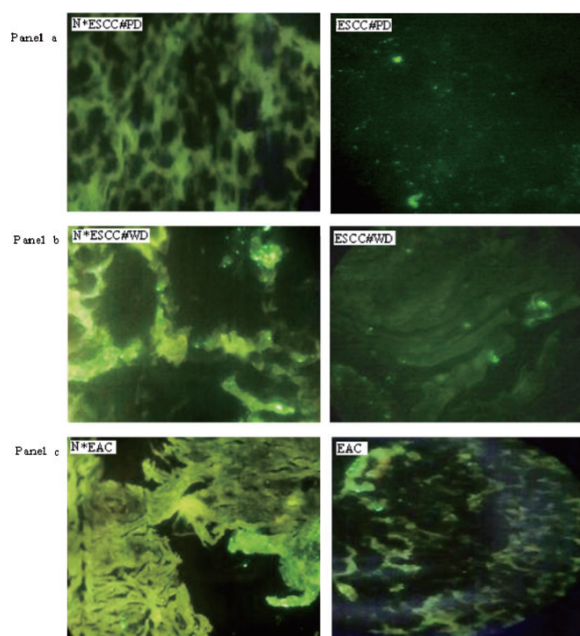


Fig. 5. Immunofluorescence staining of esophageal tissue sections. Expression of E3B1 protein a: Poorly Differentiated (ESCC#PD), b: Well Differentiated (ESCC#WD), Esophageal Squamous Cell Carcinoma and c: Esophageal Adenocarcinoma (EAC) respectively, with respect to their adjacent normals (N). (Images were captured at 40X magnification).)

forms in different forms of human cancers. We carried out Western blot analysis of E3B1/ABI-1 protein in esophageal squamous cell carcinoma and adenocarcinoma (ESCC, AC). Results indicated a consistent decrease in the expression of all the three isoforms i.e., 65 kDa, 68 kDa and 72 kDa in esophageal well and poorly differentiated when compared to their normals (Fig. 1). Normal here represents the tissue from same patient dissected 3 cm away from the site of tumor [19, 20]. We consistently observed a total absence of 65kDa isoform and also a decrease of expression of 68 and 72 kDa isoforms in esophageal squamous and adenocarcinomas (Figs 1 and 2). Western blot expression analysis was carried out on gastro-esophageal junction and colorectal cancers from independent patients. The results in gastro-esophageal junction carcinomas indicated a consistent decrease of E3B1/ABI-1 protein expression in these carcinomas when compared to their adjacent normal controls (Fig. 3). In colorectal cancers, we observed decrease in the expression of all the three isoforms of the E3B1 protein as compared to their normals (Fig. 4). The decrease in the E3B1/ABI-1 expression was observed to be independent of pathological grading, age, sex, dietary and smoking habits of the patients (Table 1). Vinculin protein was used as loading con-

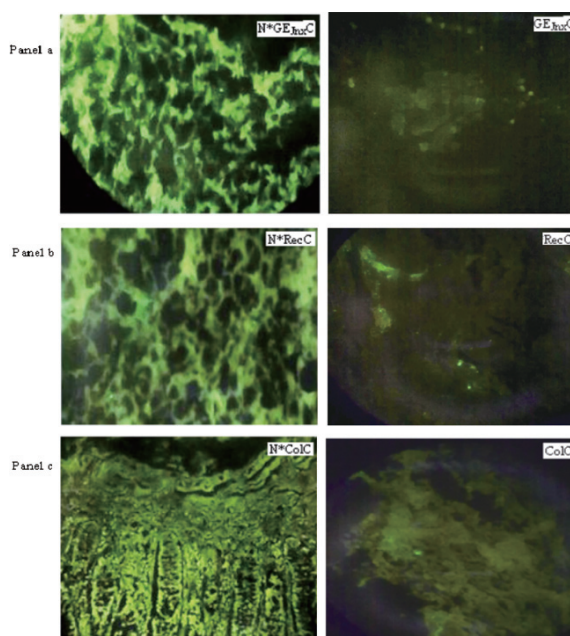


Fig. 6. Immunofluorescence staining of tissue sections. Expression of E3B1 protein a: Gastro-Esophageal Junction Cancer (GEJnC), b: Rectal Cancer (RecC) and c: Colon Cancer (CoIC) respectively, with respect to their adjacent normals (N). (Images were captured at 40X magnification).

trol in all the experiments. Furthermore, Immunofluorescence staining of the tissue sections showed lesser signal of fluorescence obtained in cancers as compared to their respective normals which is consistent with the results obtained with Western Blotting (Figs 5 and 6). Immunofluorescence studies indicate a less difference when cancer samples of esophagus were compared with their respective normals (Fig. 5a, b, c) as has been observed in Western Blotting experiments (Figs 1, 2). Hematoxylin and Eosin staining of the normal and different cancerous tissues was performed on all the samples (Figs 7 and 8) to demonstrate the authenticity of the tissue architecture.

4. Discussion

The mechanism by which E3B1/ABI-1 protein may exert its tumor suppressor activity in cancer development and progression remains unclear. Previous studies have shown that the process of actin polymerization and depolymerization is fundamental to the cellular control of cell shape, adhesion, and migration. Although the role of E3B1/ABI-1 in actin reorganization has been well described, its role in cell growth and tumorigenesis

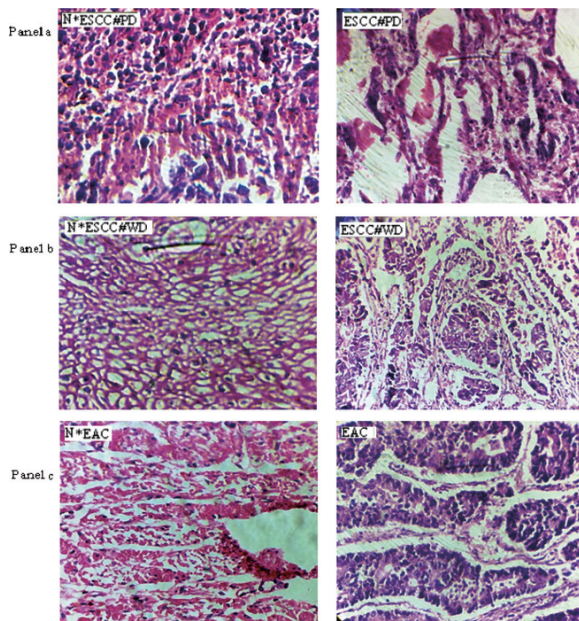


Fig. 7. Hematoxylin and Eosin staining of the normal and cancerous tissues. a: Poorly Differentiated (ESCC#PD), b: Well Differentiated (ESCC#WD), Esophageal Squamous Cell Carcinoma and c: Esophageal Adenocarcinoma (EAC) respectively, with respect to their adjacent normals (N). (Images were captured at 40X magnification).

remains unexplored. Some studies have reported that E3B1/ABI-1 promotes oncogenesis and cancer progression. E3B1/ABI-1 has been found to positively regulate breast cancer cell proliferation, migration, and invasion, as well as annulment of lamellipodia formation, and this role may be mediated via the phosphatidylinositol 3-kinase pathway [5]. E3B1/ABI-1 gene silencing by short hairpin RNA attenuated Bcr-Abl-induced abnormal actin remodeling and membrane-type-1 matrix metalloproteinase clustering, as well as inhibiting cell adhesion and migration on fibronectin-coated surfaces has been reported [21]. Knockdown of E3B1 expression in Bcr-Abl-transformed Ba/F3 cells was shown to limit the leukemogenic potential of these cells in NOD/SCID mice, and revealed that E3B1 contributes to Bcr-Abl-induced leukemogenesis partly through Src family kinases. Conversely, E3B1 also appears to be a tumor suppressor. Some reports have indicated that overexpression of E3B1 inhibits the growth and transforming activity of v-Abl in NIH3T3 cells, as well as the epidermal-growth-factor- and v-Abl-induced activation of extracellular signal-regulated kinase in 293T cells [2, 22–25]. This suggests a potential negative regulatory role for E3B1 in the signaling mediated by epidermal growth factor. Other studies have found that loss of

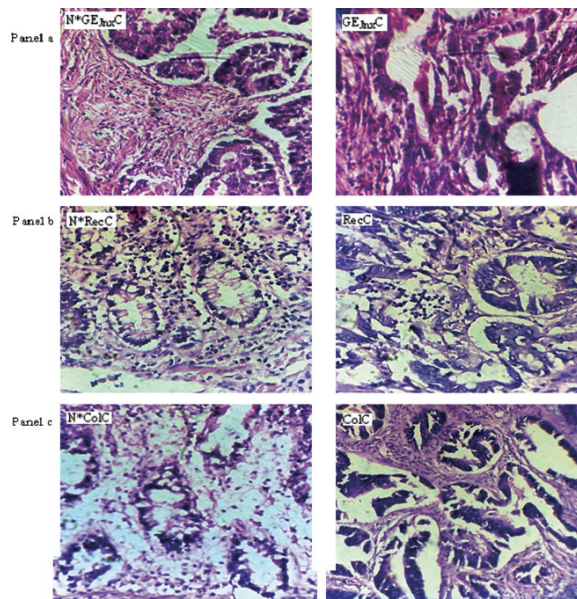


Fig. 8. Hematoxylin and Eosin staining of the normal and cancerous tissues. a: Gastro-Esophageal Junction Cancer (GEJnC), b: Rectal Cancer (RecC) and c: Colon Cancer (ColC) respectively, with respect to their adjacent normals (N). (Images were captured at 40X magnification).

E3B1 may play a role in human prostatic adenocarcinoma and the development of human Bcr-Abl-positive leukemia [16,17]. This apparently contradictory expression pattern of E3B1 across different tumors suggests that this protein may play multiple diverse roles, potentially by participating in the formation of diverse macromolecular complexes and in multiple signal pathways. It is known that E3B1 forms complex with Eps8 and sos1 in activating rac1 RhoGTPase [26] it is possible that its loss or downregulation might help cells to evade apoptosis and hence leading to proliferation.

Nevertheless, our results are consistent with the proposed role of E3B1/ABI-1 in other malignancies, and provide the first evidence that nearly lost or down-regulated or differentially expressed forms of E3B1/ABI-1 may play a similarly important role in advanced esophageal, gastro-esophageal and colorectal cancers. However, because the prognosis of cancer patients is affected by a complex array of factors, the specific utility of differences in phosphorylation status of E3B1/ABI-1 and its expression in evaluating prognostic and/or predictive value must await further *in-vivo* and stratified clinical studies that include a larger number of samples. Further studies are necessary to more precisely define the molecular mechanisms of E3B1/ABI-1 signaling pathways in the development and progression of these cancers.

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Conflict of interest

The authors declare that no conflict of interest exists.

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