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Original Articles

Prevention of epithelial to mesenchymal transition in colorectal carcinoma by regulation of the E-cadherin-β-catenin-vinculin axis

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ABSTRACT

Epithelial to mesenchymal transition (EMT) is compulsory for metastatic dissemination and is stimulated by TGF-β. Although targeting EMT has significant therapeutic potential, very few pharmacological agents have been shown to exert anti-metastatic effects. BI-69A11, a competitive Akt inhibitor, displays anti-tumor activity toward melanoma and colon carcinoma. This study provides molecular and biochemical insights into the effects of BI-69A11 on EMT in colon carcinoma cells *in vitro* and *in vivo*. BI-69A11 inhibited metastasis-associated cellular migration, invasion and adhesion by inhibiting the Akt-β-catenin pathway. The underlying mechanism of BI-69A11-mediated inhibition of EMT included suppression of nuclear transport of β-catenin and diminished phosphorylation of β-catenin, which was accompanied by enhanced E-cadherin-β-catenin complex formation at the plasma membrane. Additionally, BI-69A11 caused increased accumulation of vinculin in the plasma membrane, which fortified focal adhesion junctions leading to inhibition of metastasis. BI-69A11 downregulated activation of the TGF-β-induced non-canonical Akt/NF-κB pathway and blocked TGF-β-induced enhanced expression of Snail causing restoration of E-cadherin. Overall, this study enhances our understanding of the molecular mechanism of BI-69A11-induced reversal of EMT in colorectal carcinoma cells *in vitro*, *in vivo* and in TGF-β-induced model systems.

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer and is the second primary instigator of cancer-related mortality, despite significant current therapeutic advances [1]. Among the therapeutic interventions-surgery, chemotherapy and adjuvant chemotherapy have shown some efficiency in improving colon cancer prognosis. However, the treatment of metastatic colon cancer with adjuvant chemotherapy has not always proven successful. The progression of the metastatic cascade involves an obligatory process- Epithelial Mesenchymal Transition (EMT) that endows cancer cells with enhanced migratory and invasive potential [2–4]. Loss of epithelial markers (such as E-cadherin, claudin, and ZO1) [5], up-regulation of mesenchymal markers (including N-cadherin, vimentin, and α-smooth muscle actin), extracellular matrix components (including collagens α1 and α2) and nuclear translocation of specific transcription factors (i.e., Snail, Slug, ZEB1/2, and Twist1/2) [6] are proposed as characteristic biochemical and molecular markers of EMT induction [7,8]. Akt-mediated activation and nuclear accumulation of β-catenin is detected in 80% of CRC causing invasion and metastasis, which promotes carcinogenesis [9,10]. Aberrant activation, nuclear translocation of β-catenin and Akt-mediated β-catenin–dependent transcription advances the EMT process leading to CRC initiation, progression and metastasis [11,12].

β-catenin plays an important role in Wnt signaling and

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corresponding signaling functions. It is firmly established that activation of β-catenin-mediated TCF/LEF-1 signaling is promoted by growth factor stimulation, including insulin-like growth factor (IGF)-I, IGF-II, and insulin. In response to growth factor stimulation, phosphatidylinositol (PI) 3-kinase-activated AKT phosphoryates GSK-3β at Ser9, which leads to inactivation of GSK-3β and enhanced β-catenin-TCF/LEF-1 transcriptional activity [13]. Fang el al. [14] demonstrated Wnt-independent, Akt-dependent phosphorylation at the Ser552 residue of β-catenin, thereby guiding nuclear accumulation of β-catenin. To maintain cellular homeostasis, in normal epithelial cells, an association between β-catenin and E-cadherin has been observed frequently in the adherens junction. This association mainly prevents translocation of β-catenin to the nucleus and impedes its role as a transcriptional activator of the TCF/LEF1 system [15–18].

Recent studies demonstrate that loss of membranous β-catenin and vinculin correlate with poor prognosis of CRC patients [19]. Vinculin is a ubiquitously expressed protein, an established component of cell-matrix adhesion that localizes on cytoplasmic domains of cadherins [20,21]. Vinculin is a regulator of cell proliferation [22], focal adhesion formation [23] and maintenance of the actin skeleton [21,24]. Additionally, vinculin exhibits several antitumor activities including induction of apoptosis, and reduction in cell invasion and migration [25]. Accordingly, targeting β-catenin and vinculin complex formation through enhanced levels of E-cadherin could be a potential endpoint of EMT-based anti-cancer therapy.

TGF- β promotes initiation of EMT, which is responsible for several biological processes including cell differentiation, proliferation, and migration [26]. Mutation in TGF-β receptors in CRC [27] induces EMT by canonical Smad signaling [28] and/or non-canonical TGF-β/TAK1/IKK/NF-κB signaling [29]. Moreover, inhibition of NF-κB in metastatic cells results in EMT reversal suggesting that the IKK-2/IκBα/NF-κB pathway is required for the induction and maintenance of EMT in epithelial cancer cells [30].

Development of novel drugs targeting EMT may provide therapeutic approaches for anti-cancer therapy. BI-69A11, a competitive Akt inhibitor, showed efficacy in eliciting cell death in melanoma and colon cancer cells [31]. Previous studies also suggest that antitumor efficacy of BI-69A11 resulted from both the inhibition of NF-κB and Akt pathways [32]. Our groups have also shown that BI-69A11 induces apoptosis by dephosphorylation of Akt at Ser473 in colon carcinoma *in vitro* and *in vivo* [33]. Presently, we assessed the expression profile of EMT markers and the mechanism underlying cadherin switching and EMT-reversal induced by BI-69A11. This study also draws attention to the impact of BI-69A11 on TGF-β/Akt/NF-κB-dependent EMT progression.

2. Materials and methods

2.1. Cell lines and reagents

Cell lines and reagents are discussed in supplemental methods.

2.2. Co-culture on 3D matrices

The co-culture of HCT116/Fibroblasts-HCT116 was performed by seeding 1×10^5/matrice (6 mm × 2 mm) fibroblasts on fibroin matrices previously soaked with DMEM media on day 0. On day 2, HCT116 (1×10^5) cells were directly seeded on the opposite side of fibroin structure to create a co-culture model. The construct was cultured for an additional 21 days in DMEM medium supplemented with 10% FBS. After 21 days the co-culture matrices were raised to the air-liquid (A/L) interface to allow stratification of the HCT116 on the matrices and treated with BI-69A11. Cells were released from 3D matrices by incubating with lysis buffer and Western blotting was done with appropriate primary and secondary antibodies as previously described [33].

2.3. Transient transfection

For migration, invasion and adhesion assays, cells were transfected with empty plasmid vector and β-catenin-overexpressing plasmid, and incubated for 16–24 h before treatment with BI-69A11.

HCT116 cells were transiently transfected with all plasmids (GSK-3β (wild, KD, S9A), myr-Akt-pcDNA, NF-κB overexpressing-cDNA, pcDNA) following the same procedure as described above and treated with BI-69A11. Cells were subjected to lysis and Western blotting was performed. For RNA interference experiments, HCT116 and HT29 cells were transfected using Dharmafect and with 50–100 nM siRNA. The siRNA constructs used were (1) mismatched siRNA control (2) siRNA against β-catenin (3) Signalsilence®Akt1 siRNA.

2.4. Migration, invasion and adhesion assays

Migration, invasion and adhesion assays were carried out as described previously [34].

2.5. Immunohistochemistry

For immunohistochemical analysis, the same tumor specimens were collected and used as described in our earlier paper [33], fixed in formalin, embedded in paraffin and sectioned. Immunostaining was performed as described previously [35] with different antibodies including β-catenin, E-cadherin and Vinculin. Images were taken in a bright field microscope with 20X magnification and analyzed with Image J software. The details about other assays are described in the Supplementary Materials and Methods.

2.6. Statistical analysis

All data represent at least three independent experiments and are expressed as means ± SD. For statistical analysis, the Student's t-test and ANOVA were appropriate. An association was considered significant when the exact significance level of the test had a $p < 0.05$.

3. Results

3.1. BI-69A11 inhibits epithelium to mesenchymal transition (EMT) in vitro in colon carcinoma

Tumor cells acquire increased metastatic potential by assuming characteristics of a mesenchymal-like state enhancing their migratory and invasive capacity. Western blotting analysis was performed to quantify the effect of BI-69A11 on EMT-related markers in colorectal cancer cells (HCT116, HT29 and SW480). Expression of the epithelial marker E-cadherin was significantly ($p < 0.05$-0.0001) elevated after treatment with BI-69A11, whereas the mesenchymal markers vimentin and slug were significantly ($p < 0.05$-0.001) decreased (Fig. 1A and B). However, the inhibitory effect of BI-69A11 appeared relatively less significant on snail as compared to other mesenchymal markers (including slug and vimentin) in HCT116, HT29 and SW480 cells. To mimic an ideal 3D tumor microenvironment we investigated 3D co-culture models using HCT116 and fibroblast cells that recapitulate the *in vivo* relationship of CRC/fibroblast tumor microenvironment interactions. BI-69A11 effectively diminished the mesenchymal marker vimentin ($p < 0.001$) (Fig. 1C) and significantly ($p < 0.001$) increased the level of E-cadherin (Fig. 1D).

3.2. BI-69A11 inhibits β-catenin-mediated migration, adhesion and invasion

In the majority of CRC, β-catenin plays a prominent role as an oncogenic protein accompanied by mutations in the *APC* protein leading to abnormally high levels and stabilization of β-catenin. It has been

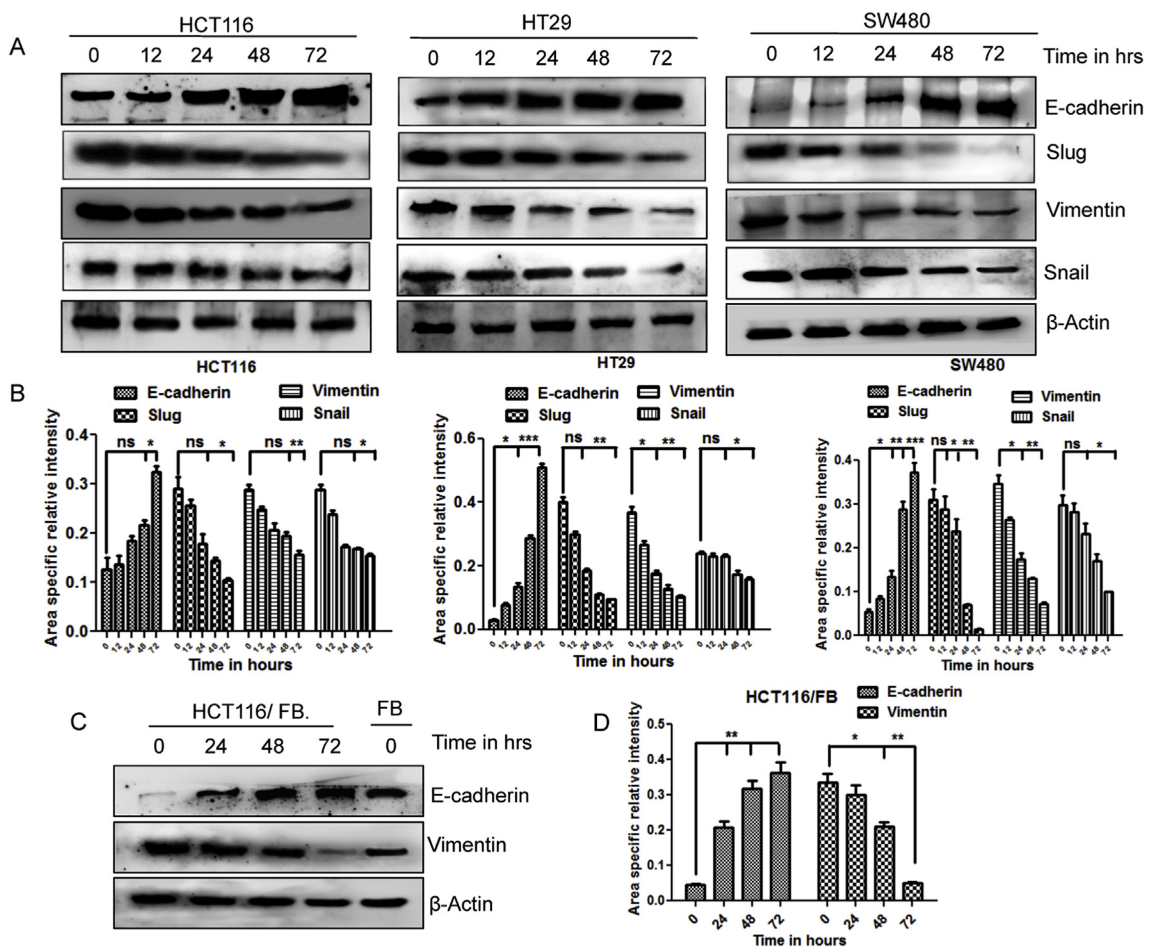

Fig. 1. Effect of BI-69A11 on EMT-associated markers using *in vitro* cell culture and 3D co-culture models. (A) Western blotting analysis of EMT-related markers, E-cadherin, Snail, Slug and vimentin, with β-actin serving as an internal loading control in HCT116, HT29, and SW480 cells after treatment with 0.5 μM BI-69A11, respectively, in a time-dependent manner. The relative protein levels were determined by densitometry analysis of the specific protein bands normalized to β-actin levels. All Western blotting results are representative of at least three independent experiments. (B) Graphical representation of densitometry analysis of relative protein levels of EMT markers in HCT116, HT29, and SW480 cell lines. Data plotted as mean ± SE (n = 3). Asterisks represent statistically significant differences from the control group (*, $p < 0.05$; **, $p < 0.01$; ***,$p < 0.001$). (C) HCT116 and fibroblast cells were co-cultured on a silk fibroin model mimicking 3D *in vivo* cell culture models for 21 days and further treated with 0.5 μM BI-69A11 in a time-dependent manner. The relative protein levels were determined by densitometry analysis of the specific protein bands normalized to β-actin levels. All Western blotting results are representative of at least three independent experiments. (D) Graphical representation of densitometry analysis of relative protein levels of EMT markers in the 3D co-culture model. Data were plotted as mean ± SE (n = 3). Asterisks represent statistically significant differences from the control group (*, $p < 0.05$, **, $p < 0.01$).

suggested that the transcriptional activation of β-catenin assists progression of EMT [8]. Here, we demonstrate that BI-69A11 inhibited β-catenin-mediated cell migration, adhesion, and invasion. As shown in Fig. 2A and B, we found that scratch length in wound healing assays decreased only ~27% and 24% in BI-69A11-treated samples, whereas wound healing was ~67% and 70% in untreated samples after 48 h. Next, we transfected SW480 cells (low β-catenin expression) with a β-catenin-overexpressing plasmid and performed wound healing assays with/without BI-69A11. Overexpression of β-catenin decreased the inhibitory potential of BI-69A11 and caused healing of the wound faster in the BI-69A11-treated sample compared to the pcDNA transfected BI-69A11-treated sample (Fig. 2C). Transwell migration assays were used to assess the effect of BI-69A11 on the cell migratory capacities of HCT116 cells (Fig. 2D) and Matrigel invasion assays monitored HCT116 cell invasion (Fig. 2F). As shown in Fig. 2E and G, migration and invasion of HCT116 cells in BI-69A11-treated samples were significantly reduced (migration: ~52% and invasion: ~56%, respectively) compared to the untreated cells. Integrin-mediated adhesion to extracellular proteins, such as collagen, is a key molecule by which mesenchymal cells acquire increased motility [36]. Cell-matrix adhesion assays confirmed that BI-69A11 significantly reduced HCT116 and HT29 cell adhesion to collagen (Fig. 2H).

3.3. BI-69A11 suppresses β-catenin nuclear localization along with a reduction in cytoplasmic β-catenin, hinders β-catenin function and restores membrane bound β-catenin

BI-69A11 reduced the nuclear translocation of β-catenin in a time-dependent manner. Elevated cytoplasmic beta-catenin in colorectal cancer is suggested to be a predictor of hematogenous metastasis [37,38]. The decrease in cytoplasmic beta-catenin by treatment with BI-69A11 indicates an ability of BI-69A11 to inhibit hematogenous metastasis (Fig. 3A). In addition, BI-69A11 also suppressed total β-catenin levels in HCT116, HT29 and SW480 cells (Fig. 3A). Immunofluorescence was carried out to determine the subcellular distribution of β-catenin after treatment with BI-69A11. BI-69A11 treatment reinstated membrane bound β-catenin and reversed the distribution of nuclear translocation (Fig. 3B).

To determine whether the reduction in cytoplasmic β-catenin levels was due to increased degradation or inhibition of synthesis, we treated HCT116 and HT29 cells with cycloheximide (CHX) alone or in combination with BI-69A11. Under these conditions, the β-catenin level was

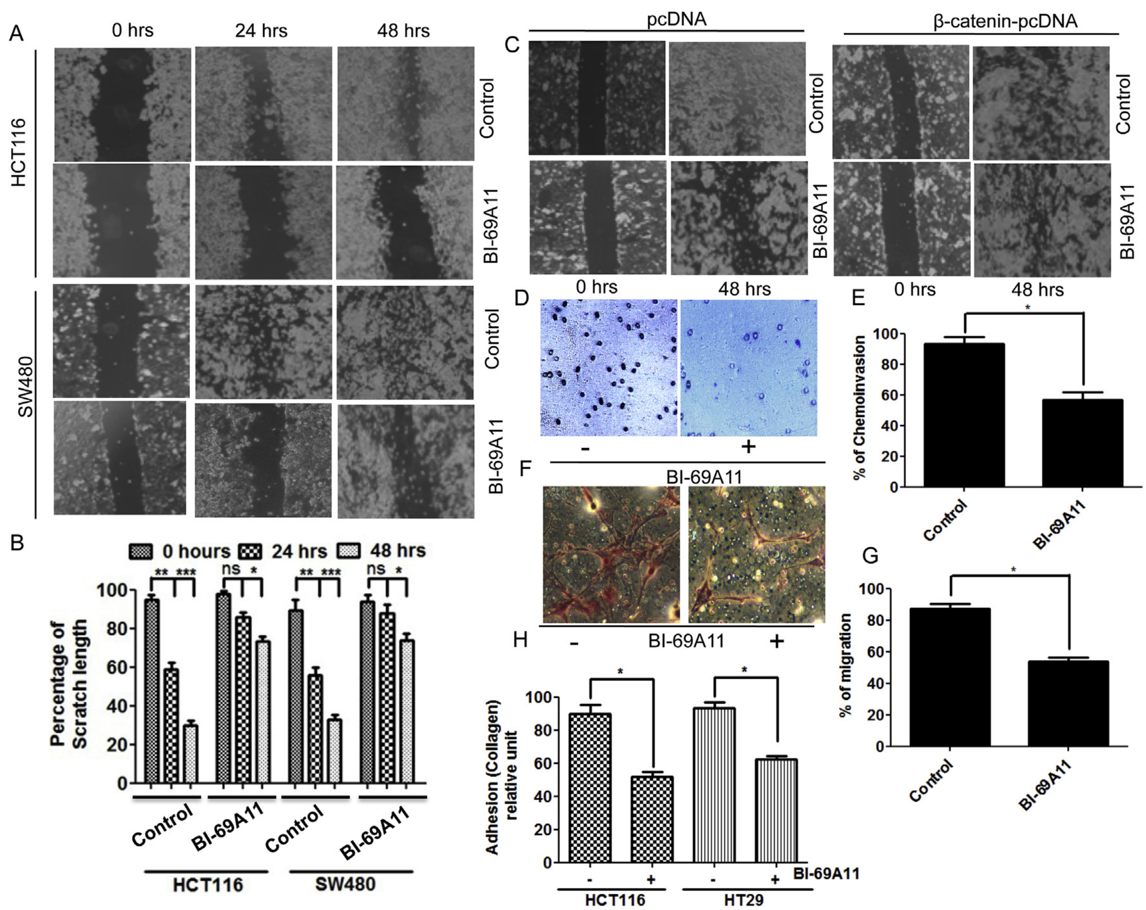

Fig. 2. BI-69A11 inhibits cell migration, invasion and adhesion blocking the β-catenin pathway. (A) Confluent monolayers of HCT116 and SW480 cells were scratched with a 1 mL pipette tip, treated with 0.5 μM BI-69A11 for 24 and 48 h. Photos of the wound were captured by microscope. (B) Wound areas of control and treated cells were quantified by using Adobe Photoshop software. Results are expressed as the mean ± S.E. of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. the 0 h control. (C) Confluent monolayer of SW480 cells was transfected with empty-vector or a β-catenin overexpressing vector. After 24 h of transfection, cells were scratched with a 1 mL pipette tip, treated with 0.5 μM BI-69A11 for 24 h. Photos of the wound were captured by the microscope. (D) and (F) HCT116 cells were seeded in a Transwell upper chamber with/without Matrigel and after attachment, they were treated with BI-69A11 for 24 h. Bright field photographs of migrated cells at the lower surface of the transwell chamber. (E) and (G) Graphical representation of cell invasion and cell migration assay with/without treatment with BI-69A11. For the adhesion assay, HCT116 and HT29 cells were treated with BI-69A11 and seeded on 24-well dishes coated with type I collagen (10 μg/mL), and cultured for 1 h. Adherent cells were assessed by colorimetry. (H) Graphical representation of relative cell adhesion with/without BI-69A11. Each bar represents mean ± S.E (n = 3). Asterisks represent statistically significant differences from the control group (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

further down-regulated after 24 h of treatment with BI-69A11 in combination with Cycloheximide as compare to BI-69A11 alone. These results suggest that the decrease in total β-catenin level occurred due to the degradation of β-catenin (Fig. 3C). BI-69A11 also reduced the protein expression levels of the β-catenin target genes c-Myc and cyclin-D1 in both HCT116 and HT29 cells (Fig. 3D). To study the effect of BI-69A11 on the transactivation function of β-catenin, HCT116 cells were transiently transfected with TOP/FOP FLASH reporter constructs. The reporter activity was significantly ($P < 0.05$) inhibited by 0.5 μM BI-69A11 (Fig. 3E). The inhibition of TOPFlash/FOPFlash reporter activity along with results described above confirm that BI-69A11 inhibits β-catenin–mediated transcriptional activity.

3.4. BI-69A11-induced reduction in nuclear translocation occurs by exploiting Akt/β-catenin pathways

Previous studies suggest a role of GSK3β in the ubiquitination and degradation of β-catenin. In the case of the Wnt-dependent signaling pathway, GSK3β phosphorylates β-catenin on the Ser33/37/41 residue leading to hypophosphorylated β-catenin that translocates into the nucleus and assists in transcribing genes such as c-Myc and cyclin D1. Wnt-independent β-catenin signaling can be activated by growth factors, such as EGF, HGF/scatter factor (SF), IGF-I, IGF-II, and insulin [18]. Desbois-Mouthon et al. suggested that both the PI3K/PKB/GSK-3 and Ras signaling pathways are involved in mediating the stimulatory effects of insulin and IGF-1 and both of these pathways are Wnt-independent [39]. Another report suggests that Akt-mediated phosphorylation of β-catenin at the ser552 residue is responsible for the enhanced accumulation of β-catenin in the cytosol and nucleus [40,41].

We performed an IGF-induced phosphor reactivity assay (Fig. 4A and Fig. 4B) and found that BI-69A11 inhibited phosphorylation of ser552 and ser45/Thr41 and exerted a minor effect on ser33/37/41 residues. These results suggest that BI-69A11 inhibits β-catenin signaling in a Wnt/GSK3β-independent manner. Additionally, pretreatment with LiCl (an agonist of canonical Wnt signaling) followed by BI-69A11, did not exert a greater effect on β-catenin reduction as compared to BI-69A11 alone (Fig. 4C). Based on this observation we confirmed that BI-69A11 mediated inhibition of β-catenin signaling through Akt/GSK3β hypophosphorylation. To validate GSK3β involvement in the BI-69A11-mediated inhibition of β-catenin, we transfected HCT116 cells with GSK3β-WT, GSK3β-KD (kinase deficient) mutant or GSK3β-S9A mutant vectors. S9A mutant is unable to be

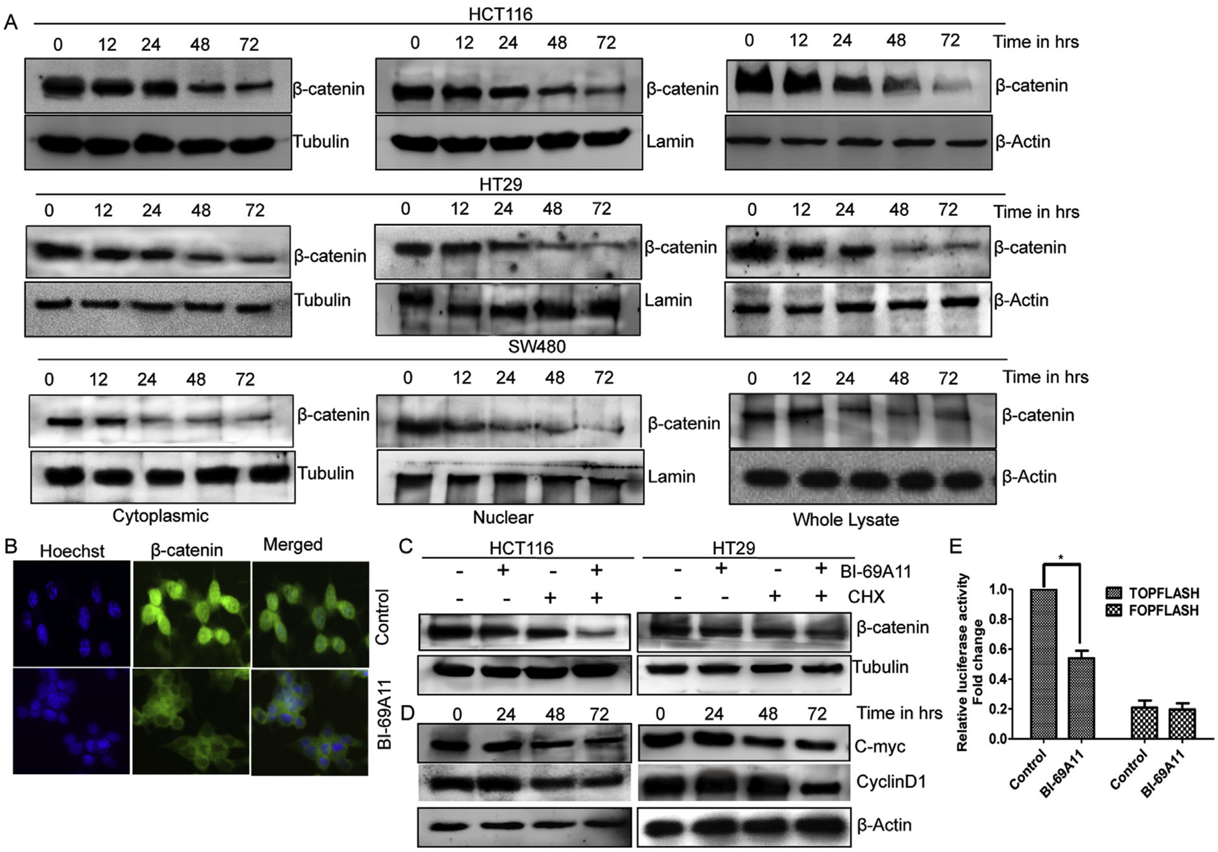

Fig. 3. BI-69A11 regulates the expression levels, cellular distribution, and function of β-catenin in CRC. (A) HCT116, HT29 and SW480 cells were treated with 0.5 μM BI-69A11 for the indicated times. Whole cell lysates, cytosolic and nuclear fractions were prepared and Western blotting analysis was performed. β-actin, tubulin and lamin were used as loading controls, respectively. (B) HCT116 cells were treated with BI-69A11 for 48 h and subjected to immunostaining with β-catenin antibody for localization studies. Nuclei were counterstained with HOECHST 33258. The results provided are representative of at least three independent experiments. (C) BI-69A11-mediated inhibition of β-catenin is due to β-catenin degradation. HCT116 and HT29 cells were treated with a protein synthesis inhibitor, Cycloheximide (CHX) and BI-69A11 alone or in combination. 24 h combination treatment with CHX/BI-69A11 resulted in a similar inhibition as found after BI-69A11 treatment alone. (D) Protein expression levels of β-catenin/TCF-target genes, c-Myc, and Cyclin-D1, were determined by Western blotting after treatment with BI-69A11 for the indicated times in HCT116 and HT29 cells. (E) HCT116 cells were transfected with TOPFlash or FOPFlash construct and TK-renilla luciferase plasmid. After 6 h, they were treated with DMSO (0.5%) or BI-69A11 (1 μM) for 24 h, lysed and assayed for luciferase activity. Transfection efficiency was normalized by calculating the relative ratio of firefly to renilla luciferase activities and the results were plotted as fold-change upon BI-69A11 treatment (n = 3, mean ± S.E.M.).

phosphorylated at Ser9 and, thus β-catenin expression is reduced; KD mutant is kinase deficient and increases β-catenin expression. Overexpression of GSK3β (Wild type and kinase-deficient) effectively suppressed BI-69A11-mediated inhibition of GSK-3β phosphorylation at the Ser9 residue. Conversely, ectopic overexpression of GSK3β-S9A (negative control) owing to inability in S9 phosphorylation, did not exhibit any increased inhibitory effect on BI-69A11-mediated inhibition of GSK3β phosphorylation (Fig. 4D). However, in these samples, overexpression of GSK3β-WT and KD mutant resulted in an increase in the expression of endogenous β-catenin and prevented BI-69A11-mediated inhibition of β-catenin (Fig. 4D and E). But a noticeable decrease in β-catenin expression was seen in the S9A dominant mutant treated sample, which was further decreased in S9A + BI-69A11-treated sample due to GSK3β-independent, Akt-mediated β-catenin degradation. This data infers that GSK-3β mediates overall β-catenin overexpression ensuring limited efficacy of BI-69A11.

Further transfection using a myr-Akt-pcDNA supports the conclusion that overexpression of Akt reduces the BI-69A11-mediated decrease in β-catenin (Fig. 4E). This confirms our previous hypothesis that BI-69A11 predominantly inhibits β-catenin activity by inhibiting the Akt/β-catenin pathway.

3.5. BI-69A11 reconstitutes the altered interaction between β-catenin and E-cadherin

E-cadherin works closely with β-catenin and maintains the functional role of adherens junction. Earlier we showed that BI-69A11 contributes to the amplification of the epithelial marker E-cadherin. Here we confirm that BI-69A11 augments the expression of E-cadherin at both protein and mRNA levels (Figs. 1A and 5A). The densitometric analysis also supports this conclusion (Fig. 5B). Co-immunoprecipitation proved that treatment with BI-69A11 caused enhanced accumulation of E-cadherin-β-catenin complexes (Fig. 5C and D). Phosphorylation of β-catenin at tyrosine 654 residue contributed to the increased ability of β-catenin to bind the basal transcription factor TATA binding protein. Phosphorylation at tyrosine 654 may result in dissociation of β-catenin/E-cadherin complexes and promotes its activity as a co-transcription factor [42]. We determined that BI-69A11 effectively reduced IGF-mediated phosphorylation of β-catenin at the tyrosine 654 residue in both HCT116 and HT29 cells (Fig. 5E). An *in vivo* study [33] followed by IHC investigated the cellular distribution of β–catenin and E-cadherin. Reduced nuclear staining was evident in the treatment group as compared to the control group (Fig. 5F). However, an elevated level of E-cadherin and membrane bound β-catenin was observed in the treatment group (Fig. 5G). These observations confirm that hindering EMT by BI-69A11 occurred by altering nuclear transport of β–catenin and

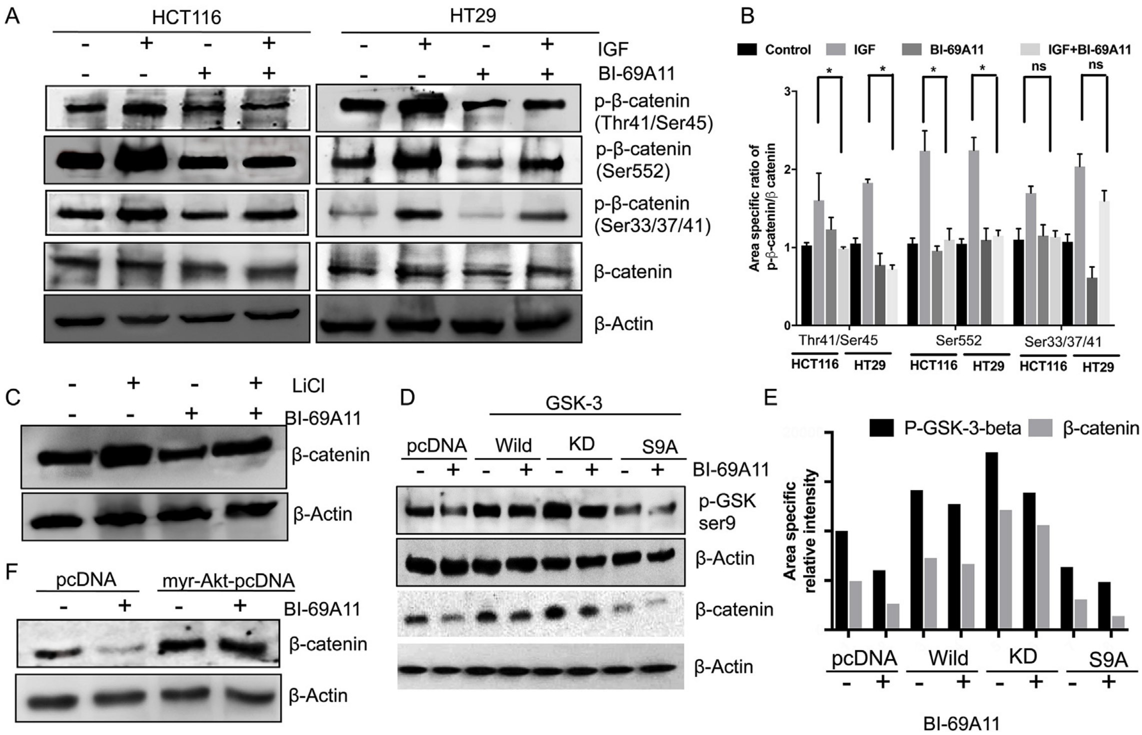

Fig. 4. BI-69A11 decreases active β-catenin levels by inhibiting phosphorylation of both Akt/GSK and Akt pathway mediated changes in β-catenin. (A) HCT116 and HT29 colon cancer cells were untreated or treated with IGF (100 ng/ml) for 30 min and/or BI-69A11 (5 μM) for 1 h and equal amounts of protein cell lysates were analyzed by Western Blotting with the indicated antibodies. (B) Representative densitometric analysis of Western blots as described in A. Data are means ± S.E. of three independent experiments; ($p < 0.05$). (C) HCT116 cells were pretreated with LiCl for 4 h followed by treatment with 0.5 μM BI-69A11 for 48 h and total level of β-catenin was measured. (D) Subconfluent HCT116 cells were transiently transfected with Empty Vector, GSK3β-WT, GSK3β-KD mutant or GSK3β-S9A mutant vectors and after 24 h of transfection, cells treated with 1 μM BI-69A11 for 24 h and Western blotting analysis was performed with the indicated antibodies.(E) Graphical representation of densitometry analysis of relative protein levels of 4D. (F) HT29 cells were transfected with pcDNA or myr-Akt-pcDNA and after 24 h of transfection, cells treated with 1 μM BI-69A11 for 24 h and Western blotting analysis was performed with the indicated antibodies. All experiments were performed three times, respectively. Asterisks represent statistically significant differences from the control group.

stabilizing β–catenin and E-cadherin complex formation.

3.6. BI-69A11 enhances the expression of membrane bound vinculin

Prior reports indicate a close association between vinculin and membrane bound β–catenin in normal colon that becomes disrupted in colon carcinoma leading to CRC metastasis [19]. Treatment with BI-69A11 caused a time-dependent increase in expression of total and membrane bound vinculin (Fig. 6A and B) along with a significant increase in the membrane bound β–catenin (Fig. 6B). The nuclear accumulation of β–catenin was decreased with BI-69A11 treatment, as we observed previously (Figs. 6B and 3A). To document the correlation with β–catenin, HCT116 cells were transfected with a β–catenin-siRNA. We found that knockdown of the total β–catenin level increased the total expression level of vinculin. Moreover, combinatorial treatment of BI-69A11 and β–catenin-siRNA increased the level of vinculin compared to treatment with β–catenin-siRNA/BI-69A11 alone (Fig. 6C). In contrast, overexpression of β–catenin in HT29 cells diminished the vinculin level in both BI-69A11-treated/untreated sample confirming the hypothesis that vinculin decreases when the total cellular level of β–catenin increases (Fig. 6D). Immunofluorescence showed that BI-69A11 treatment increased the expression of membrane bound vinculin (Fig. 6E). Immunohistochemistry also showed that the BI-69A11-treated group exhibited higher levels of vinculin and the increase in vinculin level was significant as compared to the control group (Fig. 6F and G).

3.7. BI-69A11 suppressed TGF-β mediated EMT in a non-canonical pathway targeting NF-KB

Emerging evidence indicates that the TGF-β-mediated transformation of epithelial cells into a fibroblast phenotype involves the phosphorylation of Akt [43] and Akt may be a therapeutic target for promoting resistance towards TGF-β-induced EMT. Here, we hypothesized that BI-69A11 suppresses TGF-β-induced EMT by regulating the Akt/NF-κB-mediated pathway. Morphological analysis showed that BI-69A11 converted a TGF-β-induced fibroblast-like shape to a more epithelial-type shape with decreased lamellipodia and decreased elongated cell structures (Fig. 7A). Furthermore, cell invasion studies also suggested that BI-69A11 inhibited TGF-β-induced cell invasion (Fig. 7B). Dose-dependent treatment with BI-69A11 caused reduced levels of vimentin and snail and increased E-cadherin levels (Fig. 7C). A decreased level of β-catenin was also observed suggesting BI-69A11 reduced TGF-β-induced EMT by modulating expression levels of key elements of adherens junctions. Since Akt and NF-kB were prerequisites for TGF-β-induced EMT, we investigated the effect of BI-69A11 on the Akt/NF-κB signaling pathway. Our results indicated that BI-69A11 significantly inhibited phosphorylation of Akt (Ser473 residue) and consequently, phosphorylation of IKB (Ser-32 residue) and NF-κB (Ser-536 residue) in HCT116 cells without affecting total protein levels (Fig. 7D). Moreover, BI-69A11 also inhibited total NF-κB and nuclear transport of NF-κB (Fig. 7E). To provide further validation that BI-69A11 represses TGF-β-induced EMT through the NF-κB pathway, HCT116 cells were transfected with a p65 overexpressing vector and Western blotting was carried out to identify changes in EMT markers. As anticipated, the level of snail and β-catenin were up-regulated and the level of E-

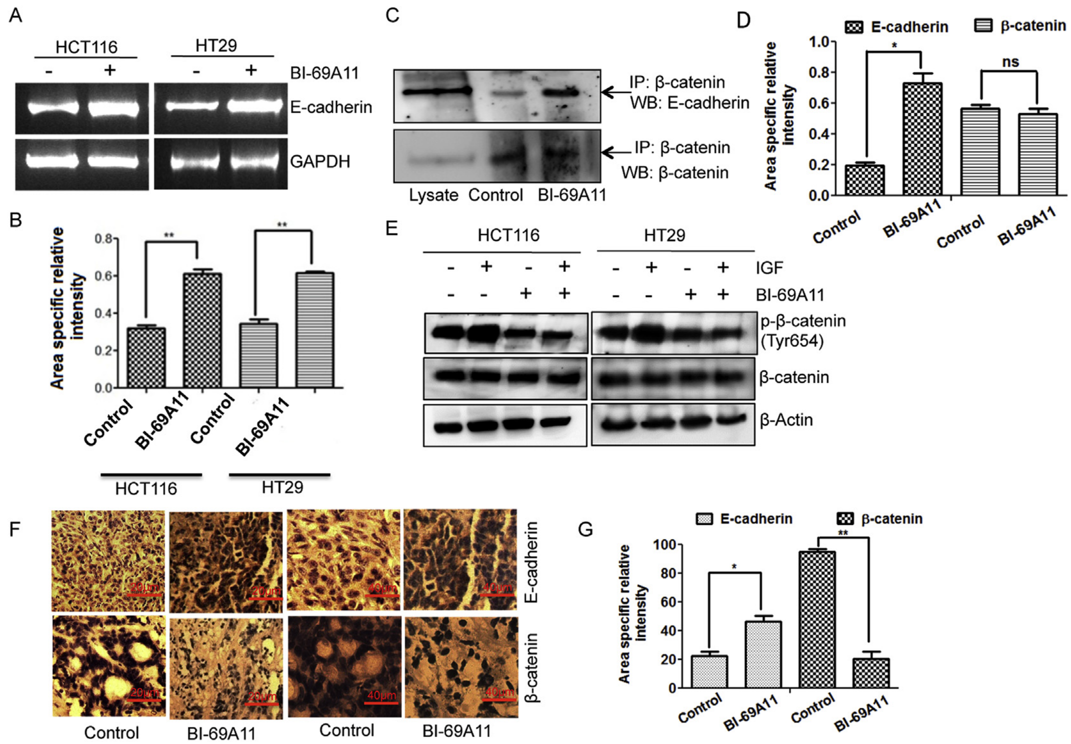

Fig. 5. BI-69A11 re-establishes E-cadherin expression by augmenting the formation of E-cadherin/β-catenin complex formation. (A) RT-PCR was performed to analyze the mRNA expression level of E-cadherin in HCT116 and HT29 cells treated with BI-69A11 for 48 h. (B) Graphical representation of densitometric analysis of control and BI-69A11-treated samples. (C) Equal protein from control and BI-69A11-treated cells were immunoprecipitated with β-catenin antibody and immunoblotted for E-Cadherin. (D) Graphical representation of densitometric analysis of control and BI-69A11-treated samples. (E) HCT116 and HT29 colon cancer cells were untreated or treated with IGF (100 ng/ml) for 30 min and/or BI-69A11 (5 μM) for 1 h and equal amounts of protein cell lysates were analyzed by Western Blotting with the indicated antibodies. (F) Immunohistochemical analysis with the indicated antibodies on tissue sections from HT29 xenografts of control and BI-69A11-treated groups. Pictures were taken at 20X and 40X magnification (G) Quantification of IHC analysis was done using ImageJ software. All the experiments were performed three times, respectively. Asterisks represent statistically significant differences from the control group (*, $p < 0.05$; **, $p < 0.01$).

cadherin was down-regulated in NF-κB overexpressed conditions and both TGF-β-treated or untreated samples and BI-69A11 treatment did not significantly change these markers and no reversion of EMT was observed (Fig. 7F).

4. Discussion

Akt inhibitors represent new classes of therapeutic agents that complement traditional cancer therapies. Akt is a central regulatory node in the majority of cancer promoting events. Thus, targeting Akt could provide immense therapeutic benefit by affecting multiple cancer-associated cellular signaling pathways. Previous studies suggested that BI-69A11 promotes apoptosis in melanoma, breast, prostate and colon carcinoma [31–33]. BI-69A11 suppresses the phosphorylation of Akt and its downstream targets thereby inducing apoptosis [33]. We hypothesize that BI-69A11 could also be employed as a potential candidate molecule to inhibit EMT-mediated cancer invasion. BI-69A11 inhibited mesenchymal markers and elevated the level of epithelial markers *in vitro* in colon cancer cells, indicating that BI-69A11 abrogated EMT in colon cancer. Immunohistochemistry using HT29 xenografts also confirmed the elevation of the epithelial marker E-cadherin in the BI-69A11-treated group *in vivo*. Moreover, the effect of BI-69A11 on the focal adhesion junction regulatory protein vinculin confirmed that BI-69A11 has a pronounced effect on both tight and focal adhesion junctions. We now establish that BI-69A11 downregulates the non-canonical NF-κB signaling pathway to repress snail expression and induce E-Cadherin leading to the inhibition of TGF-β-mediated EMT.

Akt overexpression is associated with 57% of sporadic colon tumors, which is higher than that in many other cancers. Overexpression of Akt is associated with increased cell migration and cell invasion along with augmentation of several mesenchymal markers including snail, slug, β-catenin, and vimentin [44]. Loss of E-cadherin, a tight junction protein, is also observed in Akt overexpressing tumors enhancing the extent of cell migration and metastatic dissemination [45]. Moreover, recent studies reveal an important role for the PI3K–AKT-mediated activation of β-catenin in CRC development and in progression to invasion and metastasis [46]. β-catenin plays a dual role through its interaction with the cytoplasmic surface of E-cadherin or as a transcription co-activator for T-cell factor (TCF)/lymphoid enhancer factor-responsive genes that lead to the activation of the Wnt signaling pathway. Emerging pieces of evidence also suggest that Akt/β-catenin promotes the repression of E-cadherin through nuclear-β-catenin transport and trans-activation of slug [47]. Activation of β-catenin and its transport are considered major events in EMT progression. Here, we show that BI-69A11 impedes cell migration, adhesion, and invasion through inhibition of the β-catenin-mediated pathway, whereas overexpression of β-catenin mitigates the BI-69A11-mediated inhibition. The functional activity and cellular distribution of β-catenin were regulated by BI-69A11. These events are controlled by BI-69A11 by the downregulation of β-catenin through inhibition of two distinct pathways-downregulation of the Akt/GSK/β-catenin pathway and the Akt/β-catenin pathway by decreasing the phosphorylation of specific residues.

As a tumor suppressor gene, a pivotal role of E-cadherin is to form a functional complex with β-catenin for cellular adhesion and maintenance of the epithelial layer. In addition, this complex formation tends to extinguish the functional role of β-catenin by keeping it membrane bound. The disruption of E-cadherin expression is associated with the increased nuclear localization and activity of β-catenin. In fact, the critical factor controlling nuclear translocation is the increased concentration of free β-catenin in the cytoplasm that promotes its

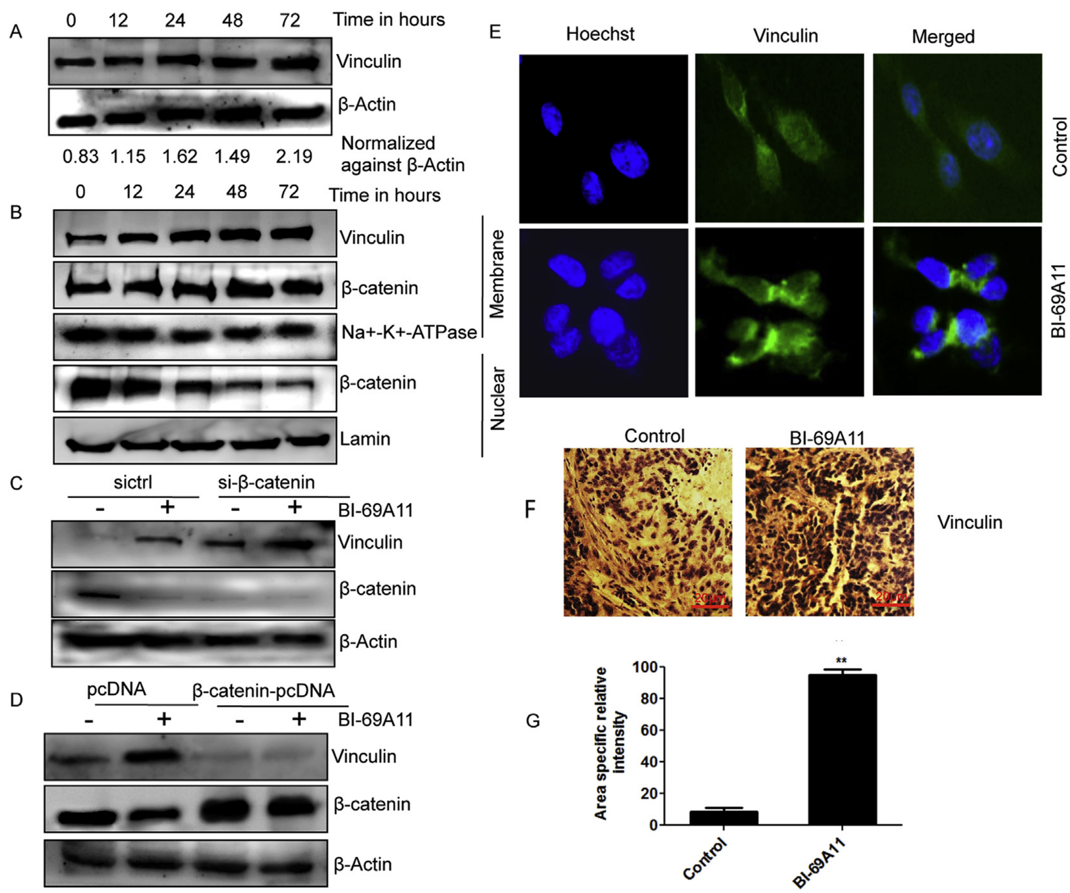

Fig. 6. BI-69A11 enhances the expression of membrane bound vinculin that helps to strengthen focal adhesion. (A) HCT116 cells were treated with BI-69A11 for the indicated times and Western blotting analysis was performed to assess the changes in vinculin levels in a time-dependent manner. (B) HCT116 cells were treated with BI-69A11 for the indicated times. Membrane and nuclear fractions were prepared and Western blotting analysis was performed. β-actin, tubulin, and lamin were used as loading controls, respectively. (C) Effect of β-catenin knockdown on Vinculin protein expression. After transfection with β-catenin siRNA, Western blotting was performed to determine the protein expression levels of β-catenin and Vinculin. (D) Effect of β-catenin overexpression affects the expression of Vinculin. After transfection HCT116 cells with β-catenin overexpressing vector, Western blotting was performed to determine the protein expression levels of β-catenin and Vinculin. (E) HCT116 cells were treated with BI-69A11 for 48 h and Immunofluorescence was performed to check the expression of Vinculin. Nuclei were counterstained with HOECHST 33258. (F) Paraffin-embedded sections of HT29 bearing tumors in nude mice were processed and IHC was done after staining with vinculin. Pictures were taken at 20X magnification. (G) Quantification of IHC analysis was done using ImageJ software. All the experiments were performed three times, respectively. Asterisks represent statistically significant differences from the control group (*, $p < 0.05$; **, $p < 0.01$).

binding to the TCF/LEF family of DNA binding proteins. Additionally, the phosphorylation of β-catenin at the tyrosine 654 residue helps to dissociate the interaction between β-catenin/E-cadherin complex formation and helps in releasing β-catenin to act as a transcription factor with the TCF/LEF family of DNA binding proteins [42]. As previously mentioned, increased levels of E-cadherin in the plasma membrane led us to hypothesize that BI-69A11 increases the complex formation between β-catenin and E-cadherin that further sequesters β-catenin to be membrane bound and perturbs the nuclear translocation of β-catenin. The most likely mechanism behind these interactions is the dephosphorylation of β-catenin at the tyrosine 654 residue that helps to remove the hindrance caused by phosphorylation at the tyrosine 654 residues.

Vinculin is localized on the cytoplasmic face of focal adhesion complexes and is a prerequisite for cell-cell adhesion [48]. In addition, close interaction between vinculin and β-catenin help to increase the association between the cytoskeleton and cell cortex to avoid excess tension [49]. This interaction is also required for the stabilization of E-cadherin in the cell surface [50]. Here, we investigated the effect of BI-69A11 on vinculin due to its close association with β-catenin. As predicted, BI-69A11 enhanced the expression of vinculin *in vitro* and *in vivo* along with the increase with membrane bound β-catenin that also supports the reports in the literature that low membrane bound β-catenin and vinculin are observed in colon carcinoma [19].

Being a major inducer of EMT, TGF-β forms a complex network to promote EMT through the Smad-dependent canonical pathway and the Smad-independent non-canonical pathway. Recent studies suggest that PI3K-Akt-dependent metastasis can occur through regulation of NF-κB transcription factor which is associated with cell invasion and motility. Induction of TGF-β1 promotes the activation of the PI3K/Akt/NF-κB/MMP9 signaling pathway in Philadelphia chromosome-positive Chronic Myeloid Leukemia hemangioblasts [51]. In pancreatic cancer, HMGA1-induces cellular invasiveness and MMP-9 activity occur through a PI3K/Akt-dependent pathway [52]. Evidences also suggest that induction of EMT with TGF-β, causes loss of sensitivity towards growth inhibition in colon carcinoma. Several chemotherapeutic agents abrogate TGF-β-induced EMT through inhibition of the non-canonical pathway [30,53]. Here we propose that treatment with BI-69A11 reverts TGF-β-induced snail, vimentin and β-catenin induction and E-Cadherin down-regulation. TGF-β treatment also causes phosphorylation of Akt, Iκβ and, NF-κB along with increased nuclear transport of NF-κB in HCT116 cells. Based on this observation, it can be concluded that the Akt/NF-kB-mediated pathway is mandatory for TGFβ1-mediated EMT.

Treatment with BI-69A11 diminished the phosphorylation of Akt, IκB, and NF-κB with reduced expression in nuclear and total NF-κB. For further confirmation, overexpression of NF-κB induced snail expression

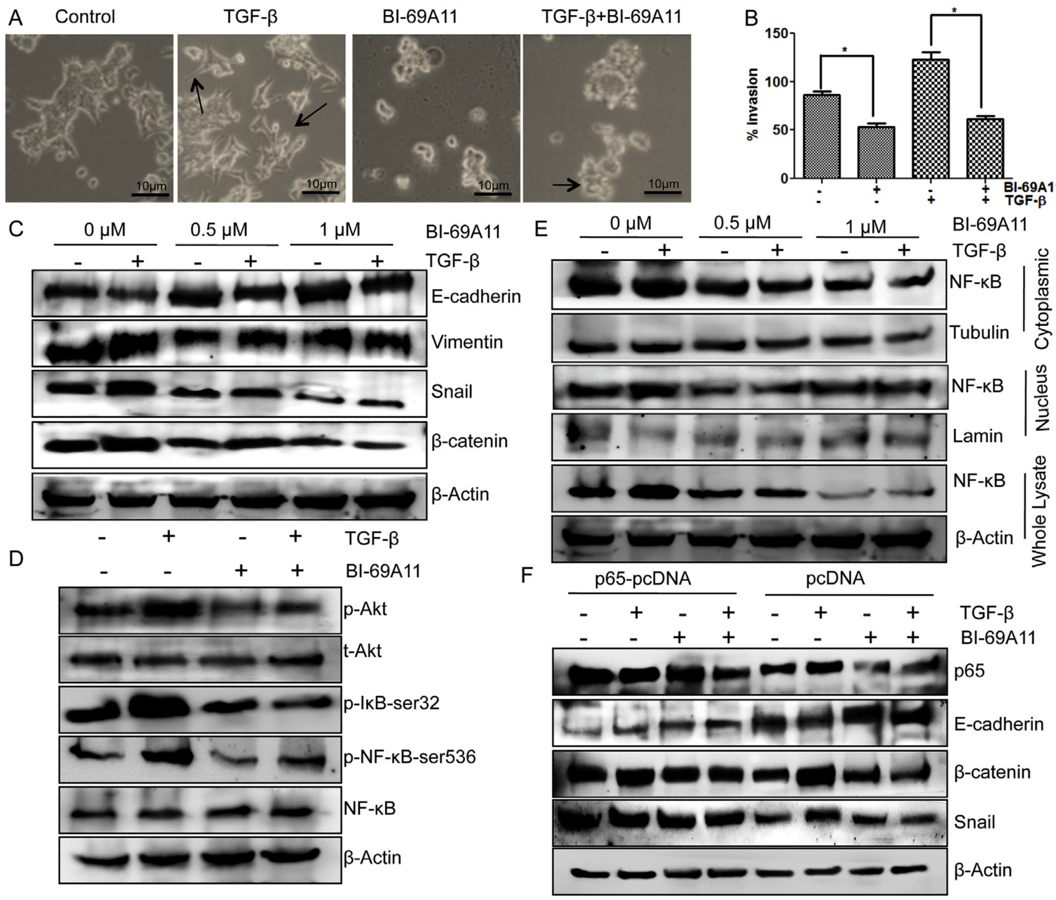

Fig. 7. BI-69A11 abrogates TGF-β-mediated EMT through an Akt/Iκβ/NF-κB-mediated pathway. (A) Morphological analysis of HCT116 cells after pretreatment with 20 ng/ml TGF-β, BI-69A11 was added to HCT116 cells and they were incubated for 48 h. Pictures were taken with a bright field microscope with 10X magnification. Arrows show lamellipodia. (B) HCT116 cells were pretreated with 20 ng/ml TGF-β followed by BI-69A11 for 48 h and cell invasion assays were performed. Graphical representation of invasion assays. All the experiments were performed three times, respectively. Asterisks represent statistically significant differences from the control group (*, $p < 0.05$; % invaded cells vs Control cells). (C) HCT116 cells were pretreated with 20 ng/ml TGF-β followed by BI-69A11 in a dose-dependent manner for 48 h. EMT markers were analyzed in a dose-dependent manner. (D) HCT116 cells were treated with 5 μM BI-69A11 for 1 h followed by treatment with TGF-β and whole cell lysates were evaluated for phosphorylation and expression of p-Akt (Ser473), p-IκB (Ser 32) and p-NF-κB (Ser 536). (E) Cytoplasmic and nuclear extract of HCT116 cells were subjected to Western blotting to measure the cellular distribution of NF-κB after the indicated dose of BI-69A11 treatment for 48 h. (F) HCT116 cells were transfected with NF-κB overexpressing plasmid to show that overexpression of NF-κB overcomes the BI-69A11-mediated resistance towards TGF-β-mediated EMT. Equal amounts of protein from control and treated cells were analyzed for EMT markers.

and downregulated E-Cadherin in HCT116 cells. Furthermore, overexpression of NF-κB completely abolished EMT inhibition by BI-69A11, indicating that BI-69A11 targets the Akt/NF-κB pathway to inhibit TGF-β-induced EMT in colon cancer.

In summary, our study elucidates a primary role of BI-69A11 in reversing EMT transition in CRC by suppressing cell migration, invasion and adhesion through targeting β-catenin. Moreover, BI-69A11 also inhibits TGF-β-induced EMT through a separate mechanism involving the Akt/NF-κB pathway. These findings confirm an ability of BI-69A11 to target multiple pathways essential in CRC initiation, progression, and metastasis. Accordingly, BI-69A11 may provide value as a therapeutic for CRC, which may be enhanced by combining this drug with other signal transduction inhibitors.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Ipsita Pal: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing - original draft, Writing - review & editing. **Y. Rajesh:** Data curation, Methodology. **Payel Banik:** Data curation, Methodology. **Goutam Dey:** Data curation, Formal analysis. **Kaushik Kumar Dey:** Project administration. **Rashmi Bharti:** Data curation, Formal analysis. **Deboki Naskar:** Data curation, Formal analysis. **Sandipan Chakraborty:** Formal analysis. **Sudip K. Ghosh:** Resources. **Swadesh K. Das:** Writing - original draft, Resources. **Luni Emdad:** Writing - original draft, Writing - review & editing. **Subhas Chandra Kundu:** Resources, Writing - original draft. **Paul B. Fisher:** Conceptualization, Resources, Writing - original draft, Writing - review & editing. **Mahitosh Mandal:** Conceptualization, Supervision, Resources.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.008.

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