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Reversal of TGF- β -induced epithelial–mesenchymal transition in hepatocellular carcinoma by sorafenib, a VEGFR-2 and Raf kinase inhibitor



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ABSTRACT

The epithelial–mesenchymal transition (EMT) is considered an essential process for cancer development and metastasis. Sorafenib, a RAF kinase and VEGFR-2 inhibitor, exhibits efficacy against advanced hepatocellular carcinoma (HCC), renal carcinoma, and thyroid cancer. It is well established that transforming growth factor- β (TGF- β) activated EMT is involved in the invasion and metastasis of Hep G2 cells in HCC. In this study, we investigated the effects of sorafenib on various biomarkers associated with EMT using flow cytometry. We found that sorafenib upregulated the epithelial marker E-cadherin and downregulated the mesenchymal marker vimentin. Furthermore, sorafenib downregulated the level of the EMT-inducing transcription factor SNAIL. Our findings provide insights into the mechanisms associated with the anti-EMT effects of VEGFR-2/RAF kinase inhibitors.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most commonly occurring malignancy worldwide and the third main cause of cancer-related mortality (Kew, 2014; Rawla et al., 2018). Metastasis and the development of new tumors are fundamental causes of the death of patients with HCC (Yamamoto et al., 1996). Furthermore, most patients are diagnosed at an advanced stage of hepatocarcinogenesis due to a lack of early-stage detection and the silent progression of the disease. The epithelial–mesenchymal transition (EMT) involves the transient conversion of epithelial cells into quasi-mesenchymal cells, where they acquire the spindle-shaped morphology of mesenchymal cells by losing their epithelial cell appearance (Fig. 1) (Nieto, 2009, 2017). The formation of the epithelial state is characterized by apical–basal polarity, which is organized and tightly connected by various tight junction proteins (zonula) and adherence junctions (occludins and claudins), where the latter are formed by the cell surface protein E-cadherin (Dongre and Weinberg, 2019). After activation, EMT leads to the suppression of E-cadherin and the loss of epithelial cell morphology. As tumor development progresses, the cancerous cells (early stage) are in the epithelial-like stage and they acquire mesenchymal cell characteristics. Quasi-mesenchymal cells are characterized by changes in mesenchymal

markers, such as fibroblast-specific protein (FSP-1), vimentin, N-cadherin, α -smooth muscle actin (α -SMA), and EMT-inducing transcription factors (EMT-TFs) comprising SNAIL (SNA 1), SLUG (SNA 2), TWIST, and ZEB1 (Ye et al., 2015; Fontana et al., 2019). At the molecular level, metastasis begins with EMT, which is followed by remodeling of the extracellular matrix, cancer cell invasion via intravasation into the bloodstream, the persistence of cancer cells in the bloodstream, extravasation of cells from capillaries into distinct organs, and the settlement and replication of cancerous cells in a more favorable environment (Banyard and Bielenberg, 2015). The transforming growth factor- β (TGF- β) signaling cascade is involved in metastatic events and it has been shown to elevate the capacity of tumors to spread throughout the host. The TGF- β -induced SMAD complex activates the mesenchymal markers vimentin and fibronectin as well as EMT-TFs comprising SNAIL, SLUG, ZEB1, and TWIST, which then suppress the expression of E-cadherin (Padua and Massagué, 2009; Nagai et al., 2011). Therefore, the reversal of EMT under stimulation by TGF- β is essential for preventing metastasis in cancer. In the present study, we assessed the antiproliferative activity of sorafenib in the Hep G2 cell line, as well as its effects on the epithelial marker E-cadherin, mesenchymal marker vimentin, and EMT-TF SNAIL using flow cytometry.

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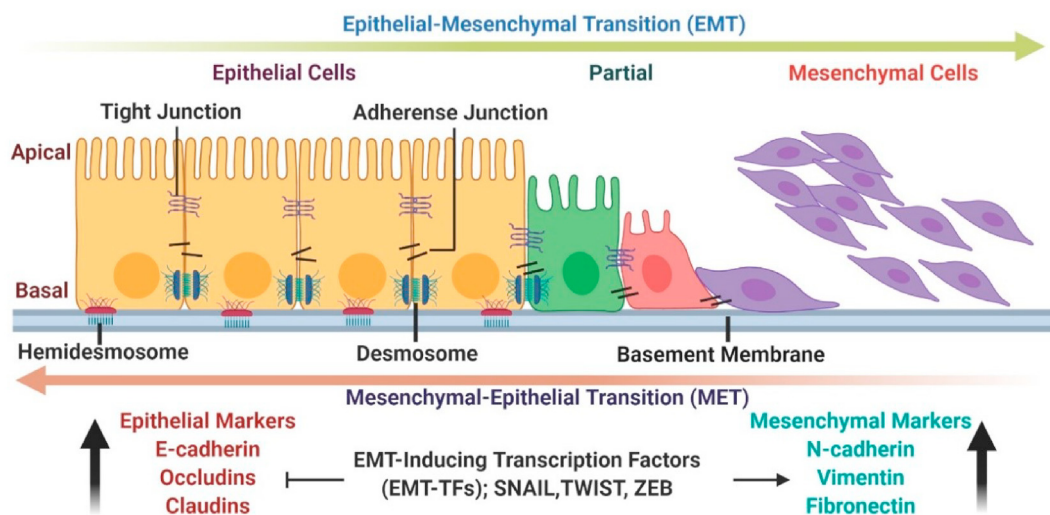


Fig. 1. Epithelial–mesenchymal transition (EMT) process. Epithelial cells exhibit apical–basal polarity and they are attached tightly via tight junctions and adherence junctions, and desmosomes are attached to the basement membrane via hemidesmosomes. Induction of EMT leads to the expression of EMT-inducing transcription factors (EMT-TFs), i.e., SNAIL, TWIST, and ZEB. These factors suppress epithelial genes and activate mesenchymal genes. Expression of these genes result in cellular changes such as disassembly of epithelial cell–cell junctions and loss of apical–basal cell polarity (color of the cell indicates EMT progression). Loss of the epithelial phenotype results in the gain of a mesenchymal phenotype, where cells become motile with the capacity for invasion. Mesenchymal cells can convert into epithelial cells and this process known as the mesenchymal–epithelial transition is more common in malignancy. During EMT, the loss of epithelial markers such as E-cadherin, occludins, and claudins is observed, whereas mesenchymal markers such as N-cadherin, vimentin, and fibronectin are overexpressed.

2. Experimental section

2.1. Materials

The required quantities of fetal bovine serum (FBS), antibiotic solution, and Dulbecco's modified Eagle's medium (DMEM) were procured from Gibco (BRL, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide were obtained from Sigma-Aldrich (St Louis, MO, USA). $1 \times$ Phosphate-buffered saline (PBS), Anti-Human vimentin PE (# 562337), and Anti-Human E-cadherin FITC (#612130) were purchased from BD Biosciences (USA). Tissue culture plates (six, 24, and 96 wells), wash beakers, centrifuge tubes, serological pipettes, and tips were purchased from Tarsons Products Pvt. Ltd (India). All other chemicals used were analytical reagent grade and they were procured from Sigma-Aldrich.

2.2. Cell line and transfection

The Hep G2 cell line was procured from the National Center for Cell Science (NCCS), Pune, Maharashtra, India. Hep G2 cells were cultured in liquid DMEM containing 10% FBS, penicillin, and streptomycin, and they were maintained in a controlled environment (37 °C under 5% CO₂ atmosphere).

2.3. In vitro antiproliferative activity assay

The antiproliferative activity of sorafenib tosylate (ST) was determined using the MTT assay (Fig. 2). Trypsinization was conducted to harvest the cell culture, which was pooled in a 15-mL tube. Cells at a density of 1×10^5 cells/mL were plated in tissue culture plates (96-well) containing DMEM with 10% FBS and 1% antibiotic solution, and incubated for 24–48 h at 37 °C. Sterile PBS was used to wash the wells and various concentrations (0.01 μ M–100 μ M) of ST in DMEM (free of serum) were added to treat the cells, before incubating at 5% CO₂ and 37 °C for 24 h. After incubation, MTT was added to each well and the cell cultures were incubated for 2–4 h. Next, the medium containing MTT was aspirated from the wells and the wells were washed with $1 \times$ PBS solution. Dimethyl sulfoxide was then added and the plate was shaken for 5 min. A

microplate reader was employed to quantify the absorbance of each well at 570 nm (Zhou et al., 2017).

2.4. EMT marker expression studies

Hep G2 cells at a density of 3×10^5 cells per 2 mL were plated in tissue culture plates (six-well) containing DMEM and incubated for 24 h at 37 °C and 5% CO₂. After incubation, the medium was removed from each well, before treating with ST at its IC₅₀ concentration (214.80 nM) and incubating again for 48 h. The medium was removed from each well, which was then treated with trypsin-EDTA solution (200 μ L). Next, 2 mL of culture medium was added to each well and the cells were pooled in polystyrene tubes (12 \times 75 mm). The tubes were then centrifuged (5 min at 300 rpm and 25 °C). The cell concentration was adjusted to $1-2 \times 10^6$ cells/mL in ice-cold 70% ethanol (Mali et al., 2018).

2.4.1. Cytoplasmic marker and cell surface marker staining

Anti-vimentin PE antibody (10 μ L) was added to each tube, before mixing thoroughly and incubating in the dark at 25 °C for 30 min. Anti-SNAIL primary antibody (50 μ L; 1:100) was also added, before mixing thoroughly and incubating in the dark at 25 °C for 30 min. Dulbecco's phosphate-buffered saline (D-PBS) was added to wash the cells and Goat Anti-Rabbit IgG H&L (FITC) (100 μ L; 1:200) was added to the cells in each tube, before incubating for 1 h at 25 °C. Finally, $1 \times$ PBS and 0.1% sodium azide were added to wash the cells, which were then analyzed by flow cytometry. In addition, the E-cadherin FITC antibody (10 μ L) was added to polystyrene culture tubes, before mixing and incubating at 25 °C for 30 min. After incubation, the cells were analyzed by flow cytometry.

2.5. Statistical analysis

All experiments were conducted in triplicate and repeated three times. The data were expressed as the mean \pm standard error of the mean. GraphPad Prism 5.0 (San Diego, CA) was used to perform statistical analyses. One-way analysis of variance followed by Turkey's test was used to assess significant differences among groups and differences were considered significant at $P \leq 0.05$.

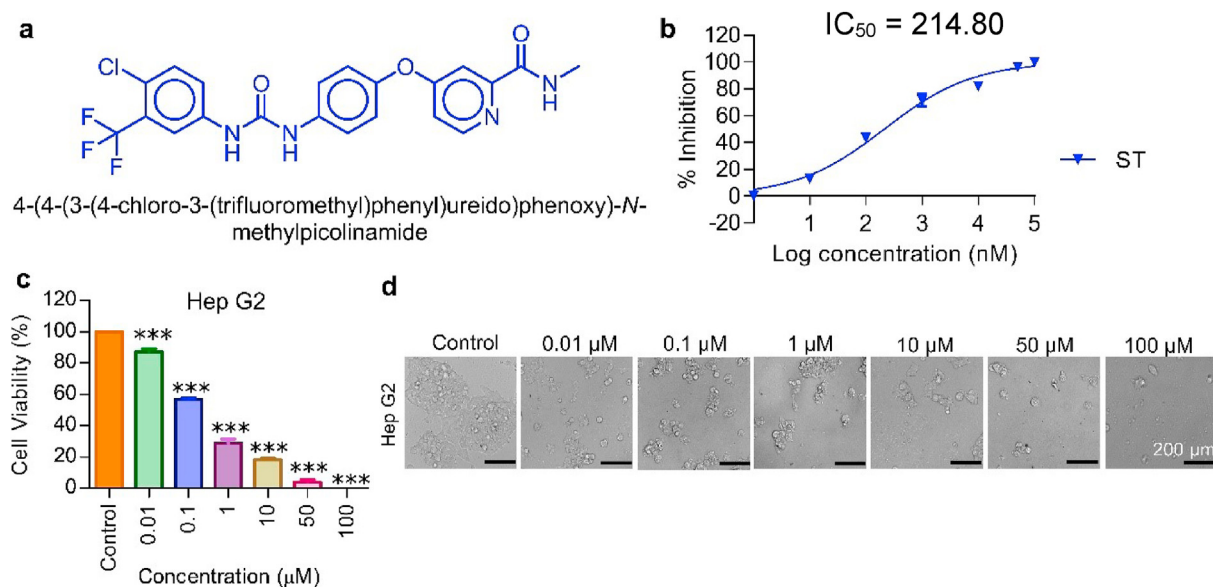


Fig. 2. *In vitro* antiproliferative activity determined using the MTT assay. (a) Chemical structure of sorafenib. (b) Graph of log concentration vs. percentage inhibition against Hep G2 cell line after treatment with various concentrations of sorafenib tosylate (ST) (0.01 µM–100 µM). Determination of IC₅₀ value; (c) graph of concentration (µM) vs. cell viability (%) in Hep G2 cell line after treatment with various concentrations of ST (0.01 µM–100 µM), where a dose-dependent reduction in cell viability was observed. (d) Microscopic images (10×) of Hep G2 cells after treatment with ST. Experiments were performed in triplicate ($n = 3$) and results are expressed as the mean \pm standard error of the mean. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ indicate statistically significant differences.

3. Results

3.1. *In vitro* antiproliferative activity assay

The MTT assay is a quantitative, sensitive, and reliable colorimetric method for measuring the viability, proliferation, and activation of cells. Moreover, receptor tyrosine kinases (Raf and VEGFRs) have essential roles in the proliferation of cancer cells. In the present study, the antiproliferative activity was determined to evaluate the IC₅₀ value for ST in Hep G2 cells. In the Hep G2 cell line, ST achieved dose-dependent inhibition with a highly potent IC₅₀ value of 214.80 nM.

3.2. Sorafenib reversed TGF- β -induced EMT in HCC

The effect of ST on EMT was analyzed using the Hep G2 cell line. After treatment with TGF- β , morphological changes indicative of EMT were observed in Hep G2 cells (Fig. 3a). TGF- β -stimulated Hep G2 cells underwent EMT and developed a fibroblast-like mesenchymal appearance, but treatment with ST for 48 h reversed these changes and a more epithelial cell-like morphology was observed. The reversal of EMT was confirmed by the effects of ST treatment on the expression patterns of the epithelial biomarker E-cadherin and the mesenchymal biomarker vimentin by using flow cytometry. The TGF- β -induced Hep G2 cells exhibited reduced expression levels of E-cadherin (down to ~2.92%), whereas ST significantly increased the expression of E-cadherin in TGF- β -induced cells to ~97.84% ($P < 0.001$) compared with that in TGF- β -induced cells alone (Fig. 3d). Furthermore, we analyzed the expression of the mesenchymal marker vimentin. The expression level of vimentin increased remarkably (~98.19%; $P < 0.001$) after treatment with TGF- β compared with the control Hep G2 cells. Treatment with ST decreased the expression level of vimentin (~0.14%) in TGF- β -induced cells ($P \leq 0.001$; Fig. 4c). The expression of the EMT-TF SNAIL was also examined to confirm the reversal of EMT. ST significantly decreased the expression of SNAIL (~0.20%) compared with that in TGF- β -induced cells alone (~97.78%; $P < 0.001$; Fig. 5c).

4. Discussion

Vascular endothelial growth factor-mediated VEGFR-2/KDR transmembrane receptor tyrosine kinases are highly overexpressed in advanced HCC, renal carcinoma, and thyroid cancer (Modi and Kulkarni, 2019). The current anticancer drugs are ineffective in clinics due to aberrant metastasis and resistance (Voulgari and Pintzas, 2009). Metastasis induced by EMT is a crucial factor that determines mortality, tumor recurrence, and treatment failure in patients with advanced HCC. EMT is a complex physiological process associated with tissue regeneration, embryonic development, and wound healing (Kim et al., 2018). In the EMT process, the epithelial cell phenotype converts into the mesenchymal cell phenotype. The plasticity of epithelial cells is an essential component of hepatocarcinogenesis. A recent report suggested that EMT is associated with the sensitivity of anticancer drugs; for example, resistance was observed in pancreatic cancer after treatment with gemcitabine due to the presence of mesenchymal type cancer cells, and this mechanism may have been attributable to the induction of the NOTCH signaling pathway, which is associated with a mesenchymal cell phenotype (Arumugam et al., 2009). Thus, inhibiting EMT may suppress metastasis as well as having beneficial effects on the sensitivity of anticancer agents. The cytokine transforming TGF- β , which is an inducer and promoter element in EMT that regulates various transcription factors, leads to the suppression of epithelial characteristics and induction of mesenchymal features in HCC. In the present study, we found that ST can potentially reverse TGF- β -stimulated EMT by suppressing E-cadherin and inducing vimentin and SNAIL. Recent studies also suggested that ST inhibits the migration and invasion of liver cancer cells (Ha et al., 2015). Furthermore, after treatment with TGF- β , epithelial cells lost their baso-apical polarity, gained plasticity, became invasive, acquired a self-renewal capacity, and developed mesenchymal characteristics, but ST significantly reversed these mesenchymal characteristics to yield a more epithelial cell-like morphology.

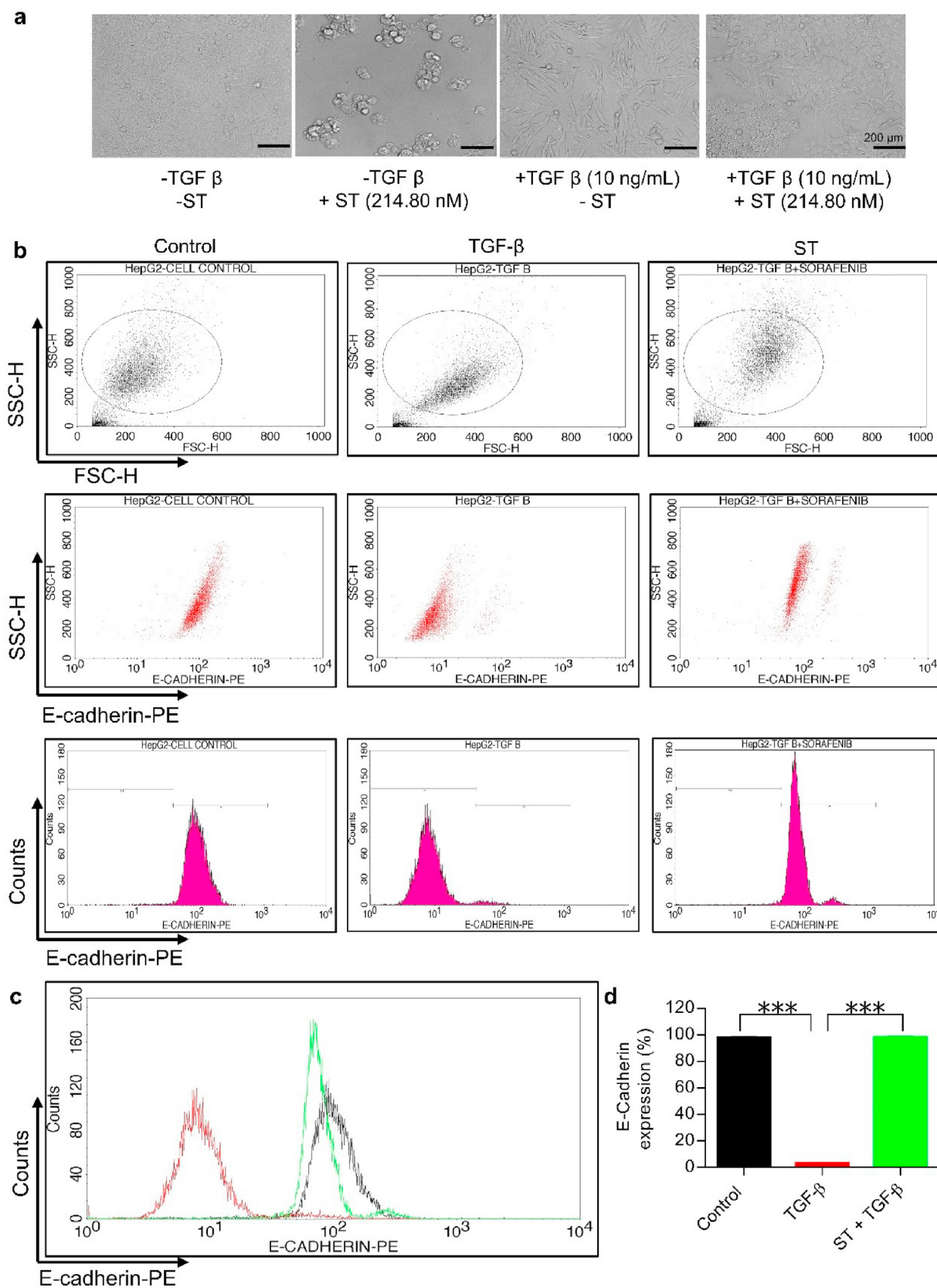


Fig. 3. (a) Inverted microscopy images showing the effects of sorafenib tosylate (ST). Induction of epithelial–mesenchymal transition by TGF- β (10 ng/mL) characterized by fibroblast-like morphology, which was reversed to yield a more epithelial-like appearance. (b) Graph of E-cadherin vs. cell count after treatment with TGF- β and ST. (c) Graphical representation of E-cadherin expression against control, TGF- β , and ST determined using flow cytometry. Decreased expression of E-cadherin was observed after induction of TGF- β . The decreased expression of E-cadherin was reversed after treatment with ST. Experiments were performed in triplicate ($n = 3$) and results are expressed as the mean \pm standard error of the mean. *** $P \leq 0.001$ indicates statistically significant differences.

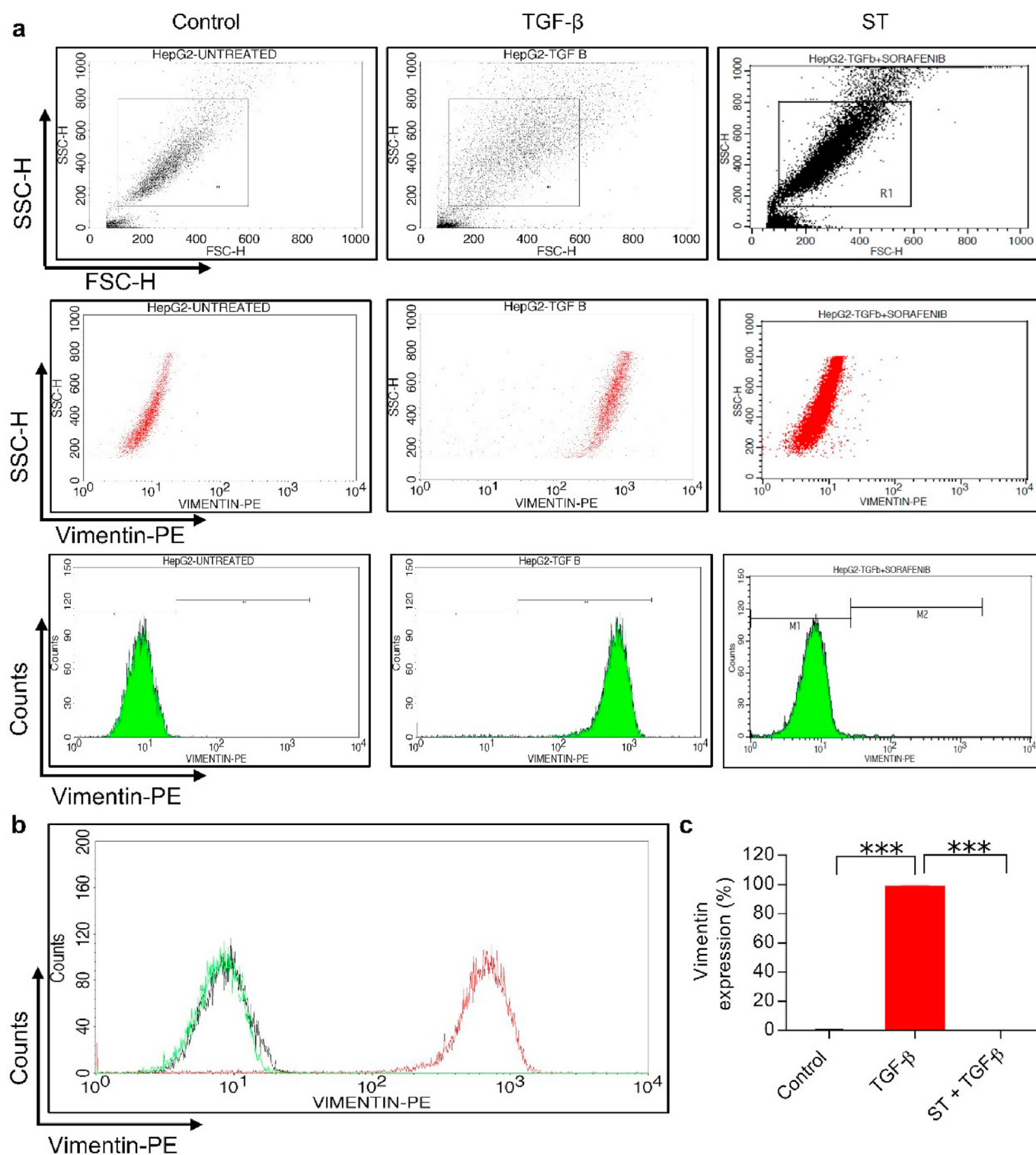


Fig. 4. Vimentin expression. (a) Graph of vimentin vs. cell count after treatment with TGF- β and sorafenib tosylate (ST). (b) Graphical representation of vimentin expression against control, TGF- β , and ST determined using flow cytometry. Expression of vimentin increased after induction of TGF- β . The increased expression of vimentin was reversed after treatment with ST. Experiments were performed in triplicate ($n = 3$) and results are expressed as the mean \pm standard error of the mean. *** $P < 0.001$ indicates statistically significant differences.

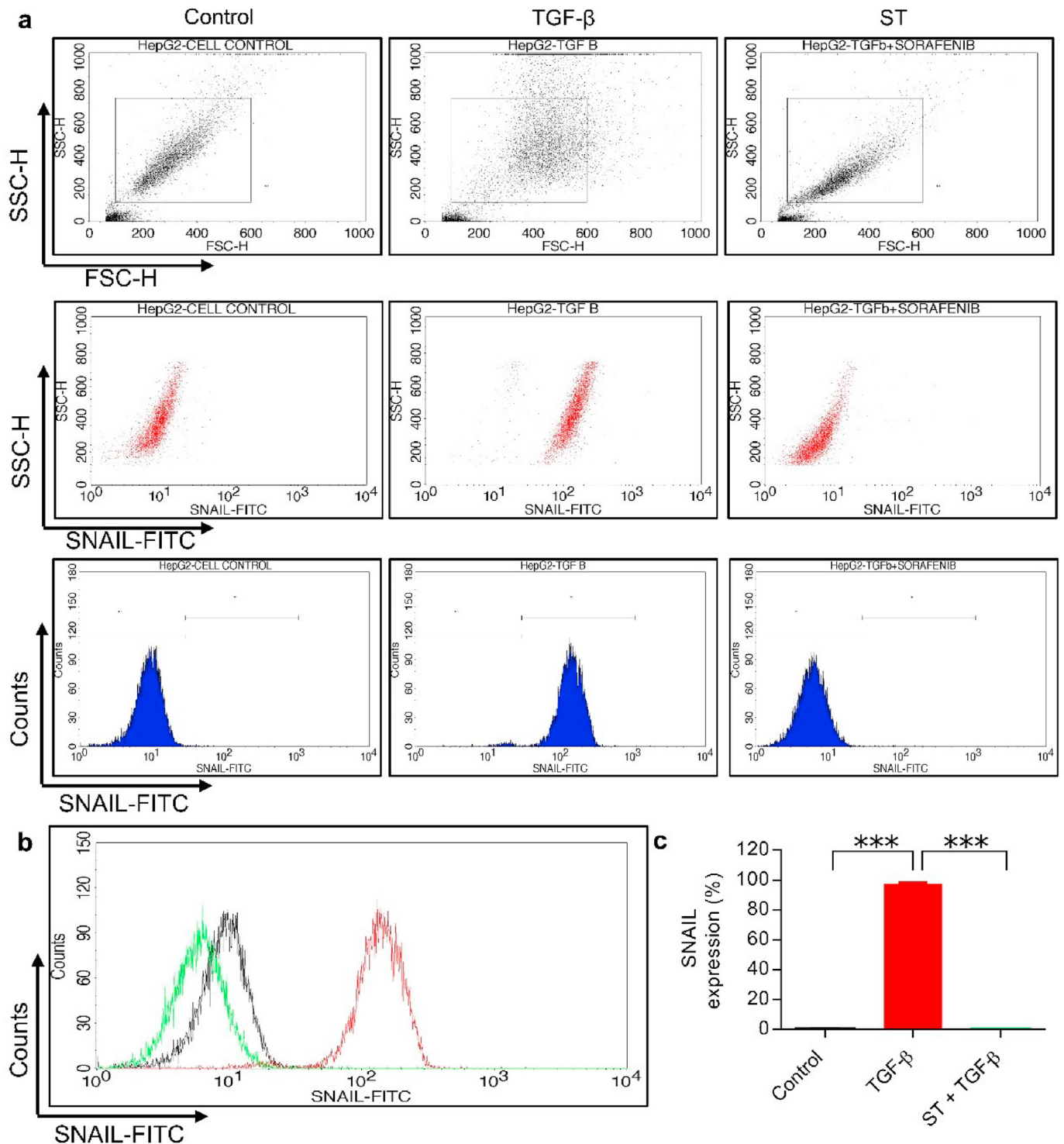


Fig. 5. SNAIL expression. (a) Graph of SNAIL vs. cell count after treatment with TGF- β and sorafenib tosylate (ST). (b) Graphical representation of SNAIL expression against control, TGF- β , and ST determined using flow cytometry. Expression of SNAIL increased after induction of TGF- β . Increased expression of SNAIL reversed after treatment with ST. Experiments were performed in triplicate ($n = 3$) and results are expressed as the mean \pm standard error of the mean. *** $P \leq 0.001$ indicates statistically significant differences.

5. Conclusion

In the present study, we investigated the effects of sorafenib on TGF- β -induced EMT in HCC using flow cytometry. Our results suggest that ST can potentially upregulate the epithelial biomarker E-cadherin and downregulate the mesenchymal biomarker vimentin. Furthermore, ST was effective against the EMT-inducing transcription factor SNAIL. These findings indicate that ST may be able to convert EMT into the mesenchymal-epithelial transition. Finally, our results obtained using ST suggest that VEGFR-2 inhibitors may be effective against malignant cells with the mesenchymal phenotype, and thus they could have greater potential in the treatment of metastatic cancer.

Author contributions

The authors Siddharth J. Modi, Anshuly Tiwari, and Vithal M. Kulkarni have approved the final version of the manuscript and they contributed equally.

Conflict of interest

The authors declare no conflict of interest pertaining to this manuscript.

Funding source

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CRedit authorship contribution statement

Siddharth J. Modi: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Project administration. **Anshuly Tiwari:** Methodology, Software, Formal analysis, Investigation, Writing - original draft. **Vithal M. Kulkarni:** Conceptualization, Validation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

ST	sorafenib tosylate
HCC	hepatocellular carcinoma
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
TGF- β	transforming growth factor-beta
EMT	epithelial-mesenchymal transition
EMT-TF	EMT-inducing transcription factor
KDR	kinase insert domain receptor

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