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Increased expression of GEF-H1 promotes colon cancer progression by RhoA signaling

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ABSTRACT

Colorectal cancer (CRC) is the third most common malignancy and a leading cause of cancer-related death worldwide. GEF-H1 is considered a RhoA-specific guanine nucleotide exchange factor. GEF-H1 upregulation may contribute to cancer cell migration and invasion and tumor progression. However, the expression and role of GEF-H1 in CRC have not yet been elucidated. This study attempted to elucidate how GEF-H1 drives tumor formation, motility, invasion and metastasis in colon cancer (CC). The expression of GEF-H1 in CC tissue microarrays (TMAs) was analyzed by immunohistochemistry (IHC). GEF-H1 was upregulated in CC tissues compared with adjacent non-tumoral tissues. In addition, we found that high GEF-H1 expression correlated with shorter overall survival and distant metastasis. Migration and invasion assays showed that GEF-H1 upregulation increased CC cell motility, invasion and metastasis. In contrast, functional knockdown of GEF-H1 by RNAi rescued the effects caused by GEF-H1 overexpression in CC cells. Overexpression of GEF-H1 re-organized the actin cytoskeleton, with increased punctate paxillin staining and F-actin stress fibers. Furthermore, western blotting showed that RhoA activation triggered by GEF-H1 overexpression caused phosphorylation of its downstream target, MLC2, in CC cells. In summary, the present study revealed that GEF-H1 is upregulated in CC tissues and plays a key role in CC metastasis through the GEF-H1-RhoA-MLC2 signaling pathway.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors [1] and ranks as the second leading cause of cancer-related death in Western countries [2] and the third leading cause of cancer-related death in China [3]. CRC is now a significant global health problem. Carcinogenesis is a multistep process that requires the accumulation of various genetic and epigenetic aberrations, which in turn drive the progressive malignant transformation of normal human cells. Although recent molecular studies have elucidated some of the mechanisms underlying the development and progression of CRC, the etiology of this malignancy remains unknown.

GEF-H1 (and its murine homologue lfc) is a guanine nucleotide exchange factor that colocalizes with microtubules through its carboxy-terminal coiled-coil domain in nonpolarized cells [4]. GEF-H1 is inactive when bound to microtubules and becomes activated after being released from microtubules upon microtubule depolymerization, either as a result of inherent instability or after treatment with microtubule-depolymerizing drugs [5]. Activated GEF-H1 promotes the exchange of GDP for GTP and the binding of GTP to RhoA (a small GTP-binding protein). Activated RhoA functions as a molecular switch that regulates various signal transduction pathways governing processes such as cytoskeleton rearrangement, cell proliferation, cell invasion and tight junctions, which contribute to cancer progression [6]. A truncated form of GEF-H1 lacking microtubule-binding ability was discovered in the U937 monocytic leukemia cell line and found to induce tumor formation in nude mice [7]. NIH-3T3 cells expressing truncated GEF-H1 were also reported to induce xenograft tumor formation in nude mice within nine weeks [8]. GEF-H1 overexpression in hepatocellular carcinoma promotes cell motility via activation of RhoA signaling [9]. However, the expression and function of GEF-H1 in CRC progression remain unknown.

The aims of this research were to explore GEF-H1 expression in colon cancer (CC) and the effects of GEF-H1 on CC motility and invasion and to elucidate the potential underlying molecular mechanisms. In this research, we found that GEF-H1 is upregulated in CC tissues

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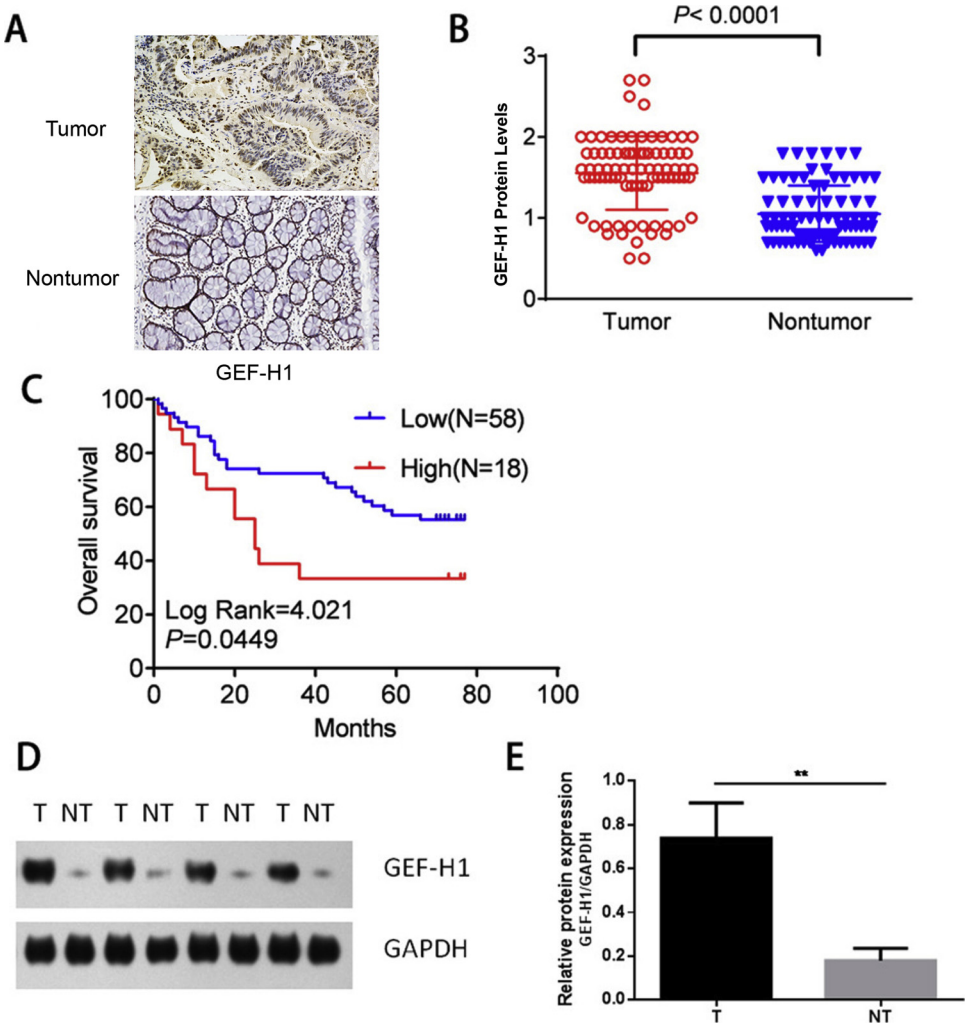

Fig. 1. GEF-H1 protein expression in CC. A, B: GEF-H1 expression level was detected in 78 pairs of colon tumor and non-tumoral adjacent colorectal tissues. Immunochemical staining showed that GEF-H1 expression was significantly higher in CC than in adjacent non-tumoral colorectal tissues (×200, $P < 0.0001$). C: Kaplan–Meier plots of GEF-H1 expression in 78 cases of CC. Log-rank test was performed to evaluate overall survival based on low or high GEF-H1 expression ($P = 0.0448$). D, E: GEF-H1 protein was detected in 20 pairs with colorectal tumor and adjacent non-tumoral colorectal tissues. However, GEF-H1 protein level was higher in 20 colorectal tumor specimens (NT: non-tumoral adjacent colorectal, T: colorectal tumor). Colorectal tumor samples exhibited higher GEF-H1 expression (by more than two-fold) than adjacent non-tumoral samples (n = 20). *$P < 0.05$; **$P < 0.001$.

Table 1
Clinicopathological information for the 78 CC cases studied. High expression of GEF-H1 in CC was associated with distant metastasis ($P = 0.011$), while no significant associations were found between GEF-H1 protein expression level and patient age, gender, tumor size, grade or stage.

Characteristics	No.s of patients	GEF-H1 expression		P value
		Low (%)	High (%)	
Age				
≤55	11	9 (81.8)	2 (11.2)	1.000
>55	67	50 (74.6)	17 (25.4)	
Gender				
M	45	31 (68.9)	14 (31.1)	0.105
F	33	28 (84.8)	5 (15.2)	
Tumor, d/cm				
≤5	48	36 (75.0)	12 (25.0)	0.868
>5	30	23 (76.7)	7 (23.3)	
Distant metastasis				
Yes	10	4 (40.0)	6 (60.0)	0.011*
No	68	55 (80.9)	13 (19.1)	
AJCC T stage				
1	4	3 (75.0)	1 (25.0)	0.911
2	9	6 (66.7)	3 (33.3)	
3	44	34 (77.3)	10 (22.7)	
4	21	16 (76.2)	5 (23.8)	
Grade				
Low	5	3 (60.0)	2 (40.0)	
Moderate	51	37 (72.5)	14 (27.5)	0.242
High	22	19 (86.4)	3 (13.6)	

compared with adjacent non-tumoral tissues. In addition, clinical data showed that GEF-H1 overexpression predicts more aggressive tumor phenotypes and shorter recurrence-free survival of patients. We also revealed that GEF-H1 upregulation can promote CC metastasis via the activation of RhoA signaling. Our findings may suggest new strategies for the therapeutic treatment of CC.

2. Methods

2.1. Cell culture and reagents

HCT-116 and HT-29 cells (The Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Biological Industries, Cromwell, CT, USA), penicillin (100 U/ml, Thermo Fisher Scientific) and streptomycin (100 U/ml, Thermo Fisher Scientific). All cells were cultivated at 37 °C with 5% CO_2.

2.2. Reagents and antibodies

The following commercial antibodies were used: GEF-H1 (ab90783, ABCAM, USA), β-actin (A5441, Sigma, USA), F-actin (ab205, ABCAM, USA), paxillin (12065, rabbit, CST, USA), MLC (M4401, Sigma), and pMLC (sc-12896, Santa Cruz Biotechnology, USA). A RhoA Activation Assay Kit was purchased from NewEast Biosciences (26904, USA).

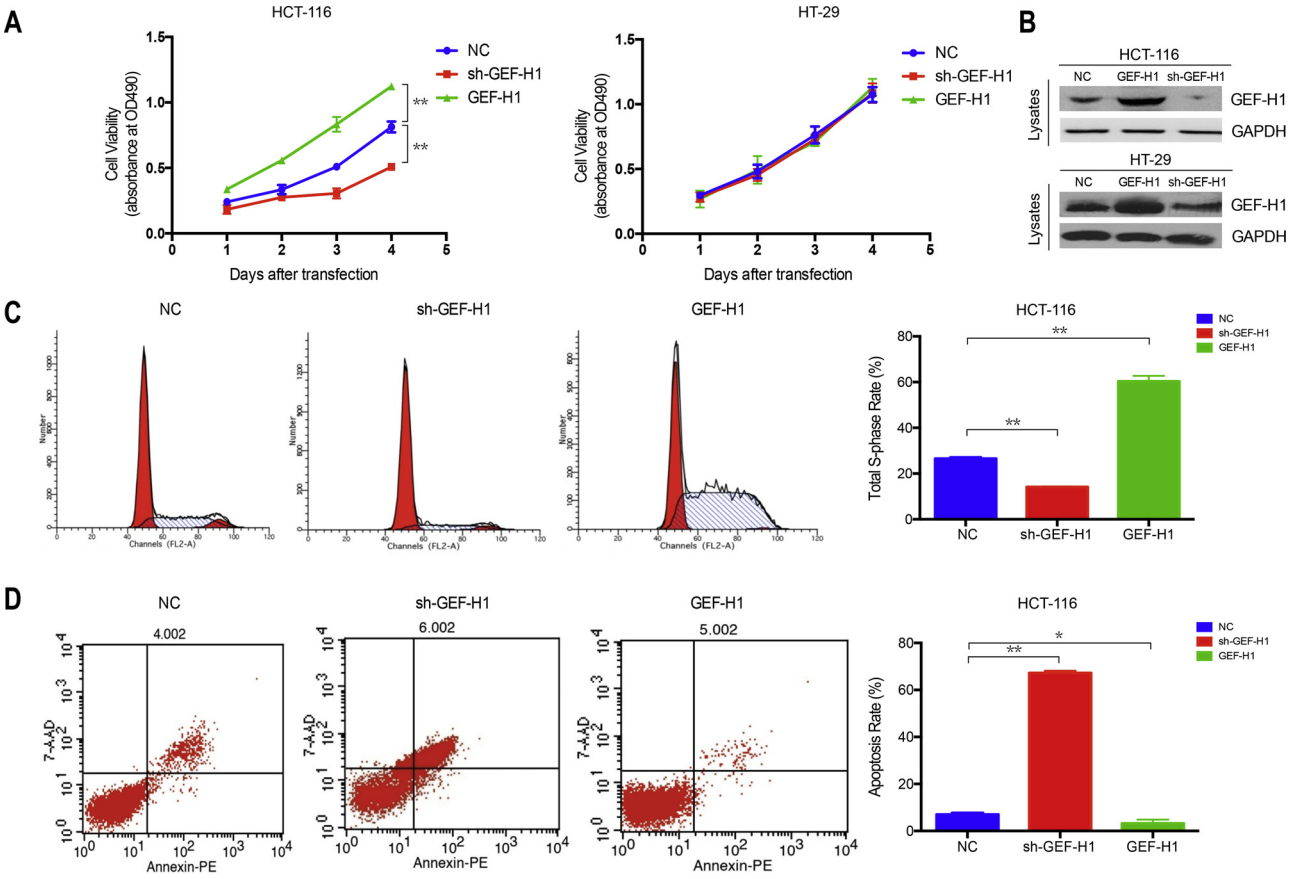

Fig. 2. Effects of GEF-H1 on colon cancer cell proliferation, apoptosis and cell cycle. A: The proliferative ability of HCT-116 cells that transfected with exogenous GEF-H1 or sh-GEF-H1 was detected by MTT (left). The proliferative ability of HT-29 cells that transfected with exogenous GEF-H1 or sh-GEF-H1 was detected by MTT (left). B: Exogenous GEF-H1 or sh-GEF-H1 was transfected to colon cancer cells, and then protein samples were collected and GEF-H1 was detected via western blotting. C: Cell cycle was assayed by flow cytometry in every group of cells. Apoptosis rate was assayed by flow cytometry in every group of cells. $*P < 0.05$; $**P < 0.001$. Experiments were performed in triplicate.

2.3. Tissue microarrays and immunohistochemistry

Tissue microarrays (TMAs) and immunohistochemistry (IHC) reagents from Shanghai Outdo Biotech Co., LTD., were used to analyze GEF-H1 expression. For the evaluation of GEF-H1 staining, a semi-quantitative scoring system that notes signal strength was used: 0 (no signal), 1 (weak, light yellow), 2 (moderate, yellow-brown), and 3 (strong, brown). Percentage scores were assigned as 1, < 10%; 2, 11–35%; 3, 36–70%; or 4, > 70% [10]. The staining index of each sample was derived by multiplying the signal score by the percentage score to give a final score from 0 to 12. The average of all scores served as the optimal cutoff value that was used to classify expression as low (0–5) or high (6–12) and to analyze the related clinical characteristics. In addition, 20 pairs of fresh surgical CRC and non-tumoral adjacent colon tissues were collected in Nanjing Drum Tower Hospital. Written informed consent for CC resection and research was obtained from each patient before the resection procedure. The study protocol was approved by the Medical Ethics Committee of Nanjing Drum Tower Hospital.

2.4. shRNA and siRNA constructs

The LV5-GEF-H1 plasmid was prepared using the LV5 mammalian expression vector according to a previous study [11]. The following shRNA sequences targeting GEF-H1 were designed and termed LV3-GEF-H1-RNAi: GEF-H1 shRNA (shGEF-H1): 5′-GATCC-(GN18)-(TTCAA GAGA)-(N18C)-TTTTTTG-3′ and 3′-G(CN18)-(AAGTTCTCT)-(N18 G)-AAAAAACTTAA-5′. The shNC sequence is 5′-TTCTCCGAACGTGTCA CGT-3′.

For rescue experiments, specific siRNAs directed against human RhoA were designed through scanning to identify AA(n19)TT sequences. Candidate sequences were compared with RhoA cDNA sequences, and their specificity was verified in the non-redundant human DNA database using a BLAST algorithm (accession through NCBI). A control siRNA was also tested and selected because it exhibited no cellular toxicity. The following sequences were selected: anti-RhoA siRNA, sense 5′-GACAUGCUUGCUCAUAGUCTT-3′, antisense 3′-TTCU GUACGAACGAGUAUCAG-5′; control siRNA, sense 5′-CAGUCAGGAGG AUCCAAAGTG-3′, antisense 3′-TTGUCAGUCCUCCUAGGUUUC-5′. All of the small RNAs were synthesized by Western Biomedical Technology Co., LTD., China.

2.5. Lentivirus production

Lentivirus expressing LV5-GEF-H1, LV5, LV3 or LV3-GEF-H1-RNAi were produced in HEK-293 T cells using the corresponding LV5 or shRNA-expressing LV3 vector and the packaging plasmids PG-p1-VSVG, PG-P2-REV and PG-P3-RRE. Viruses (LV3, LV5-Lentiviral, GEF-H1 and siGEF-H1) were concentrated by using PEG-it Virus Precipitation Solution (System Biosciences, Mountain View, CA) and stored at −80 °C.

2.6. Cell proliferation assay

Cell proliferation was evaluated by MTT assay. Briefly, cells were seeded in triplicate at a density of 2×10^3 cells/well in 96-well culture

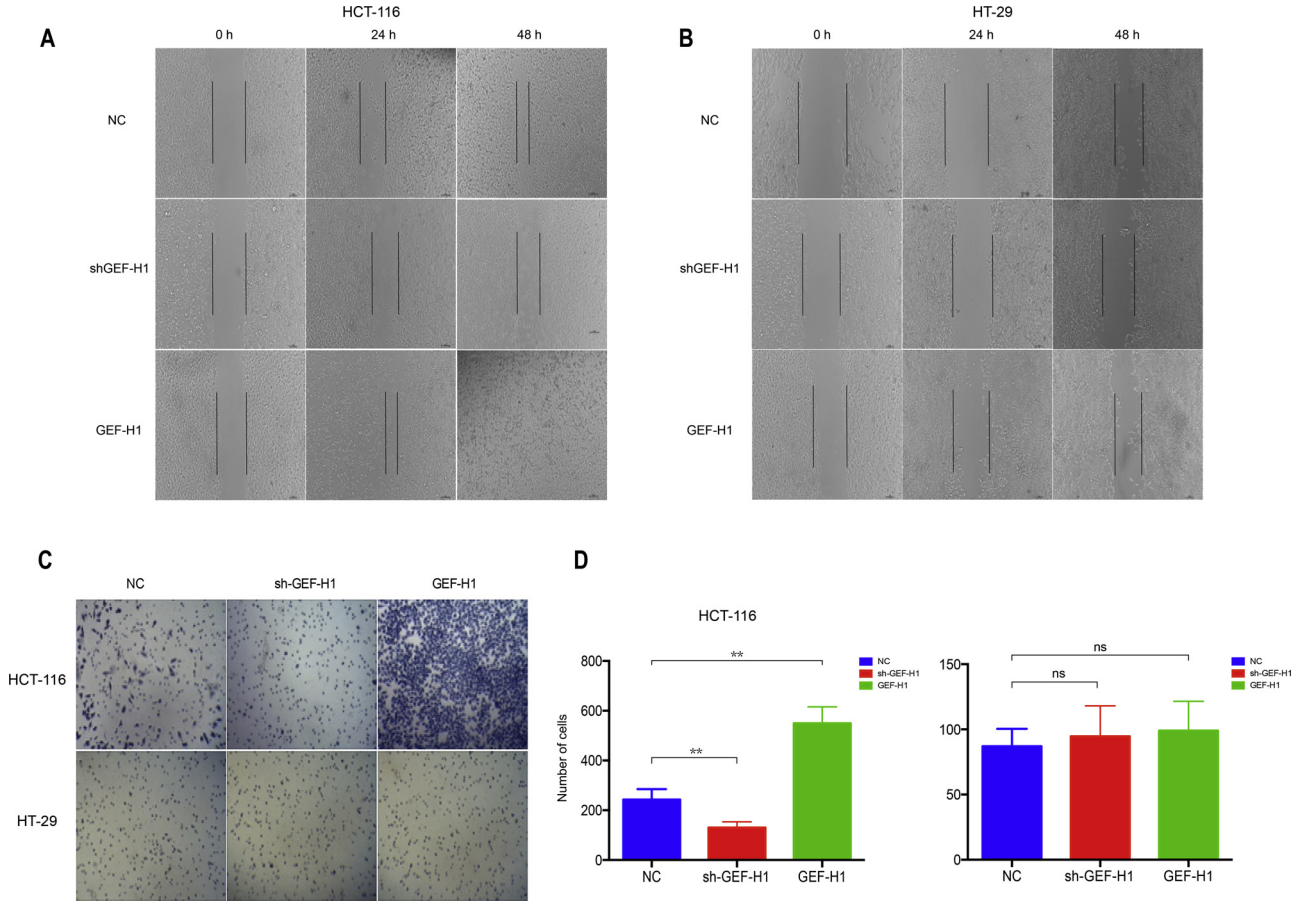

Fig. 3. GEF-H1 modulates HCT-116 cell migration and invasion. A: Migration: Wound healing assays were analyzed at 0 h, 24 h and 48 h in HCT-116. B: Migration: Wound healing assays were analyzed at 0 h, 24 h and 48 h in HT-29. C&D: Invasion: Transwell assays were analyzed at 96 h in HCT-116 and HT-29 (right). The number of cells were quantied (right).

plates and cultured for 72 h. DMSO was used as a vehicle control. Reduced formazan was dissolved in DMSO and quantified by measuring the absorbance at 450 nm using a microplate reader, and the OD values are reported as the mean ± SD.

2.7. Cell cycle and apoptosis

Cell cycle analysis was performed with a cell cycle detection kit (KeyGen Biotech, Nanjing, China) and flow cytometry. Cells were harvested, washed, re-suspended, incubated with propidium iodide in the dark for 15 min, and subjected to flow cytometry analysis using FloMax software (Partec, Munich, Germany). The various cell populations were assessed using quadrant statistics.

2.8. Wound healing assay

Cells were seeded in triplicate in 6-well culture plates. When the cells reached 90% confluence, a straight scratch of approximately 1 mm in width was created in the middle of the well using a pipette tip, and the cells were washed with phosphate-buffered saline (PBS). Wound closure was monitored, and images were taken at 0 and 24 h after wounding under an inverted microscope. Each experiment was repeated three times.

2.9. Invasion assay

Cellular invasive ability was evaluated by using Matrigel (BD Biosciences, San Jose, CA) invasive chambers. Cells (1×10^4) were suspended in 200 µl of culture media (DMEM/4% FBS) and placed in the upper Transwell chambers (Corning Lowell, MA); 500 µl of DMEM/10% FBS was added to each lower Transwell chamber. After incubation for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2, non-invading cells were removed from the upper side of the membrane using cotton swabs. Invading cells on the lower side of the membrane were fixed with 100% methanol, stained with crystal violet, and mounted on glass slides. All the invading cells were counted and imaged by light microscopy. More than 10 views were analyzed by light microscopy (×200 magnification), and all the invading cells were counted. The mean value of three independent experiments is expressed as a percentage.

2.10. Western blotting

Proteins were separated using SDS–PAGE, transferred to PVDF membranes, and blocked with 5% (w/v) nonfat milk (for most antibodies) or 3% (w/v) bovine serum albumin (BSA; for anti-Rac1 and anti-RhoA antibodies) in TBS with 0.5% (v/v) Tween®-20 before being probed with the appropriate antibodies. For protein detection, membranes were incubated overnight at 4 °C with specific antibodies diluted 1:1000 in TBS-T containing 5% nonfat milk. Primary antibodies bound to immunoreactive bands were visualized by ECL detection with secondary anti-rabbit or anti-mouse horseradish peroxidase antibodies (1:5000 dilution).

2.11. Immunofluorescence microscopy

Cells were cultured on poly-L-lysine-coated slides for 48 h, fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in

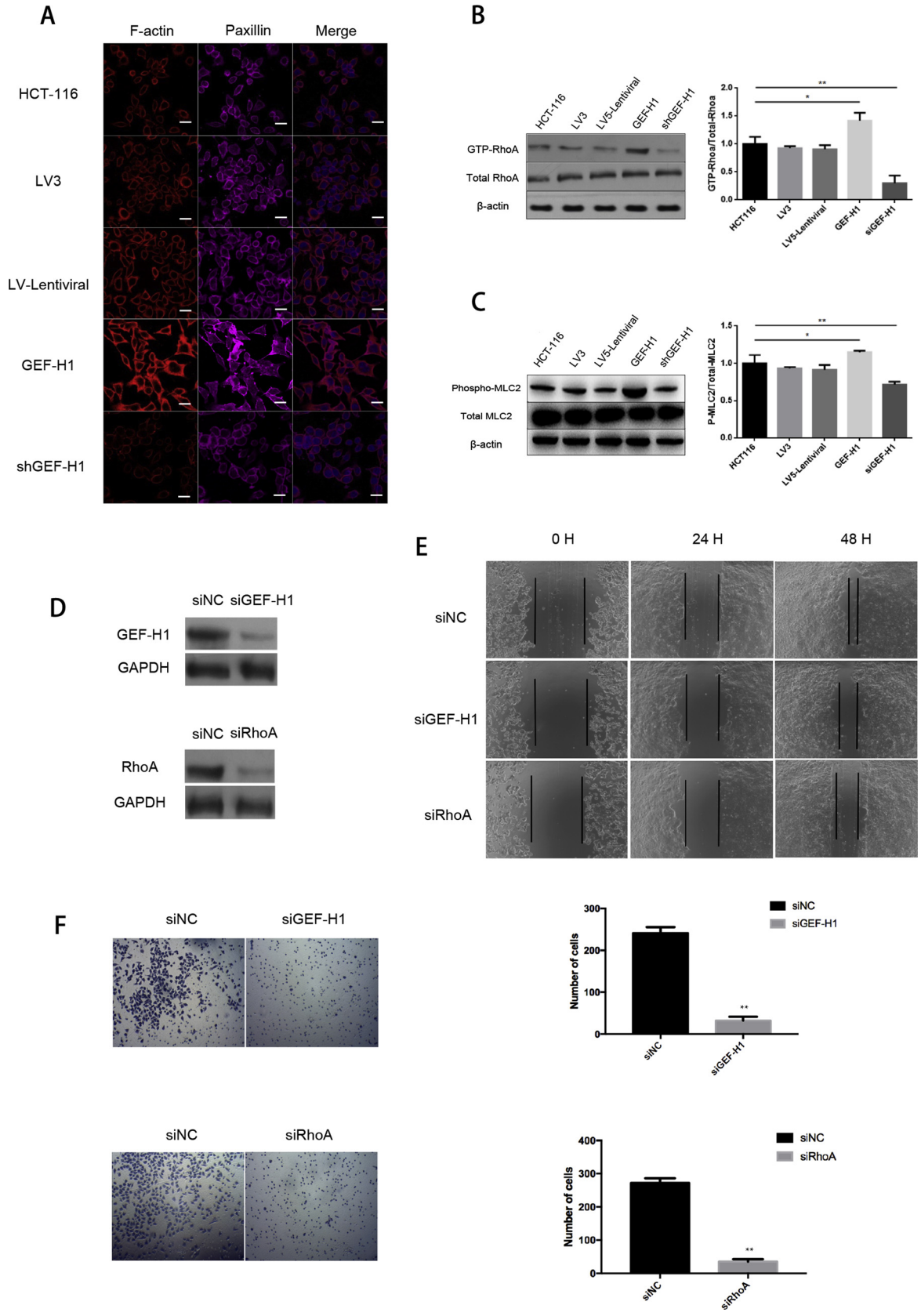

Fig. 4. GEF-H1 regulates actin cytoskeleton reorganization in HCT-116 cells.
A: Cells overexpressing GEF-H1 were immunostained with anti-paxillin antibody (purplish red) and phycoerythrin antibody (red) to observe focal adhesions and F-actin stress fibers, respectively. GEF-H1-overexpressing cells acquired an elongated, polarized shape with narrow, finger-like projections along the cell edge. Upregulation of GEF-H1 increased punctate paxillin staining and F-actin stress fibers compared with other treatments and controls. Merged images of paxillin and F-actin are shown. DAPI (blue) was used to visualize nuclei. B, C: Upregulation of RhoA and phosphorylation of its downstream effector, MLC2, were decreased in GEF-H1-depleted cells, and total MLC2 and RhoA levels did not obviously change. E, F: siRhoA-transfected cells exhibited a delay in wound closure and impaired invasion, similar to siGEF-H1-treated cells. Both siGEF-H1 and siRhoA similarly inhibited cell migration and invasion. $*P < 0.05$; $** P < 0.001$.

PBS. Fixed cells were blocked with 1% BSA in PBS and incubated with anti-paxillin antibody (1:200, Cell Signaling) at room temperature for 1 h. After being washed three times with PBS, cells were incubated with a secondary antibody conjugated to fluorescein isothiocyanate (FITC) and Alexa Fluor 555-conjugated phalloidin (Invitrogen) for 1 h at room temperature. Nuclei were visualized with 4-6-diamino-2-phenylindole (DAPI). Morphological alterations, actin filament formation and paxillin puncta in the cells were observed, and images were captured with confocal microscopy (Leica, Germany).

2.12. RhoA activity assays

RhoA small GTPase activity assays were performed using GLISA kits according to the manufacturer's instructions. We first determined the cell number to cell lysis buffer volume ratio that would reproducibly result in protein concentrations of 0.5 μg/μl. Cell lysates were then snap-frozen in liquid nitrogen to preserve GTP-bound GTPase (RhoA) activity. GTP-bound RhoA was bound by RhoA-GTP-binding protein conjugated to the wells of 96-well plates. Bound active RhoA was detected with a RhoA-specific antibody. Absorbance readings at 490 nm were indicative of Rac1 and RhoA activity.

2.13. Statistical analysis

Data are presented as the mean ± standard deviation. Differences in GEF-H1 expression between CC and corresponding uninvolved mucosal tissues were compared by the paired Student's *t*-test or Wilcoxon matched-pairs signed-rank test where appropriate. Significance values were obtained by paired Student's *t*-test (*$P < 0.05$; **$P < 0.01$) when compared to untreated controls. The associations between GEF-H1 expression and clinicopathological characteristics were analyzed using the Chi-square test. Kaplan–Meier survival curves were constructed according to GEF-H1 expression using the log-rank test. All figures are representative of at least three independent experiments. *P* values of < 0.05 indicated statistical significance.

3. Results

3.1. GEF-H1 is upregulated in colon cancer

The TMA included 180 tissue cores from 90 patients with CC who underwent surgical resection in April to November 2008 and were followed up until August 2014. Patients without an adequate tissue sample, lacking clinicopathological information, or lost to follow-up were excluded from the study. GEF-H1 staining was significantly different in CC tissue compared to adjacent non-tumoral colon tissue. The GEF-H1 expression level was detected in 78 CC and 78 non-tumoral adjacent colon tissues (Fig. 1A). GEF-H1 expression levels were higher in CC tissue than in adjacent non-tumoral colon tissue ($P < 0.001$, Fig. 1B). High GEF-H1 expression correlated with shorter overall survival ($P = 0.0448$) by Kaplan–Meier analysis (Fig. 1C). According to AJCC staging [12], 4 cases were graded as stage T1, 9 as stage T2, 44 as stage T3, and 21 as stage T4. No significant associations were found between GEF-H1 protein expression level and patient age, gender, tumor size, grade or stage. However, high GEF-H1 expression in CC was associated with distant metastasis ($P = 0.011$, Table 1). We also explored GEF-H1 protein expression in fresh surgical tissues from another 20 patients with CC by immunoblotting. GEF-H1 protein levels were also significantly elevated in CC tissues compared to adjacent normal tissues ($P < 0.05$, Fig. 1D, E).

3.2. Effect of GEF-H1 on colon cancer proliferation, apoptosis and cell cycle

To elucidate the cellular functions of GEF-H1 in CC cells, we used two colon cancer cell lines (HCT-116 and HT-29) to proceed our research. As is known to us, HCT-116 is a cell line with KRAS mutation (G13D) and HT-29 is a KRAS WT cell line [13]. Lentivirus-mediated GEF-H1 upregulation and shRNA-mediated GEF-H1 knockdown were carried out, and cell viability, apoptosis and cell cycle were analyzed. The proliferation of GEF-H1-overexpressing cells was significantly increased ($P < 0.05$) compared with the control group in HCT-116. In addition, functional knockdown of GEF-H1 caused a marked reduction in cell proliferation ($P < 0.05$, Fig. 2A) in HCT-116. But there was no significant difference among different groups in HT-29 cells. To further evaluate the cellular functions of GEF-H1 in CRC, flow cytometry was performed to assess apoptosis and cell cycle. Knockdown of GEF-H1 markedly increased apoptosis (Fig. 2D). In terms of cell cycle, a smaller proportion of cells were in the S phase of the cell cycle when GEF-H1 was silenced by shRNA ($P < 0.01$), but GEF-H1 upregulation may reverse this effect (Fig. 2B). The results suggested that GEF-H1 upregulation may increase proliferation.

3.3. GEF-H1 enhances HCT-116 cell migration and invasion

To examine whether GEF-H1 affects cell migration and invasion, we performed wound healing and Matrigel transwell invasion assays. We found that GEF-H1 overexpressed HCT-116 cells showed significantly enhanced migration ability (Fig. 3A). After 48 h of incubation, cells with GEF-H1 upregulation exhibited faster wound closure than control cells, which showed a delay in wound closure, with a clear gap remaining at the end of the experiment. The invasion assay results also indicated that GEF-H1-overexpressing cells were more invasive than control cells in Matrigel-coated transwells ($P < 0.05$) (Fig. 3C and D). However, as we can see, cell migration and invasion of HT-29 only marginally changed after the transfection of sh-GEF-H1 and GEF-H (Fig. 3B–D). The above results suggested that GEF-H1 overexpression could efficiently enhance the migratory and invasive capability of CC cells, while shGEF-H1 cells showed a significant impairment of wound healing and invasive ability, indicating that shRNA-mediated depletion of GEF-H1 markedly reduced cell migratory and invasive ability.

3.4. GEF-H1 regulates cell morphology and actin cytoskeleton rearrangement

GEF-H1 is a RhoA-specific guanine nucleotide exchange factor that links microtubule dynamics and RhoA GTPase regulation of the actin cytoskeleton [14]. RhoA acts through the downstream effector Rho-associated kinase to regulate cell retraction through the assembly of actin stress fibers and focal adhesions, two key cytoskeletal events in migrating cells [15,16]. To ascertain whether GEF-H1 regulates changes in the actin cytoskeleton to enhance CC cell motility, we examined the assembly of contractile stress fibers and associated focal adhesion complexes by F-actin and paxillin staining, respectively. Paxillin is a focal adhesion-associated adaptor protein that plays an important role in controlling cell spreading and migration [17–19]. Our data showed that cells with high GEF-H1 levels acquired an elongated, polarized shape with narrow, finger-like projections along the cell edge (Fig. 4A). In addition, these cells adopted a spreading morphology with an increase in punctate paxillin staining and stress fibers compared with control cells (Fig. 4A). In addition, cells with upregulated GEF-H1 were polarized and formed numerous projections along the cell edge, while cells with low GEF-H1 expression due to GEF-H1 knockdown had fewer focal adhesions and were less polarized. In determining whether GEF-H1 regulates cell morphology and actin cytoskeleton rearrangement by the RhoA/MLC2 signaling pathway, we demonstrated that overexpression of GEF-H1 may upregulate RhoA and the phosphorylation of its downstream effector, MLC2, which were decreased in GEF-H1-depleted cells (Fig. 4B and C). In addition, total MLC2 and RhoA levels showed no apparent change under all tested conditions.

To elucidate whether GEF-H1 modulates cell migration and invasion by RhoA activity, we knocked down RhoA in GEF-H1 cells and compared the results to those obtained with siGEF-H1 (Fig. 4D–F). Wound

healing and invasion assays were analyzed at 0, 24 and 48 h (Fig. 4E). Cells transfected with siRhoA and siGEF-H1 exhibited a delay in wound closure and impaired invasion, similar to shGEF-H1-treated cells (Fig. 4F). Both siGEF-H1 and siRhoA similarly inhibited cell migration and invasion (Fig. 4E and F). This strongly indicated that GEF-H1 could accelerate the migration and invasion of CC cells through the activation of RhoA.

4. Discussion

GEF-H1 is a microtubule-associated guanine nucleotide exchange factor that activates RhoA upon release from microtubules. During cell migration, GEF-H1 release and microtubule depolymerization are followed by increased actin stress fiber formation and myosin II-dependent contraction mediated by RhoA activation. Then, activated RhoA influences cell cycle progression by controlling the transition from G1 phase to S phase [8]. GEF-H1 activity is also associated with tumorigenesis. A cell line transformed by GEF-H1 transfection can induce tumor development after injection into nude mice [6,8]. In the present study, we detected GEF-H1 expression in all CC and non-tumoral adjacent colon tissue samples with cytomembrane localization, and found that GEF-H1 was overexpressed in CC. In addition, high GEF-H1 expression was closely correlated with shorter overall survival. CC cells with KRAS mutation transfected with exogenous GEF-H1 have better viability, a lower apoptosis rate and faster cell cycle progression. The above effects were reversed by transfection of shRNA against GEF-H1 into these cells. But we found that in HT-29, a KRAS WT cell line, the proliferation of the cells after transfection only marginally changed.These results suggested that GEF-H1 plays a role in colon carcinogenesis. However, functional knockdown of GEF-H1 by shRNA reduced HCT-116 cell proliferation in this study, while in Hep3B cells, GEF-H1 suppression did not have an apparent effect on cell viability or cell proliferation [9]. This difference is perhaps due to the different type of tumor cells. Moreover, GEF-H1 expression is not associated with CC size, while GEF-H1 promoted CC cell proliferation in vitro. This difference probably results from the use of distinct assay systems.

Recent studies indicated that GEF-H1 overexpression may promote the migration, invasion and metastasis of hepatocellular cells [9] and breast cancer cells [15,20] and the brain metastatic behavior of melanoma cells [21]. Our study also found that GEF-H1 could endow CC cells with motile and invasive properties. HCT-116 cells expressing exogenous GEF-H1 exhibited faster wound closure and greater invasive capacity than control cells. However, shRNA-mediated depletion of GEF-H1 markedly reduced cell migration and invasion. However, we did not observe any significant changes among HT-29 cells after transfection. We performed a RhoA rescue assay to prove whether GEF-H1 mediates cell migration and invasion via RhoA activity. The results indicated that siRhoA- and shGEF-H1-transfected cells exhibited a delay in wound closure and impaired invasion, similar to siGEF-H1-treated cells. Both shGEF-H1 and siRhoA similarly inhibited cell migration and invasion. The RhoA rescue study suggested that GEF-H1 modulates cell migration and invasion by RhoA activity.

RhoA activation by GEF-H1 and microtubule depolymerization regulates actin cytoskeleton remodeling and focal adhesions during cell migration [14]. To validate whether GEF-H1 regulates the actin cytoskeleton in CC cells, we observed the alterations in stress fibers and focal adhesions by staining for F-actin and paxillin, a focal adhesion-associated protein required for regulating cell spreading and motility. HCT-116 cells transfected with lentivirus expressing GEF-H1 exhibited a spreading morphology and an increase in punctate paxillin staining and stress fibers compared with control HCT-116 cells. Moreover, GEF-H1-overexpressing HCT-116 cells had a polarized shape and formed numerous projections along the edge. ShGEF-H1-treated HCT-116 cells had reduced assembly of stress fibers and focal adhesions and were less polarized. RhoA activation leads to phosphorylation of its downstream effector, MLC2 [14], which is required for the assembly of actomyosin complexes. In this study, lentivirus-mediated GEF-H1 upregulation increased MLC phosphorylation by RhoA, while shGEF-H1 treated and control HCT-116 cells had low MLC phosphorylation levels. To further validate our findings, we knocked down RhoA in GEF-H1 cells and compared the results to those obtained with siGEF-H1and found that both siGEF-H1 and siRhoA similarly inhibited cell migration and invasion. This significantly suggested that GEF-H1 could accelerate the migration and invasion of CC cells through the activation of RhoA.

In conclusion, we illustrated how GEF-H1 regulates KRAS mutated CRC cell motility, invasion and metastasis and how microtubule-released GEF-H1 activates RhoA, which in turn activates its downstream effectors in CC cells. CC pathogenesis, invasion and metastasis are triggered by GEF-H1/RhoA/MLC2 signaling. Moreover, GEF-H1 expression level is closely correlated with overall survival in patients with CRC.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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