

Vasorin/ATIA Promotes Cigarette Smoke–Induced Transformation of Human Bronchial Epithelial Cells by Suppressing Autophagy-Mediated Apoptosis



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Abstract

BACKGROUND: Escaping cell death pathways is an important event during carcinogenesis. We previously identified anti-TNF α -induced apoptosis (ATIA, also known as vasorin) as an antiapoptotic factor that suppresses reactive oxygen species (ROS) production. However, the role of vasorin in lung carcinogenesis has not been investigated. **METHODS:** Vasorin expression was examined in human lung cancer tissues with immunohistochemistry and database analysis. Genetic and pharmacological approaches were used to manipulate protein expression and autophagy activity in human bronchial epithelial cells (HBECs). ROS generation was measured with fluorescent indicator, apoptosis with release of lactate dehydrogenase, and cell transformation was assessed with colony formation in soft agar. **RESULTS:** Vasorin expression was increased in human lung cancer tissues and cell lines, which was inversely associated with lung cancer patient survival. Cigarette smoke extract (CSE) and benzo[a]pyrene diol epoxide (BPDE)–induced vasorin expression in HBECs. Vasorin knockdown in HBECs significantly suppressed CSE-induced transformation in association with enhanced ROS accumulation and autophagy. Scavenging ROS attenuated autophagy and cytotoxicity in vasorin knockdown cells, suggesting that vasorin potentiates transformation by impeding ROS-mediated CSE cytotoxicity and improving survival of the premalignant cells. Suppression of autophagy effectively inhibited CSE-induced apoptosis, suggesting that autophagy was pro-apoptotic in CSE-treated cells. Importantly, blocking autophagy strongly potentiated CSE-induced transformation. **CONCLUSION:** These results suggest that vasorin is a potential lung cancer–promoting factor that facilitates cigarette smoke–induced bronchial epithelial cell transformation by suppressing autophagy-mediated apoptosis, which could be exploited for lung cancer prevention.

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Introduction

Lung cancer is a major health concern that is closely associated with cigarette smoke exposure [1,2]. While cigarette smoke carcinogens induce lung cancer via damaging DNA [3], only a small fraction of DNA-damaged cells become malignant, partly because apoptosis eliminates precancerous cells to prevent tumor formation and growth. Meanwhile, carcinogens and proliferation cues activate cell survival mechanisms to counteract cell death. As the success of carcinogenesis depends on the balance of cell death and survival pathways within the

pre-malignant and cancerous cells, evading apoptosis significantly contributes to carcinogenesis [2,4]. However, tremendous efforts in tackling currently known apoptosis pathways have had limited improvement for cancer prevention [5]. Thus, elucidating novel apoptosis evasion mechanisms in cancer is highly significant for reducing cancer incidence and mortality.

Carcinogens induce production of reactive oxygen species (ROS). Mitochondria are the main site of ROS production during the process of electron leakage along the mitochondrial respiratory chain for energy production. While ROS serve as second messengers for cellular signaling [6], they also damage DNA, lipids, and proteins, contributing to the pathogenesis of cancer. Particularly, DNA damage may generate somatic gene mutations that lead to cancer development. However, excessive ROS are highly toxic, resulting in extensive damage of cellular components and eventually cell death through apoptosis or necrosis [7]. This type of ROS-mediated cell death is assumed to be a protective mechanism against cancer [8,9]. Therefore, restraining ROS in a nontoxic range in pre-malignant and cancerous cells is crucial for carcinogenesis [7]. Although ROS scavenging by reductases such as superoxide dismutase, catalase, and the cellular redox buffer system GSH/GSSH has been extensively studied [6,10], how ROS is regulated during cigarette smoke-induced lung carcinogenesis is not yet completely elucidated.

We recently identified anti-TNF α -induced apoptosis (ATIA), also known as vasorin, as an antiapoptotic factor that protects cells against TNF α - and hypoxia-induced apoptosis [11]. While it is expressed on the cell membrane and can be secreted [12,13], vasorin also translocates to the mitochondria where it binds to thioredoxin-2 and suppresses ROS production [11]. We and others have previously reported that vasorin is overexpressed and promotes growth in glioblastoma [11,14], while an oncogenic role in hepatoma was also recently proposed [15,16]. However, the role of vasorin in lung carcinogenesis has never been examined. Thus, we hypothesized that vasorin may play an oncogenic role in cells with cigarette smoke-induced genomic damage through suppression of excessive ROS production. This hypothesis was tested by examining vasorin expression in human lung cancer tissues and cell lines and investigating the role of vasorin in cigarette smoke extract (CSE)-induced transformation of human bronchial epithelial cells. The results suggest that vasorin is a potential lung cancer-promoting factor that facilitates cigarette smoke-induced bronchial epithelial cell transformation by suppressing ROS-mediated autophagy and apoptosis.

Materials and Methods

Reagents and Antibodies

Synthesized benzo[a]pyrene diol epoxide (BPDE) was kindly provided by Dr. Shantu Amin (Department of Pharmacology, Penn State College of Medicine, Hershey, PA) [17] and dissolved in anhydrous dimethyl sulfoxide. CSE was prepared as described previously [18] and expressed as total particulate material ($\mu\text{g}/\text{mL}$) for treating cells. Chloroquine diphosphate salt (Cat. No. C6628), wortmannin (W1628), and 3-methyladenine (M9281) were obtained from Sigma (St. Louis, MO). Recombinant human transform growth factor- β (TGF- β) was purchased from eBioscience (San Diego, CA). Primary antibodies used were anti-vasorin/vasorin (MAB2140; R&D Systems, Minneapolis, MN), ATG-7 (PA5-17216; Thermo Fisher Scientific, Grand Island, NY), β -actin (A2103; Sigma), β -tubulin

(T8328; Sigma), LC3B (L7543; Sigma), p62 (610833; BD Biosciences, San Jose, CA), PARP1 (BML-SA248; Enzo Life Sciences, Farmingdale, NY), phospho-Smad2 (3101; Cell Signaling, Danvers, MA), Smad2 (3103; Cell Signaling), and GAPDH (sc-32233; Santa Cruz Technologies, Santa Cruz, CA).

Cell Culture

Immortalized human bronchial epithelial cell (HBEC) lines HBEC-1, HBEC-2, HBEC-13, and small airway epithelial cell (SAEC) line SAEC-30 were kindly provided by Drs. Jerry W. Shay and John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX) [19] and authenticated by short tandem repeat DNA profiling (Genetica DNA Laboratories, Burlington, NC). HBECs were maintained in keratinocyte serum-free medium (K-SFM) (Cat. No. 17005042; Thermo Fisher Scientific) supplemented with 5 $\mu\text{g}/\text{L}$ of human recombinant epidermal growth factor and 50 mg/L of bovine pituitary extract and antibiotics (100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin) in plates coated with FNC Coating Mix (0407; AthenaES, Baltimore, MD). The immortalized human bronchial cell line BEAS-2B was purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in K-SFM with supplements and antibiotics. All lung cancer cell lines were obtained from ATCC and authenticated by Genetica DNA Laboratories.

Establishment of Vasorin Knockdown Stable BEAS-2B Cell Lines

The vasorin shRNA-expressing plasmid was constructed by inserting a synthetic oligonucleotide encoding a hairpin sequence with a 19-nucleotide stem that is homologous to the target sequence of human vasorin, GGCCGGCAACACCCGCATT, and a 9-base loop sequence into pSilencer 4.1-CMV hygromycin at the BamHI site (OligoEngine). BEAS-2B cells were transfected with pSilencer 4.1-CMV-hygromycin-vasorinshRNA using FuGENE HD transfection reagent (Promega, Madison, WI) following the manufacturer's instruction. Cell clones with stable vasorin knockdown were selected with hygromycin (25 $\mu\text{g}/\text{mL}$) and confirmed by western blot. Cells stably transfected with pSilencer 4.1-CMV-hygromycin were pooled after hygromycin selection and used as negative control.

Small Interference RNA (siRNA) Transfection

Small interfering RNAs (siRNA; SiGenome SMARTpool) for human vasorin, ATG-7, and control were purchased from Dharmacon (Lafayette, CO). Cells were seeded in 12-well plates at about 60–70% confluence overnight and then were transfected with siRNAs using INTERFERin siRNA transfection reagent (Polyplus-transfection, New York, NY) according to the manufacturer's instructions.

Western Blot

Cells were harvested in M2 buffer (20 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 3 mM ethylene glycol-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid A, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β -glycerophosphate, 1 mM sodium vanadate, and 1 $\mu\text{g}/\text{ml}$ leupeptin) to obtain total cell lysate. The protein concentration was determined with Bio-Rad protein quantification kit (Bio-Rad, Hercules, CA). Western blot was carried out according to standard protocol with 10%–12% sodium dodecyl

sulfate–polyacrylamide gels and polyvinylidene difluoride membranes. The protein signal was detected by enhanced chemiluminescence reagent (Millipore). The band intensity was quantified using NIH ImageJ software with normalization to respective loading controls and expressed as fold changes with the control sample as 1. For LC3B expression, only LC3B-II was normalized to loading controls and quantified.

Detection of LC3B Puncta

pEGFP-LC3B–expressing plasmid was a gift from Dr. Han-Ming Shen (National University of Singapore). BEAS-2B cells were seeded on tissue slide cover glass and transfected with the plasmid using FuGENE HD. The next day, cells were further transfected with control or vasorin siRNA. Twenty hours after siRNA transfection, cells were treated with CSE for 2 hours, washed with cold phosphate saline buffer and fixed with cold methanol for 5 min in room temperature. Cells were then observed and photographed using a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). The GFP puncta were counted and the results expressed as puncta/cells.

Detection of ROS

ROS detection using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA 5 μ M; Invitrogen) as an indicator was done as described previously [10]. The indicator was added to cell culture medium for 30 min before the cells were lysed with M2 buffer. ROS levels were measured in cell lysates with a fluorescence plate reader using a fluorescein filter. The readings were normalized to respective protein concentrations.

Cell Death Assay

Cell death was detected by the release of lactate dehydrogenase (LDH) in culture medium using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit from Promega [20]. After removing a portion of the media, the lysis buffer from the kit was added to the wells to release total LDH. The average basal readings (OD490) from control cells were subtracted from all the readings and then the results expressed as percentage against individual total LDH reading set as 100. The experiments were carried out in triplicate.

Colony Formation Assay

BEAS-2B cells (10^4 cells/well in a 6-well plate) were treated with CSE (20 μ g/ml) or BPDE (0.2 μ M) for 1 hour and then incubated in fresh medium. The treatment was repeated every two days for a total of three treatments. Cells were trypsinized and seeded in soft agar (12-well plate) in duplicate. After two weeks of incubation, colonies in the agar were photographed under microscope in six random and nonoverlaid fields and counted. Colony formation was expressed as colonies/field [10].

Tissue Array and Immunohistochemistry

Immunohistochemistry staining using VECTASTAIN ABC Kit and DAB (3,3'-diaminobenzidine) Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) and result assessment of human lung cancer tissue array (Imgenex; Novus Biologicals, Centennial, CO) has been described previously [10]. Briefly, the paraffin-embedded tissue slide was deparaffinized and rehydrated, and then antigens were heat-retrieved followed by 3% H_2O_2 treatment and blocking. After overnight incubation of primary antibody (goat anti-vasorin, R&D

systems, 1:100) at 4 °C, further incubation was performed with ABC Kit and color was developed with DAB according to manufacturer's instructions. The staining of vasorin was compared between tumor and corresponding normal bronchial epithelial cells, and a tumor tissue was considered to have “normal expression” when its staining was comparable with that of bronchial epithelial cells, or as “increased expression” when its staining was higher.

Patient Survival Analysis with TCGA and Online Database

RNA-seq data for human NSCLC ($n = 957$) were retrieved from The Cancer Genome Atlas (TCGA) database. The corresponding patients were followed up to 150 months. Data were analyzed for vasorin expression and patient survival by Cox regression with adjustment for covariates that included age, sex, smoking status, and tumor stage. Estimation of the survival function between high versus low gene expression was done with Kaplan–Meier estimator. For the online data (Kaplan–Meier Plotter, kmplot.com) that combine 7 cohorts ($n = 673$, GSE19188, GSE29013, GSE30219, GSE31210, GSE3141, GSE37745, and GSE50081), the association of vasorin expression and lung cancer patient survival was analyzed by the Kaplan–Meier plotter online analysis tool [21,22].

Statistics

All quantitative data were expressed as mean \pm standard deviation. Comparison of two means was performed with two-tailed Student's *t* test. For analyzing tissue array results, Fisher's exact test was used. $P < 0.05$ was considered statistically significant.

Results

Vasorin is Overexpressed in Lung Cancer and Transformed HBECs

We first examined vasorin expression in human NSCLC by using immunohistochemistry in tissue arrays containing lung cancer and normal lung tissues. A total of 192 NSCLC cases, including 100 squamous cell carcinomas (SqCC), 74 adenocarcinomas (AC), and 18 other types of NSCLC were evaluated. The results revealed that vasorin was overexpressed in cancers compared with normal lung tissue (Figure 1A). Overall, 34.4% of all evaluated NSCLC cases regardless of tumor histology showed increased expression (Figure 1B). Notably, vasorin overexpression was significantly more prevalent in lung AC (52.7%) compared with lung SqCC (21.0%; $P < 0.0001$, Fisher's exact test, Figure 1B). This may suggest that vasorin abnormality is involved in the pathogenesis of both lung cancer types, although it is more commonly affected in lung AC. Consistently, vasorin expression was increased (two- to five-fold) in 7 of 9 tested NSCLC cell lines as compared with two immortalized HBEC lines. The increased expression of vasorin was detected for both the 110 kD and 72 kD isoforms (both antiapoptotic) we have previously reported [11] (Figure 1C).

Increased Vasorin Expression is Associated With Poor Survival of Patients With Lung Adenocarcinoma

The clinical significance of increased vasorin expression in lung cancer was investigated using data from TCGA. Cox regression analysis of lung cancer cases followed up for up to 150 months revealed a significant inverse association between vasorin expression and survival of these patients after adjustment for age, sex, smoking status, and tumor stage [hazard ratio (HR) = 1.34, 95% confidence

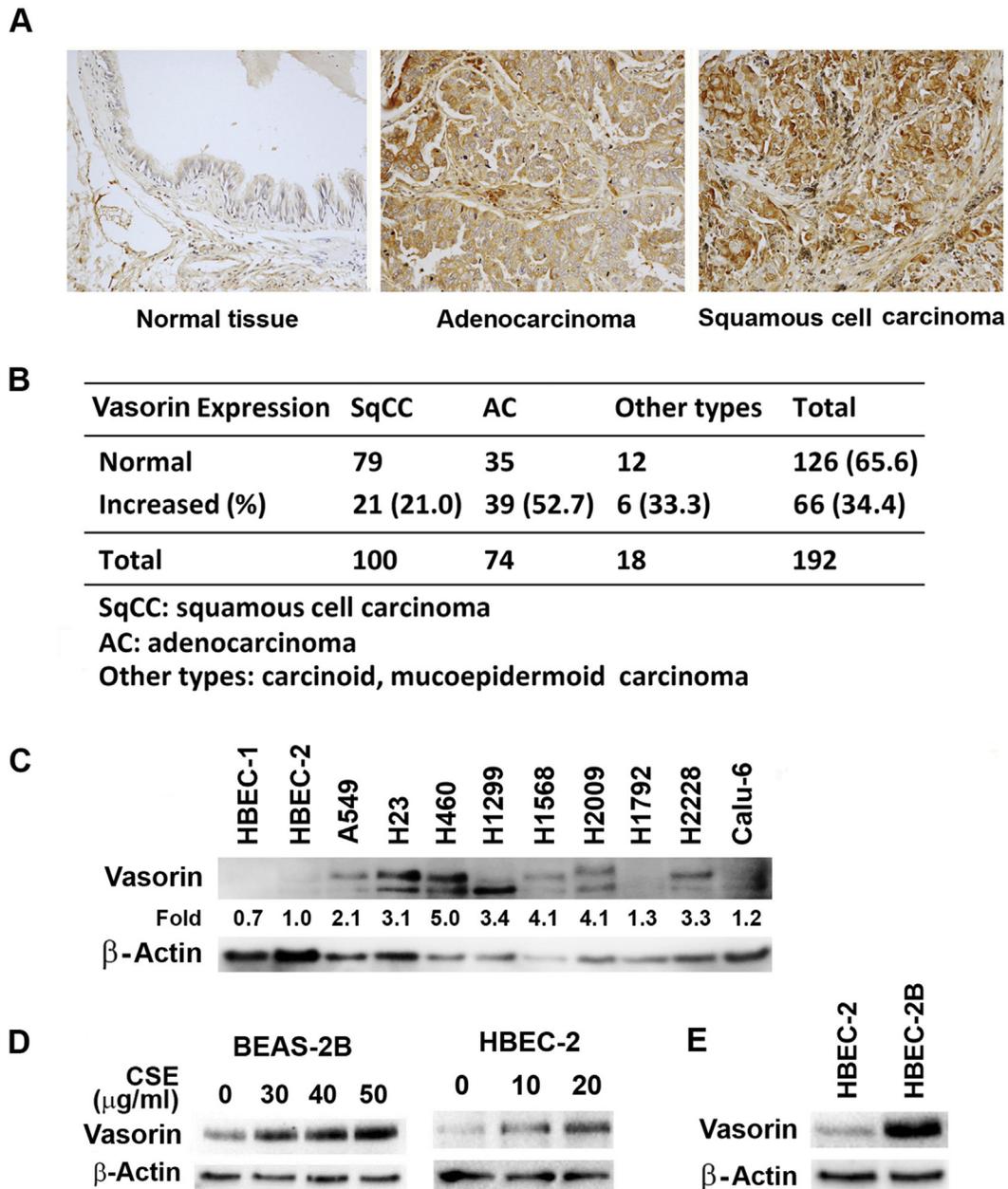


Figure 1. Increased vasorin expression in human lung cancer and HBECs exposed to tobacco carcinogens. (A) Tissue arrays were stained for vasorin with immunohistochemistry. The normal airway and alveolar epithelial cells are weakly positive while the tumor cells are strongly positive. (B) Results of tissue arrays with comparison of the staining between vasorin and normal lung epithelial cells. (C) Vasorin was examined in nontransformed (HBEC-1 and -2) and lung cancer cell lines. Relative vasorin fold increase (HBEC-2 was set to 1) was shown. (D) BEAS-2B and HBEC-2 cells were treated with CSE for 24 hours. (E) HBEC-2B (BPDE-transformed) cells were compared with HBEC-2 cells for vasorin expression. Protein was detected by western blot in C to E with β -actin as loading control.

interval (CI) 1.02–1.76, $P = 0.0356$, Figure S1A]. A similar trend was also observed in adenocarcinomas and squamous cell carcinomas from TCGA when analyzed separately using an online analytical tool [https://www.proteinatlas.org/ [25,26]]. With an independent online resource validation [Kaplan–Meier Plotter [21,22]], the inverse association between vasorin expression and survival in patients with lung adenocarcinoma was detected (HR = 1.64, 95% CI = 1.28–2.03, $P = 6.9e-5$, Figure S1B), although the association in squamous cell carcinomas was unclear. In addition, vasorin over-expression indicates a worse prognosis in both smokers (HR = 1.58, 95% CI = 1.05–2.39, $P = 0.028$) and never smokers (HR = 2.22,

95% CI = 0.95–5.19, $P = 0.05$, Figure S1B) with lung adenocarcinomas [22]. These results suggest that vasorin is a potential tumor-promoting oncoprotein in human lung adenocarcinoma.

Vasorin Expression in HBECs was Induced by Cigarette Smoke Extract and BPDE

To explore the role and mechanism of vasorin in lung carcinogenesis, we first examined if cigarette smoke affected its expression in HBECs. CSE strongly induced vasorin expression in a dose-dependent manner in BEAS-2B and HBEC-2 cells (Figure 1D). Similarly, BPDE, the active metabolite of cigarette smoke carcinogen

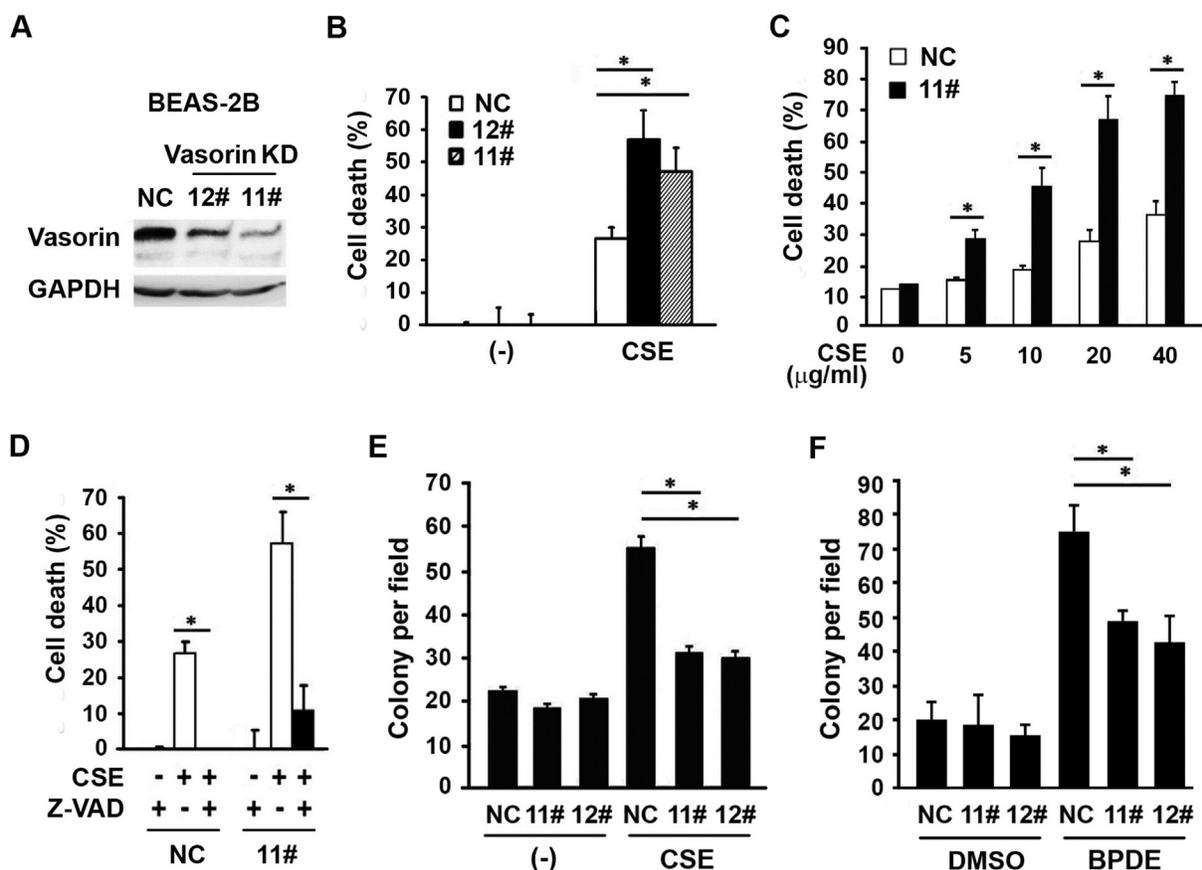


Figure 2. Vasorin knockdown potentiates CSE-induced cytotoxicity and cigarette smoke carcinogen-induced transformation. (A) Stable knockdowns of vasorin in BEAS-2B cells were generated with shRNA and expression was measured by western blot using GAPDH as loading control. (B, C, D) Cells were treated with 20 µg/ml of CSE (B), or various concentrations of CSE (C) for 48 hours, or cells were left untreated or pretreated with z-VAD (20 µM) followed by CSE (20 µg/ml) treatment for 48 hours (D). Cell death was measured by LDH release assay in triplicate samples. (E, F) Control and two clones of BEAS-2B vasorin knockdown cells (10^4 cells each) were untreated or exposed to CSE (20 µg/ml; E) or BPDE (0.2 µM; F) every two days for a total of three times and then seeded in soft agar in duplicate. Two weeks later, the colonies were photographed at 6 random fields per well and counted. * $P < 0.05$.

benzo[a]pyrene that potently transforms HBECs [10,18], strongly induced vasorin expression (Figure S2). In addition, HBEC-2B cells (HBEC-2 cells transformed by BPDE [23,24]), showed a marked constitutively increased vasorin expression (Figure 1E), indicating that the transient increase in vasorin expression by CSE and BPDE was stabilized during transformation and in lung cancer cell lines. These results suggest that cigarette smoke carcinogens induce vasorin expression, which may be involved in the early steps of lung cancer development.

Knockdown of Vasorin Expression Enhances CSE-Induced Cytotoxicity

Because vasorin was identified as an anti-apoptosis factor [11], we examined if it is involved in suppressing CSE-induced cytotoxicity. Stable vasorin knockdown was established in BEAS-2B cells (Figure 2A). While the cytotoxicity of CSE to BEAS-2B cells in general was increased in a dose-dependent manner, the cells with vasorin knockdown showed significantly increased cell death in each of the various CSE doses evaluated (Figure 2B and C). The CSE-induced cytotoxicity was suppressed by the pan-caspase inhibitor z-VAD (Figure 2D), indicating that vasorin inhibits CSE-induced apoptosis in human lung epithelial cells.

Knockdown of Vasorin Expression Suppresses CSE-Induced HBEC Transformation

Transformation of lung epithelial cells represents an early step of lung carcinogenesis, and the capacity to grow in an anchorage-independent manner is an indicator of transformation. Thus, we used the soft-agar colony formation assay and stable vasorin knockdown cells to investigate whether vasorin plays a role in CSE-induced transformation [18,23]. While CSE and BPDE effectively induced control cells to form colonies in soft agar, vasorin knockdown significantly suppressed this effect (Figure 2E and F and Figure S3). Together with the observation that vasorin inhibits CSE-induced apoptosis, these results suggest that vasorin potentiates CSE-induced HBEC transformation by suppressing apoptotic cell death.

Vasorin Knockdown Potentiates CSE-Induced Autophagy Through Cellular ROS Accumulation

Cigarette smoke exposure of lung epithelial cells activates autophagy and mediates apoptosis or survival under certain circumstances [24–29]. Thus, we investigated if vasorin knockdown impacts CSE-induced autophagy. Indeed, exposure of BEAS-2B cells to CSE strongly induced autophagy activation, as demonstrated by the increased LC3B-II and decreased p62 expression (Figure 3A) and

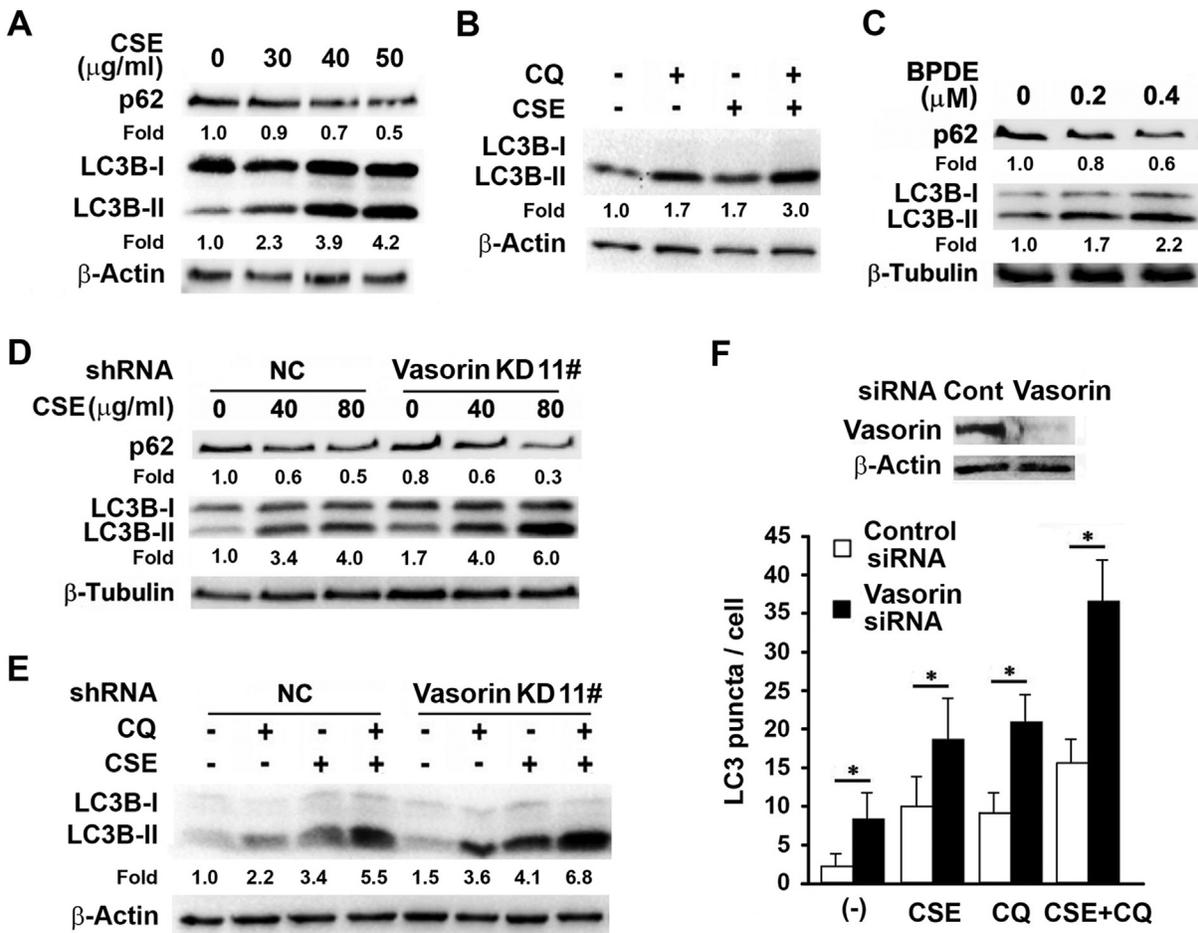


Figure 3. Vasorin inhibits autophagy. (A) BEAS-2B cells were exposed to increasing concentration of CSE for 4 hours. (B) BEAS-2B cells were treated with CSE (20 μg/ml) for 4 hours with or without pretreatment of chloroquine (CQ, 20 μM for 30 min). (C) BEAS-2B cells were treated with increasing concentrations of BPDE for 4 hours. (D, E) Control or stable vasorin knockdown cells were treated with various concentrations of CSE for 4 hours (D), or with 20 μg/ml of CSE for 4 hours without or with CQ (20 μM) pretreatment for 30 min (E). (A, B, C, D, E) Proteins were detected with western blot with β-actin or β-tubulin as loading control. (F) BEAS-2B cells were transfected with pEGFP-LC3B plasmid for 24 hours, and then were further transfected with control or vasorin siRNA (10 nM). The next day, cells were left untreated or treated with 20 μM of CQ for 30 min before CSE (20 μg/ml) exposure for 2 hours. Cells were observed and images recorded under a fluorescence microscope and LC3 puncta counted. **P* < 0.05.

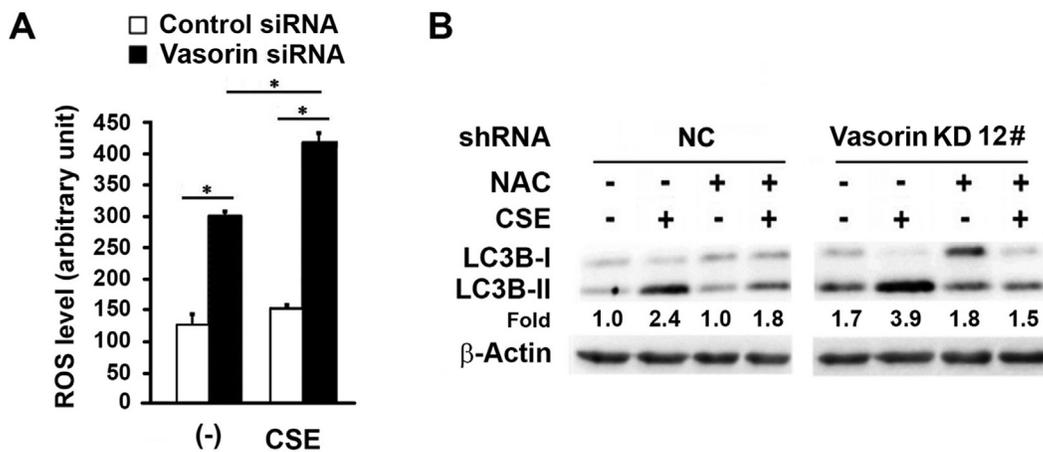


Figure 4. Involvement of ROS in vasorin-mediated autophagy. (A) BEAS-2B cells transfected with control or vasorin siRNA (10 nM) for 24 hours were left untreated or treated with CSE for 4 hours. At the last 30 min of treatment, CM-H2DCFDA (5 μM) was added to the cells. The cells were then harvested for ROS detection with a fluorescent plate reader. The readings of fluorescence were normalized to corresponding protein concentrations. Experiments were carried out in triplicate. (B) BEAS-2B cells without (control) or with stable vasorin knockdown were treated with NAC (3 mM) 30 min before CSE (20 μg/ml) incubation for 4 hours. Western blot was carried out to detect LC3B with β-actin as loading control. NAC, N-acetylcysteine.

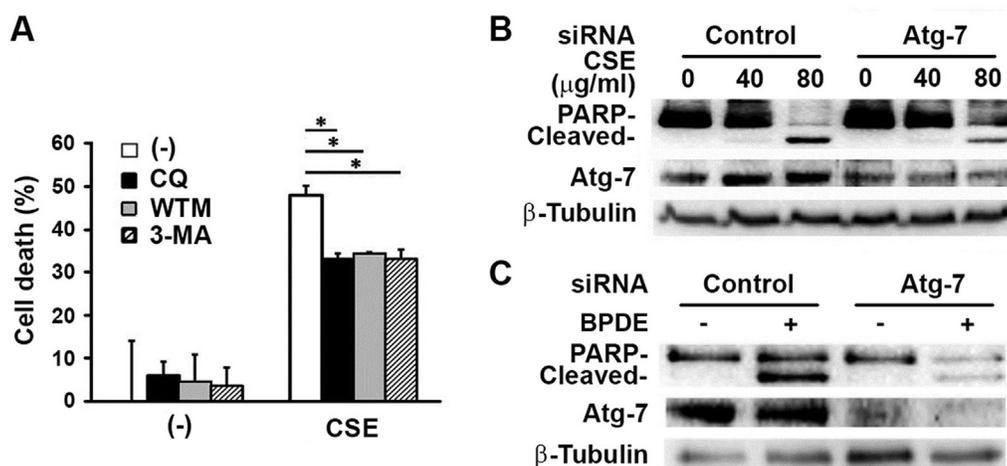


Figure 5. Autophagy contributes to CSE-induced cell death. (A) BEAS-2B cells treated with autophagy inhibitors CQ (20 μM), WTM (1 μM) or 3-MA (10 mM) for 30 min followed by incubation of CSE (20 μg/ml) for 48 hours. Cell death was determined in triplicate samples by LDH release assay. **P* < 0.05. (B, C) BEAS-2B cells were transfected with control or Atg-7 siRNA (10 nM) for 24 hours, then treated with CSE (20 μg/ml, B) or BPDE (0.4 μM) for 4 hours (C). PARP cleavage and Atg-7 levels were examined by western blot with β-tubulin as a loading control.

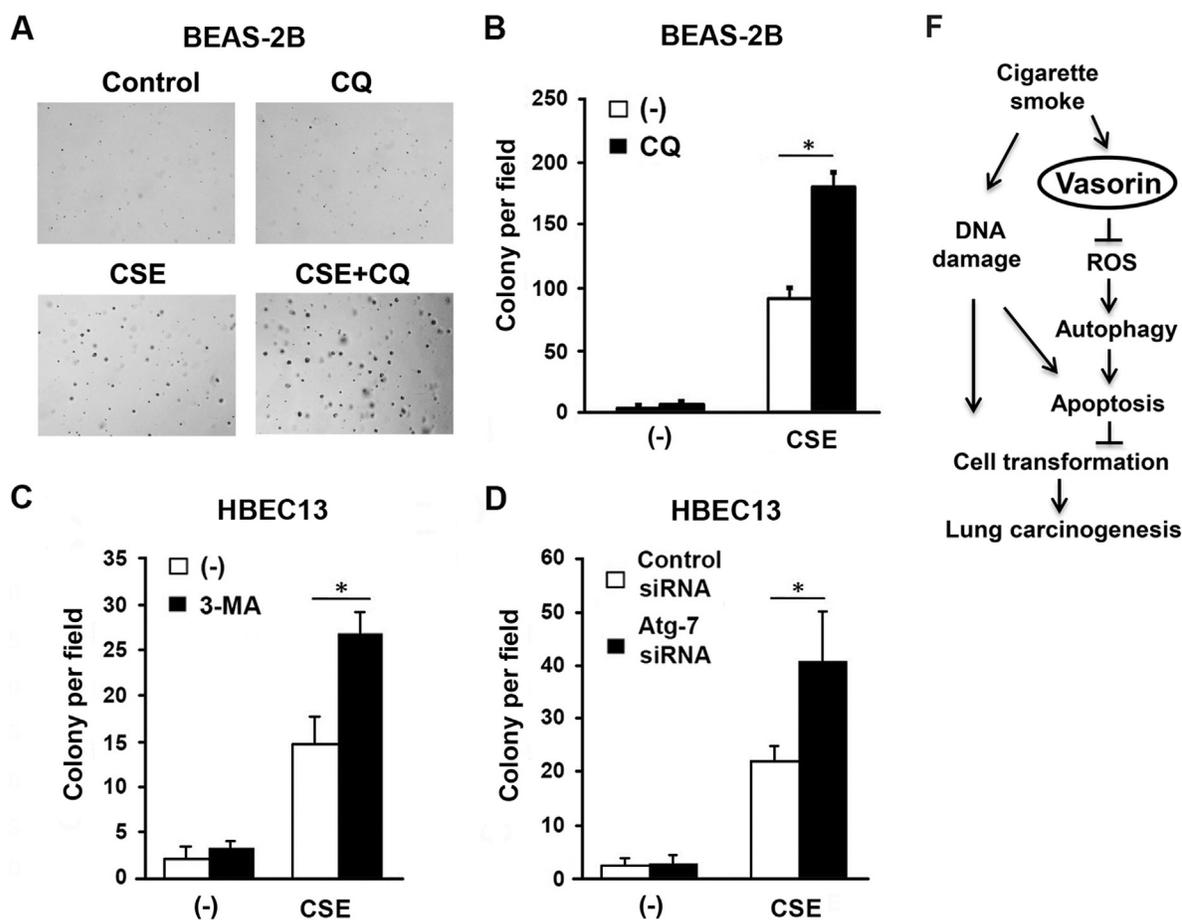


Figure 6. Inhibition of autophagy increases CSE-induced transformation. (A, B) Representative images (A) of colonies in soft agar of BEAS-2B cells untreated, treated with CQ (20 μM), CSE (20 μg/ml), or their combination. Colonies were counted after two weeks of seeding (B). (C and D) HBEC-13 cells were untreated or treated with 3-MA (10 mM, C), or transfected with control or Atg-7 siRNA before the treatment of CSE (20 μg/ml, D). Cells were then seeded in soft agar and colonies counted as in A and B. **P* < 0.05.

GFP-LC3B puncta (Figures 3F and S4). Suppressing lysosomal protein degradation with chloroquine (CQ) confirmed the increased activity of autophagy by CSE treatment (Figure 3B and F). A similar effect of BPDE on autophagy was also detected (Figure 3C). Of note, while the detection of LC3-I is not very reproducible, which may partly be due to the fact that the anti-LC3 antibody preferably detects LC3-II and that LC3-I is relatively unstable [30], the induction of LC3-II is reproducibly detected in our experiments. These findings, consistent with previous reports [26–29,31–33], indicate that CSE and BPDE activate autophagy in HBECs.

Interestingly, vasorin knockdown strongly increased CSE-induced autophagy, which was detected biochemically and morphologically (Figure 3D–F). Vasorin suppresses ROS accumulation in other cell types such as fibroblasts, and excessive ROS suppresses CSE carcinogen-induced HBEC transformation [10,11]. Thus, we further examined if ROS is involved in vasorin-mediated autophagy regulation in HBEC cells. While the basal ROS level was significantly increased in vasorin knockdown cells, the CSE-induced ROS accumulation was much higher when vasorin was suppressed (Figure 4A), suggesting that vasorin plays a role in retaining ROS at low levels in cells. The ROS scavenger N-acetylcysteine effectively reduced CSE-induced LC3B-II expression to basal levels in control and vasorin knockdown cells (Figure 4B), suggesting that CSE induces autophagy depending on ROS and that vasorin deficiency promotes CSE-induced autophagy through enhanced ROS accumulation.

Suppressing Autophagy Inhibits CSE-Induced Cytotoxicity and Potentiates Transformation

We then examined the role of autophagy in CSE-induced cytotoxicity and transformation. Pharmacological (CQ, wortmannin [WTM] and 3-methyladenine [3-MA]) or genetic (siRNA against Atg-7) autophagy suppression effectively reduced CSE-induced cytotoxicity (Figure 5A), and this was associated with decreased apoptosis as shown by decreased PARP cleavage (Figure 6B). Similarly, BPDE-induced apoptosis was inhibited by autophagy suppression (Figure 5C). These results suggest that autophagy plays a proapoptosis role in the context of CSE-induced cytotoxicity in HBECs. In addition, autophagy inhibition with either CQ and 3-MA or Atg7 siRNA effectively increased CSE-induced transformation (Figure 6A–D). Together with data that show vasorin suppresses CSE-induced autophagy and apoptosis, these results suggest that vasorin promotes transformation, at least in part, by inhibiting autophagy-mediated apoptosis.

Discussion

This study provides evidence that vasorin is a potential lung cancer-promoting factor that facilitates cigarette smoke-induced bronchial epithelial cell transformation. Vasorin expression was increased in a number of human lung cancer tissues, which was inversely associated with lung cancer patient survival. Vasorin expression was also increased in lung cancer cell lines and transformed HBECs. Vasorin knockdown in HBECs significantly suppressed CSE-induced transformation, which was associated with enhanced ROS accumulation and cytotoxicity. Mechanistically, vasorin knockdown potentiated CSE-induced autophagy and apoptosis in an ROS-dependent manner. Suppression of autophagy effectively suppressed CSE-induced apoptotic cell death and potentiated CSE-induced transformation. These results suggest that vasorin is a potential lung cancer-promoting factor, which suppresses autophagy-

mediated apoptosis to facilitate CS-induced bronchial epithelial cell transformation. Taken together, our findings establish a novel lung carcinogenesis mechanism involving vasorin, the vasorin/ROS/autophagy/apoptosis pathway, which could be exploited for preventing lung carcinogenesis.

Although an increasing number of oncogenic gene mutations are being identified in lung cancer, about one-third of patients with lung cancer have no known mutations or aberrant activation of cancer driver genes. Cellular signaling pathways regulating DNA repair, cell proliferation, survival, and death are involved in cancer development. In addition, there are potential cofactors or promoting factors that facilitate driver-mediated carcinogenesis [3]. Our findings suggest vasorin as an endogenous tumor-promoting factor. Vasorin is induced by cigarette smoke to counteract apoptosis, which maintains survival of premalignant cells during cigarette smoke-induced lung epithelial cell transformation. Notably, the inverse association between vasorin expression and patient survival seen in both smokers and nonsmokers suggests that the role of vasorin in human lung cancer development may also apply to lung cancer caused by secondhand cigarette smoke or other environmental carcinogen exposures.

Vasorin suppresses ROS and apoptosis found in mouse embryonic fibroblasts exposed to TNF α or hypoxia [11]. Consistent with this role, vasorin suppressed CSE-induced and ROS-mediated apoptosis in HBECs. It should be noted that vasorin was shown to function as a decoy ligand to suppress TGF- β signaling in vascular smooth muscle and some tumor cells [12,34,35]. TGF- β functions as a tumor suppressor in the early stages of carcinogenesis [36,37]. However, TGF- β -induced signaling was not affected by vasorin knockdown in lung cancer cells (data not shown). Thus, inhibition of TGF- β signaling is unlikely to be involved in vasorin's function in cigarette smoke-induced cell transformation. While our current results suggest an ROS-related tumor-promoting mechanism, it does not exclude the possibility that the ROS and TGF- β pathways cooperatively contribute to vasorin's cancer-promoting function. Future *in vivo* studies are needed to clarify this possibility.

Autophagy is a cellular process for degradation of exhausted, redundant, and unwanted cell components, including proteins and organelles [24,38], which can lead to either cell survival or death depending on context. In general, moderate autophagy maintains cell homeostasis and survival, whereas excessive autophagy exhausts cell components to kill cells [24,38]. In parallel with its contradictory functions in regulating cell survival or death, the role of autophagy in carcinogenesis is believed to be complex: to promote or suppress cancer development [39]. Although cell type- and insult-specific functions of autophagy are observed, it is highly likely that autophagy plays different roles in carcinogenesis in different organs. Although autophagy has been widely studied in lung cancer cell lines, there is no report to date on the role of autophagy in lung epithelial cell transformation and lung cancer initiation. Prior studies suggest the premise that cigarette smoke induces autophagy in lung epithelial cells [25–29,31–33], and our results further show that autophagy suppresses cell transformation, an early step in lung carcinogenesis. This observation is consistent with the general assumption that autophagy suppresses carcinogenesis at early stages [40–44]. Similarly, a most recent report strongly suggests that autophagy restricts chromosomal instability during replicative crisis, and that loss of autophagy function is required for the initiation of cancer [45]. However, it is also believed that autophagy promotes cancer progression at later stages [40–44]. How vasorin in concert with

autophagy activation contributes to lung carcinogenesis needs further careful delineation with temporal autophagy suppression in vasorin knockout animals.

As the so-called autophagic death is uncommon, autophagy can mediate either apoptosis or necrosis in different contexts and circumstances [46,47]. Our observations that vasorin knockdown strongly potentiated autophagy and suppressing autophagy effectively attenuated CSE-induced apoptosis suggest that autophagy mediates cytotoxicity induced by CSE and cigarette smoke carcinogens such as BPDE through apoptosis to inhibit transformation.

Our results establish a potentially oncogenic role of vasorin in lung carcinogenesis, and shed lights on the mechanism by which vasorin facilitates carcinogen-induced transformation. Further studies are warranted to map the vasorin-mediated pathway in lung cancer development, which may identify targets that could be exploited for lung cancer prevention and/or therapy.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2019.09.001>.

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