Human tumor necrosis factor alpha-induced protein eight-like 1 exhibited potent anti-tumor effect through modulation of proliferation, survival, migration and invasion of lung cancer cells

Devivasha Bordoloi[1] · Ganesan Padmavathi[1] · Kishore Banik[1] · Khwairakpam Amrita Devi[1] · Choudhary Harsha[1] · Sosmitha Girisa[1] · Constanze Buhrmann[2] · Mehdi Shakibaei[2] · Ajaikumar B. Kunnumakkara[1]

Abstract
Lung cancer represents one of the most prevalent neoplasms across the globe. Tobacco smoking, exposure to different occupational and environmental carcinogens, and various dietary factors are strongly implicated in the development of lung cancer. The 5-year survival rate of lung cancer is extremely poor which can be attributed to its propensity for early spread, lack of appropriate biomarkers and proper therapeutic strategies for this aggressive neoplasm. Emerging evidence suggests tumor necrosis factor-α-induced protein eight like 1 (TIPE1 or TNFAIP8L1), which functions as a cell death regulator, to hold high prospect as an important biomarker. Interestingly, this protein was found to be significantly downregulated in human lung cancer tissues compared to normal lung tissues. In addition, this protein exerted marked downregulation in different stages and grades of lung tumor. Further knockout of TIPE1 led to the enhancement in proliferation, survival, migration and invasion of NCIH460 human lung cancer cells through modulation of Akt/mTOR/STAT-3 signaling cascade. In addition, TIPE1 was found to be involved in nicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, N-nitrosonornicotine and benzo[a]pyrene-mediated lung cancer through enhanced proliferation, survival and migration of lung cancer cells. Altogether, this newly identified protein plays a critical role in lung cancer pathogenesis and possess enormous prospect to serve as an important tool in the effective management of this aggressive neoplasm.

Keywords Cancer · Lung · TIPE1 · Biomarker · Tobacco · Akt/mTOR · STAT-3 · Targeted therapy

Introduction

Lung cancer is one of the most prevalent neoplasm with high invasive and metastasizing potential. It develops in a multi-stage process involving a series of genetic and epigenetic alterations in the lung epithelial cells [1–3]. The regions with highest lung cancer mortality rates in the year 2012 are Central and Eastern Europe and Eastern Asia among males and Northern America and Northern Europe among females. Remarkably, the highest smoking prevalence is generally in Eastern and South-Eastern Asia and Eastern Europe among males whereas European countries, followed by Oceania and Northern and Southern America in case of females [4–6]. Apart from smoking; the predominant risk factor for lung cancer, exposure to varied occupational and environmental carcinogens and diverse dietary factors are strongly implicated in the development of lung cancer [7]. The 5-year survival rate of lung cancer is less than 15% in developed countries whereas it is 5% or even less in many developing countries [8]. Propensity for early spread, lack of suitable biomarkers and effective therapeutic strategies contribute enormously to the poor survival of the lung cancer patients [8–10].

✉ Devivasha Bordoloi
devivasha.bordoloi@gmail.com

✉ Ajaikumar B. Kunnumakkara
kunnumakkara@iitg.ac.in; ajai78@gmail.com

[1] Cancer Biology Laboratory and DBT-AIST International Center for Translational and Environmental Research (DAICENTER), Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam 781039, India

[2] Musculoskeletal Research Group and Tumour Biology, Chair of Vegetative Anatomy, Institute of Anatomy, Faculty of Medicine, Ludwig-Maximilian-University Munich, Pettenkoferstrasse 11, D-80336 Munich, Germany

Increasing lines of evidence suggest tumor necrosis factor-α-induced protein eight like 1 (TIPE1), a recently identified member of the tumor necrosis factor-α-induced protein eight (TIPE) family, which functions as a cell death regulator possess enormous prospect as a clinical cancer biomarker [11, 12]. This protein was predicted to interact with caspase-8 and F-Box and WD Repeat Domain Containing 5 (FBXW5). Furthermore, various post-translational modifications were also predicted in the case of TIPE1 [11, 13]. TIPE1 has been implicated in different cancers, including breast cancer, cervical cancer, gastric cancer, liver cancer, lung cancer, and nasopharyngeal cancer. For instance, Hu and group reported TIPE1 to cause inhibition of breast cancer cells' proliferation preferentially through downregulation of extracellular-signal-regulated kinase (ERK) phosphorylation [14]. TIPE1 was found to be involved in promoting cervical cancer progression and suppressing cisplatin susceptibility in a p53-dependent fashion [15]. In addition, TIPE1 expression was reported to be significantly lower in gastric cancer tissues, which was negatively linked with differentiation status and distant metastasis. Furthermore, TIPE1 overexpression inhibited epithelial-to-mesenchymal transition (EMT) and metastasis of gastric cancer cells both in vitro and in vivo while its silencing in well-differentiated gastric cancer cells reversed those processes. [16]. EMT is an embryonic process hijacked by epithelial-like carcinoma cells in order to attain mesenchymal-like phenotype. Oncogenic EMT is a central determining factor of metastasis and it leads to increased motility, invasiveness, stemness, aggressiveness and chemoresistance [17]. Zhang and co-workers showed that TIPE1 significantly reduced the growth and tumor weight of liver cancer homographies in mice and also inhibited the growth and colony forming ability of hepatocellular carcinoma (HCC) cells [18]. Mechanistically, interaction of TIPE1 with Rac1 led to the inhibition of Rac1 activation as well as p65 and c-Jun N-terminal kinase pathway. Thus, TIPE1 induced apoptosis in HCC cells via negative regulation of Rac1, and hence, loss of TIPE1 expression might serve as a novel prognostic indicator for patients with HCC [18]. Further, the expression and biological function of TIPE1 was also studied in lung cancer. TIPE1 overexpression led to reduced cell growth, colony forming ability, proliferation, and invasion, but induced apoptosis in TIPE1-downregulated lung cancer cells via modulation of TIPE1-mediated expression of Cyclin D1, Cyclin B1, caspase-3, -8, and matrix metalloproteinase (MMP)-2, -9. Additionally, TIPE1 also inhibited the growth and tumor weight of murine lung cancer homografts, clearly revealing the potent antitumor role of TIPE1 in lung cancer cells [19]. Further, in case of nasopharyngeal carcinoma (NPC), TIPE1 is reported to promote the proliferation of NPC cells by modulating AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway and inhibit autophagy [20].

In the present study, we determined the expression of TIPE1 in lung cancer tissues and its role in lung cancer pathogenesis. Our findings revealed that TIPE1 plays a pivotal role in regulating different hallmarks of lung cancer such as survival, proliferation, invasion and migration. This study also indicated for the first time the involvement of TIPE1 in tobacco-mediated lung carcinogenesis.

Materials and methods

Tissue microarray (TMA)

Expression evaluation of TIPE1 in different stages of lung cancer tissues as well as human lung tissues was performed using immunohistochemical analysis. TMA with normal and malignant lung tissues (Cat. No. LC1503, US Biomax, Inc., USA) with a total of 75 tissues, 150 cores (duplicated cores from the same patient in all cases) from different individuals which include 29 adenocarcinoma, 3 adenosquamous carcinoma, 29 squamous cell carcinoma, 2 bronchioalveolar carcinoma, 4 small cell undifferentiated carcinoma, 2 large cell carcinoma, 1 neuroendocrine carcinoma and 5 normal lung tissues was used for the analysis (Suppl. Table 1).

Immunohistochemistry (IHC)

IHC was carried out according to manufacturer's protocol where the tissues were initially deparaffinized followed by rehydration, peroxidase quenching, blocking and incubation with primary and peroxidase conjugate secondary antibodies. Consequently, 3,3'-Diaminobenzidine (DAB) chromogen was added, then counterstained with hematoxylin, dehydrated and finally mounted. Histostain-Plus IHC Kit, horseradish peroxidase (HRP), broad spectrum (Invitrogen, Cat. No. 859043; CA, USA) and Metal enhanced DAB Substrate Kit (Cat No. 34065; Invitrogen, CA, USA) were used for immunostaining the tissue micro array. Tissues which are stained brown were taken as positive for the presence of specific antigen and score was given as per staining intensity as well as the number of positive cells. The percentage of positive cells were scored on a scale from 0 to 4+ whereas score for staining intensity was given from 1 to 3 [21–23].

Cell culture

Human NSCLC cell line (NCIH460) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco™; Life Technologies, NY, USA), supplemented with 10% fetal bovine serum (FBS; Gibco®, NY, USA) and 1× Pen-Strep (Invitrogen, CA, USA) at 37 °C, 5% CO_2 and 95% humidity.

TIPE1 knockout using CRISPR/Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) mediated gene editing method was used for carrying out knockout of TIPE1 in NCIH460 lung cancer cells. CRISPR/Cas9 All-in-One Lentivector sets (Human) expressing human Cas9 as well as respective sgRNAs which include scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (Cat. No. K010) and TNFAIP8L1 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414605) were obtained from Applied Biological Materials, Richmond, BC, Canada. The sequences of sgRNA target are given in Suppl. Table 2. NCIH460 cells were seeded at a density of 25,000 cells/well, allowed to attain around 70–80% confluency followed by transfection with 1 μg of plasmid in incomplete opti-minimum essential media (MEM) using Lentifectin™ transfection reagent (Cat. No. G074, Applied Biological Materials, Richmond, BC, Canada). After 5–8 h, to the transfected cells, 10% FBS (Gibco®, NY, USA) was added. Following 24 h, the media was replaced with fresh DMEM media (with 10% FBS and 1× Penstrep). Subsequently, puromycin (Cat. No. P8833, Sigma-Aldrich, Missouri, USA) was used for the positive selection of cells. Finally, Western blot analysis was performed for confirmation of TIPE1 knockout of the selected clones.

Cell proliferation assay

The effect of TIPE1 knockout on the proliferation of human lung cancer cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay where 2×10^3 cells/well of both scrambled sgRNA transfected cells (CRISPR/Cas9 scramble) and TIPE1 knockout cells (CRISPR/Cas9 TIPE1) were seeded in 96-well plates and then incubated in a CO_2 regulated incubator for 24 h. The MTT assay was performed at 0 and 72 h. After each time point, MTT (Cat. No. M2128, Sigma-Aldrich, Missouri, USA) was added, incubated for 2 h, followed by addition of 100 μl of dimethylsulfoxide (DMSO) (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) after removing the culture medium. The absorbance of the colored solution was taken after 2 h incubation at room temperature with a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland) at 570 nm. The effect of TIPE1 knockout on the proliferation of human lung cancer cells was determined through normalization of the absorbance value of 72 h with 0 h, taking the absorbance of CRISPR/Cas9 scramble as 100%. In addition, this assay was performed to determine the proliferation of nicotine (Cat No. N3876, Sigma-Aldrich, Missouri, USA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or NNK (Cat No. 78013, Sigma-Aldrich, Missouri, USA), N-nitrosonornicotine or NNN (Cat No. 75285, Sigma-Aldrich, Missouri, USA) and benzo[a] pyrene or BaP (Cat No. B1760; Sigma-Aldrich, Missouri, USA) treated TIPE1 knockout cells. The MTT assay was performed at 0 and 24 h after the addition of the tobacco components (nicotine; 1 μM, NNK; 0.05 μM, NNN; 0.05 μM and BaP; 0.25 μg/ml) and the same procedure was followed thereafter.

Colony formation assay

The effect of TIPE1 knockout on the survival of lung cancer cells was determined using a colony formation assay. In this assay, CRISPR/Cas9 scramble and CRISPR/Cas9 TIPE1 cells were seeded at a density of 1000 cells/well and were allowed to grow for 2 weeks with frequent replenishing of media. The colonies formed were fixed with 70% ethanol and then stained with 0.01% (w/v) crystal violet (Cat No: 548-6209; SRL Pvt. Ltd., India). The individual clone types were identified, and images were taken. Further, the survival fractions of TIPE1 knockout cells were calculated using image J software. In addition, to determine the colony formation ability of tobacco components treated TIPE1 knockout cells, following 24 h incubation of the seeded cells, nicotine (1 μM), NNK (0.05 μM), NNN (0.05 μM) and BaP (0.25 μg/ml) were added. The media of all the wells were changed after incubating for 24 h and the same procedure was carried out henceforth.

Migration assay

Migration assay was performed to evaluate the effect of TIPE1 knockout on the migration of human lung cancer cells. Briefly, 6×10^5 cells/well (both CRISPR/Cas9 scramble and CRISPR/Cas9 TIPE1 cells) were seeded and incubated until the formation of monolayer occurred, after which serum free DMEM medium was added to the wells followed by incubation for 6–8 h. Then, a scratch or wound was created in the middle of the culture well and migration of the cells were assessed by noting the difference in the area of the wounds with an inverted microscope (Nikon T1-SM, Japan). Further, to determine the effect of tobacco components on TIPE1 knockout cells' migration, nicotine (1 μM), NNK (0.05 μM), NNN (0.05 μM) and BaP (0.25 μg/ml) were added to the cells after serum starvation followed by scratching of wound. Images captured at different time points; 0, 6, 12 h were analyzed with the help of Image J software.

Invasion assay

The invasive potential of NCIH460 cells after knockout of TIPE1 was analyzed using a Boyden chamber assay where 24-well, 8mm pore transwell inserts (Cat No. 354480,

Corning, USA) pre-coated with matrigel were used. Initially, cells were serum starved for 18 h, trypsinized and then seeded at a density of 5×10^4 cells/500 μl in serum free DMEM medium in the upper chamber of the transwell insert. Then 750 μl of DMEM medium containing 10% FBS was added to the lower chamber and incubated for 24 h in a CO_2 incubator. The cells present on the upper surface of the membrane were then scraped off whereas cells present at the bottom of the transwell insert were fixed in 70% ethanol followed by staining with 0.01% (w/v) crystal violet. The cells were visualized under a microscope (Nicon Eclipse TS100, Japan) and images were captured. The stained cells were then eluted, and absorbance was measured at 590 nm with the help of TECAN Infinite 200 PRO (Switzerland) multimode plate reader.

Western blot

Western blot analysis was carried out for confirming TIPE1 knockout in NCIH460 lung cancer cells as well as to determine the targets of TIPE1. Briefly, cells were lysed with the help of whole cell lysis buffer containing protease inhibitors and the protein concentration of the lysates were determined using Bradford reagent (Cat. No. 500-0205; Bio-Rad, California, USA). 50 μg of proteins were separated in a 12% or 8% SDS-acrylamide gel, transferred to nitrocellulose membrane (Bio-Rad, California, USA), blocked, and then probed with primary antibodies overnight (Suppl. Table 3). Subsequently, the blots were incubated with appropriate secondary antibodies conjugated with HRP (Suppl. Table 3). Finally, the bands were visualized in a ChemiDoc™ XRS System (Bio-Rad, California, USA) with Clarity Western ECL Substrate (Cat. No. 1705061; Bio-Rad, California, USA). α-tubulin served as the loading control.

Statistical analysis

Statistical analysis was carried out with the help of Student's *t* test. Data are presented as Mean ± SE. *p*-value <0.05 denotes statistical significance.

Results

This study determines the role of TIPE1 in the pathogenesis of human lung tumor. Our previous studies on TIPE and TIPE2, another two members of the TNF alpha induced protein 8 family, suggested their role in lung cancer through regulation of different hallmarks of cancer via modulation of multiple signaling molecules [9, 10]. In this study, first, expression analysis of TIPE1 in different lung cancer pathologies was done using IHC. Further, the study indicated the role of TIPE1 in regulating different processes in lung cancer cells which include proliferation, survival, migration, invasion and metastasis along with molecular mediators responsible for the modulation of the different hallmarks in lung cancer. The study also suggested TIPE1 to be involved the effective regulation of tobacco mediated lung carcinogenesis.

TIPE1 is downregulated in human lung cancer

It has been reported that TIPE1 expression inversely correlates with differentiation status and distant metastasis in gastric tumor, and high expression in lung tumor correlates positively with patient survival [16, 19]. By Immunohistochemical analysis, we found that TIPE1 expression was significantly downregulated in lung cancer tissues compared to normal lung tissues (Fig. 1A, B). Additionally, TIPE1 expression was markedly decreased in different stages of lung cancer (Stage I, II, and III) as well as different grades of lung tumor (Grade 1, 2, and 3) compared to normal lung tissues (Fig. 1C, D).

Knockout of TIPE1 increased the proliferation and survival of lung cancer cells

Proliferation plays a critical role in the development and progression of cancer and is manifested by altered activity of diverse signal transduction pathways [24]. Hence, we determined the effect of TIPE1 knockout on the proliferation of human lung cancer cells through MTT assay. Our results showed that knockout of TIPE1 led to significantly increased proliferation of NCIH460 cells compared to scrambled control cells (Fig. 2A).

Knockout of TIPE1 increased the survival of lung cancer cells

The effect of TIPE1 knockout on the survival of NCIH460 cells was determined using a colony formation assay. Clonogenic assay or colony formation assay is a cell survival assay based on the ability of a single cell to grow into a colony. This assay essentially tests the ability of a cell to undergo "unlimited" division [25]. Knockout of TIPE1 was found to increase the clonogenic potential of NCIH460 cells as evinced by the increase in the number and size of colonies compared to scrambled control (Fig. 2B).

Knockout of TIPE1 increased the migration of lung cancer cells

Cell migration is a critical mechanism which is involved in the regulation of tissue morphogenesis, epithelial wound healing as well as tumor metastasis [26]. It is usually regulated by matrix-degrading enzymes like metalloproteases, integrins,

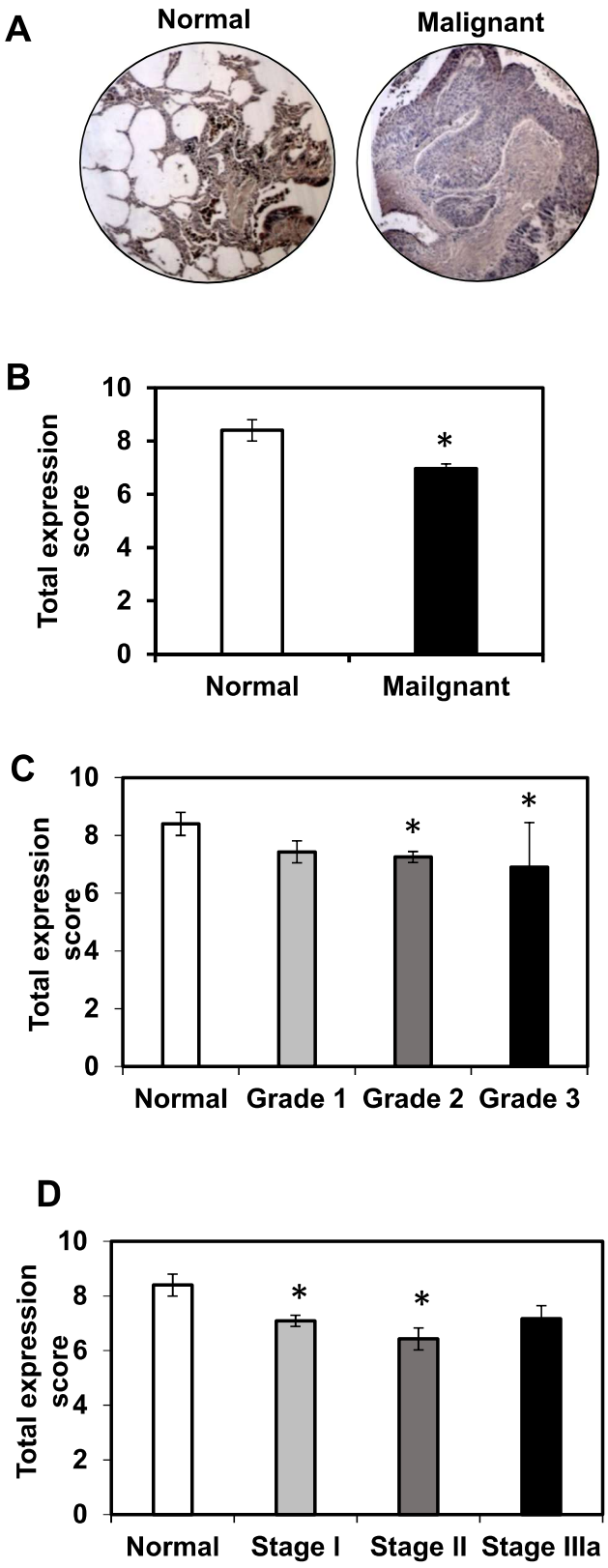


Fig. 1 Expression analysis of TIPE1 in lung cancer tissues through immunohistochemical analysis. (**A**) Representative images of TIPE1 expression in non-malignant (normal) lung tissues and lung cancer tissues; (**B**) expression of TIPE1 in normal vs. malignant lung tissues; (**C**) expression of TIPE1 in different grades of lung cancer which include Grade 1, 2 and 3; (**D**) TIPE1 expression analysis in different stages of lung tumor; stage I, II and III. Data are represented as mean ± SE, * indicates $p < 0.05$

cell-cell adhesion molecules etc. [27]. Therefore, to determine the effect of TIPE1 knockout on the migration of human lung cancer cells, wound healing assay was done. In case of TIPE1 knockout NCIH460 cells, almost complete healing of the wound was observed at 12 h itself, which was prior to the healing time of wound created in the scrambled control (Fig. 2C). Thus, knockout of TIPE1 was found to promote the migration of lung cancer cells effectively.

Knockout of TIPE1 increased the invasion of lung cancer cells

Different genetic and epigenetic alterations in cancer cells lead to the activation of multiple signaling cascades which are involved in promoting the growth, survival, migration and invasion of cancer cells [28]. Invasion of neighboring tissue is one of the most important hallmarks of cancer, which is mediated through interactions between tumor and extracellular matrix and cancer related fibroblasts [29, 30]. Therefore, to determine whether knockout of TIPE1 has any effect on the invasive potential of human lung cancer cells, we have performed a Boyden chamber assay. Interestingly, in TIPE1 knockout cells, a notably higher number of invasive cells were observed compared to control cells (Fig. 2D). Thus, this result suggested that TIPE1 might be involved in the regulation of invasive potential of lung cancer cells.

TIPE1 is involved in the tobacco mediated proliferation of lung cancer cells

We further determined the effect of nicotine, NNK, NNN and BaP on the proliferation of TIPE1 knockout lung cancer cells using the MTT assay. Treatment with different tobacco components led to the increased proliferation of TIPE1 knockout cells compared to the scrambled control cells treated with these components. Highest proliferation was observed in NNK treated TIPE1 knockout cells followed by nicotine, NNN and BaP treated TIPE1 knockout lung cancer cells respectively (Fig. 3). These results suggest that TIPE1 negatively regulates tobacco mediated proliferation of human lung cancer cells.

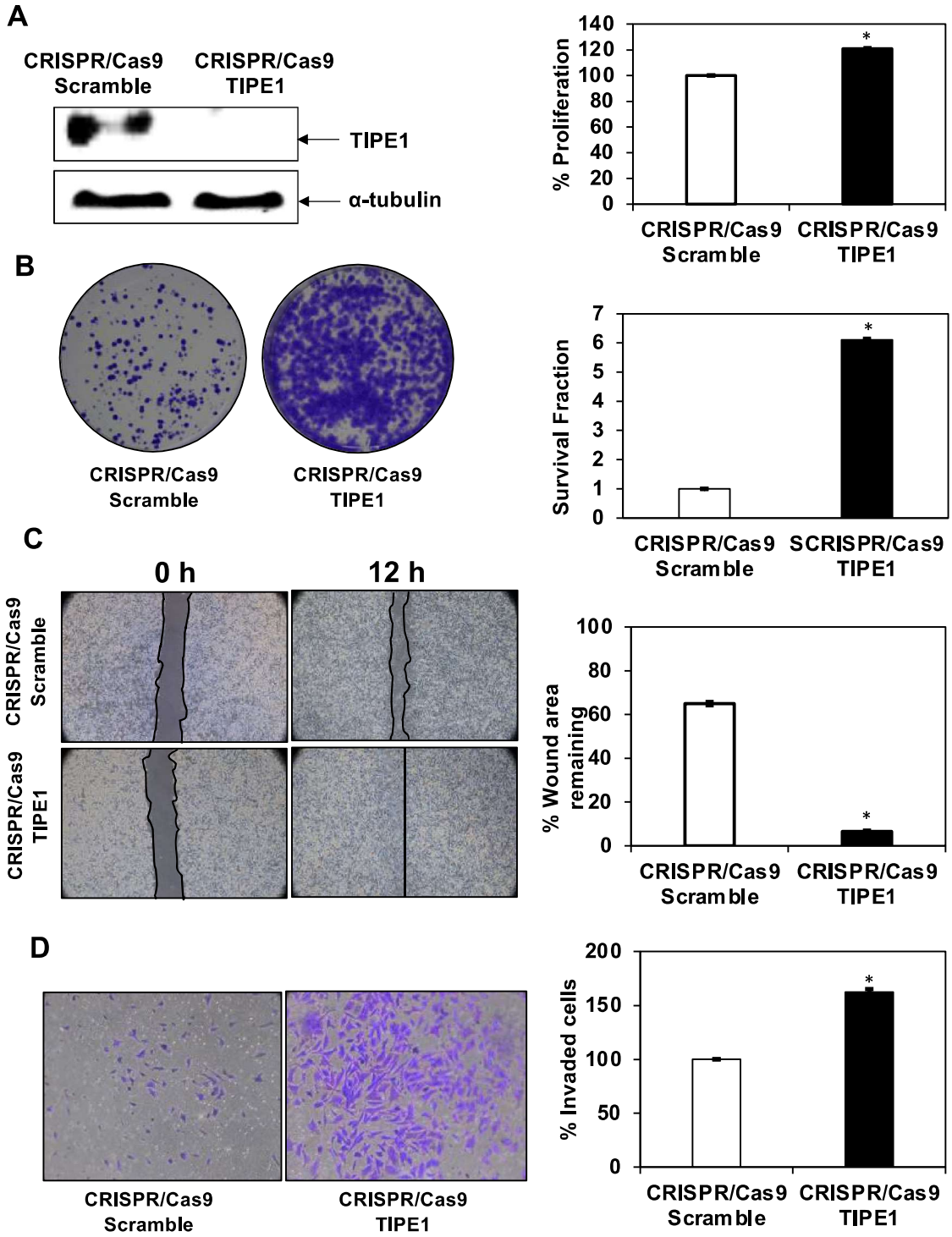


Fig. 2 Effect of TIPE1 knockout on proliferation, survival, migration and invasion of lung cancer cells. (**A**) Expression of TIPE1 in NCIH460 lung cancer cells after CRISPR/Cas9 knockout shown using Western blot (left panel); MTT assay showing the effect of TIPE1 knockout on the proliferation of lung cancer cells after 72 h (right panel). (**B**) Effect of TIPE1 knockout on the clonogenic potential of lung cancer cells shown using colony formation assay (left panel) and its graphical representation in terms of survival fraction (right panel). (**C**) Wound healing assay to determine the migration of lung cancer cells after knockout of TIPE1. Images were captured at 0 and 12 h (10× magnification) (left panel). Graphical representation of the migration potential of TIPE1 knockout cells in comparison with CRISPR/Cas9 scramble (right panel). (**D**) Effect of TIPE1 knockout on the invasion of lung cancer cells using Boyden chamber assay (left panel) and the graphical representation in terms of percent invaded cells (right panel). Data are represented as mean ± SE, * indicates $p < 0.05$

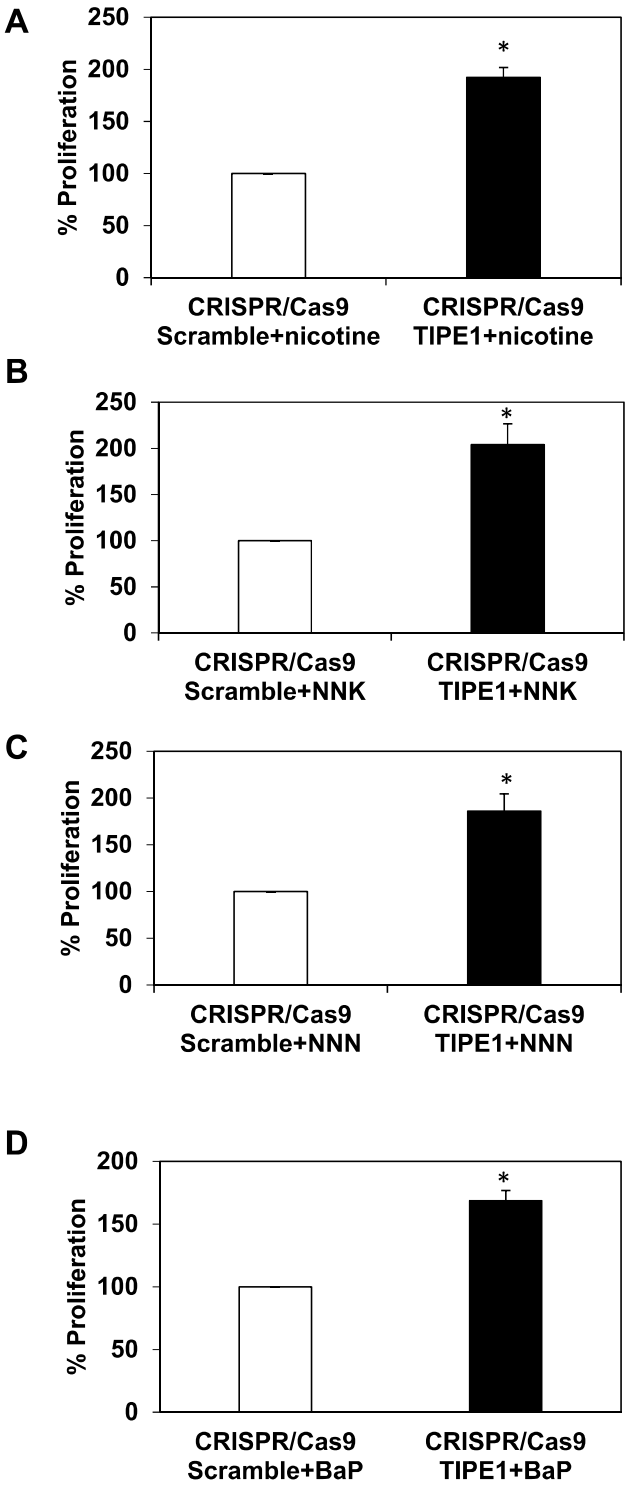


Fig. 3 Effect of TIPE1 knockout on tobacco induced proliferation of lung cancer cells. Role of TIPE1 in (**A**) nicotine, (**B**) NNK, (**C**) NNN, (**D**) BaP induced proliferation of lung cancer cells evaluated using MTT assay after 24 h. Data are represented as mean ± SE, * denotes p value <0.05

TIPE1 is involved in the tobacco-mediated survival of lung cancer cells

We also determined the effect tobacco components on the survival of TIPE1 knockout human lung cancer cells with the help of colony formation assay. The results showed that TIPE1 knockout cells treated with nicotine, NNK, NNN and BaP exerted a significantly increased survival fraction in comparison with scrambled control cells treated with the respective components. TIPE1 knockout cells treated with NNN exerted highest survival fraction followed by nicotine, NNK and BaP treated cells, respectively (Fig. 4). Thus, knockout of TIPE1 enhanced tobacco induced survival of human lung cancer cells effectively.

TIPE1 is involved in the tobacco mediated migration of lung cancer cells

The migration potential of tobacco components treated TIPE1 knockout cells was also determined via wound healing assay. The results showed that in tobacco components treated TIPE1 knockout cells, there was complete healing of the wound already after 6 h, whereas in case of tobacco components treated scrambled control cells, notable wound area remained (Fig. 5). These results demonstrate that TIPE1 is involved in the effective modulation of tobacco mediated migration of human lung cancer cells.

Knockout of TIPE1 modulated Akt/mTOR/STAT-3 signaling axis

The results of the present study revealed TIPE1 to possess anti-tumorigenic effect on human lung cancer cells. Therefore, we determined the downstream targets of TIPE1 by analyzing the expression of different proteins involved in diverse processes in cancer cells (Fig. 6). Knockout of TIPE1 upregulated the expression of apoptosis regulatory proteins such as survivin and X-linked inhibitor of apoptosis (XIAP). Further, the expression of Caspase 9 was found to be downregulated. In addition, TIPE1 knockout cells exerted increased expression of Cyclin D1, c-Myc, microtubule-associated proteins 1A/1B light chain 3B (LC-3B), vascular endothelial growth factor A (VEGF-A) and MMP-9 and downregulation of tumor suppressors' such as p53 and p21, compared to scrambled control cells. Notably, loss of TIPE1 was also found to inflect the important components of Akt/mTOR signaling cascade as TIPE1 knockout NCIH460 cells showed upregulation in the expression of Akt1, p-Akt^{S473}, p-Akt^{T308}, mTOR, p-mTOR^{S2448}, S6 and p-S6$^{S235/236}$ and downregulation in phosphatase and tensin homolog (PTEN) and regulated in development and DNA damage responses 1 (Redd1). Further, knockout of TIPE1 upregulated the expression of signal transducer and activator

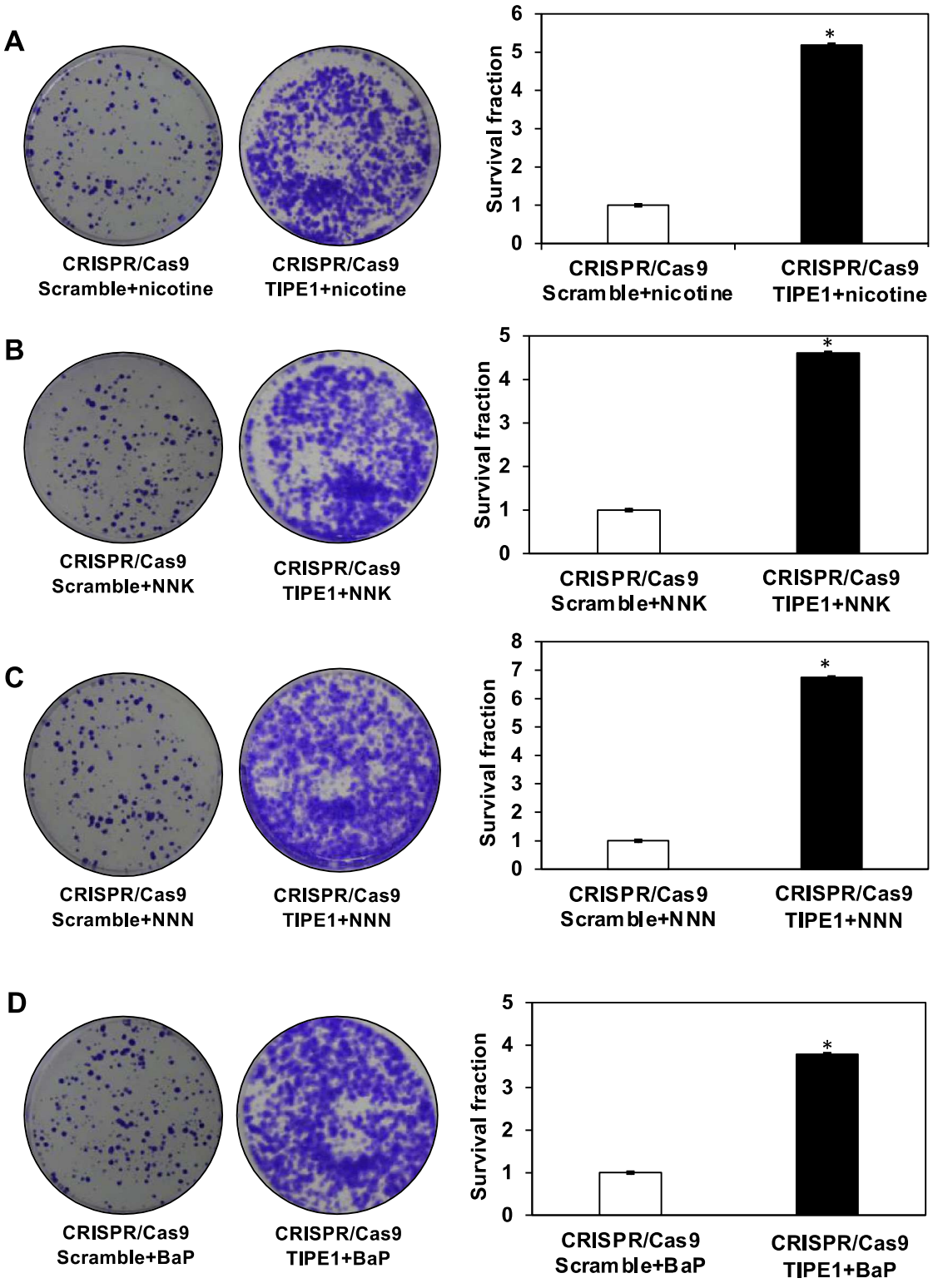


Fig. 4 Effect of TIPE1 knockout on tobacco induced survival of lung cancer cells. Images of the colonies formed in (**A**) (left panel) nicotine, (**B**) (left panel) NNK, (**C**) (left panel) NNN and (**D**) (left panel) BaP treated TIPE1 knockout cells along with scrambled control cells treated with the respective compounds for 6 h; graphical representation of clonogenic potential of (**A**) (right panel) nicotine, (**B**) (right panel) NNK, (**C**) (right panel) NNN and (**D**) (right panel) BaP treated TIPE1 knockout cells in terms of survival fraction compared to scrambled control cells treated with tobacco components. Data are represented as mean ± SE, * denotes p value <0.05

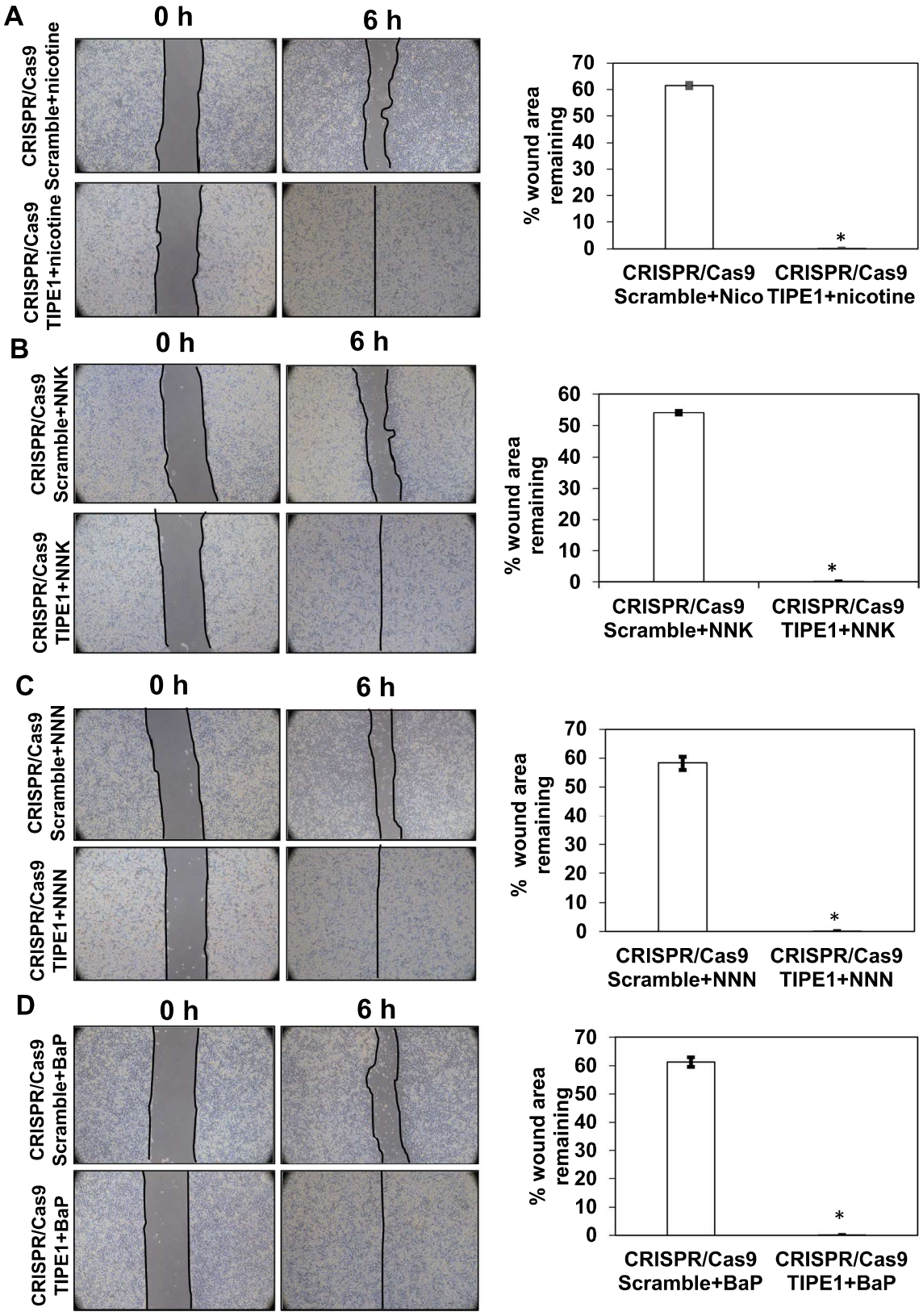
of transcription 3 (STAT-3) and p-STAT-3^{S727}. Collectively, loss of TIPE1 increased the proliferation, survival, invasion and migration of lung cancer cells plausibly through activation of Akt/mTOR/STAT-3 signaling axis (Fig. 7).

Discussion

Lung cancer represents the most common cancer as well as the most common cause of death due to cancer across the globe [31]. The poor survival rate of lung cancer can be primarily attributed to the propensity for early spread, lack of effective biomarkers for early diagnosis and prognosis and the ineffectiveness of existing lung cancer therapies [32]. Hence, there arises an urgent need to develop novel biomarkers for diagnosis and prognosis which can facilitate its effective management. Notably TIPE1, which has been reported to have important role in the regulation of cell death possess prospect in this regard [11]. Expression analysis of TIPE1 showed it to exert reduced expression in lung cancer tissues than the normal lung tissues. Further, it exhibited downregulation in different lung cancer types, pathological conditions, stages and grades of lung tumor. In line with our findings, a study conducted on gastric cancer also showed TIPE1 to be downregulated, showing inverse correlation with differentiation status and distant metastasis in gastric cancer tissues [16]. In addition, study conducted by Wu and co-workers also reported TIPE1 to be downregulated in lung cancer tissues, which positively correlated with patient survival [19]. Thus, the findings of our analysis as well as existing literature provide a basis for considering the involvement of TIPE1 in the negative regulation of lung cancer pathogenesis. Further, to decipher the exact role of TIPE1 and its downstream targets, the expression of TIPE1 was disrupted with the help of CRISPR/Cas9-mediated gene editing. Knockout of TIPE1 led to the increased proliferation of NCIH460 cells compared to scrambled control cells. In addition, TIPE1 knockout led to the increased clonogenic potential of NCIH460 cells as evinced by the increase in the number and size of colonies than the scrambled control. In a study conducted by Zhang and group, TIPE1 was reported to inhibit the growth and colony forming ability of HCC cells [18]. Besides, overexpression of TIPE1 reduced colony formation of lung cancer cells, reported by Wu and co-workers [19]. In addition, our results suggest TIPE1 to negatively regulate the migration and invasion of lung cancer cells. In line with our findings, Wu and co-workers also suggested overexpression of TIPE1 to be associated with decreased invasion of lung cancer cells, implying its antitumor role [19]. Further, TIPE1 was also reported to inhibit the proliferation of breast cancer cells through modulation of ERK [14].

It has been well evinced that smoking contributes to around 90% of lung cancer cases, which can be attributed to different chemical carcinogens present in it. Around 60 carcinogens are reported to be found in cigarette smoke among which NNK represents the strongest tobacco-specific carcinogen [33, 34]. NNK was also found to cause enhanced proliferation, inhibition of chemotherapy-induced apoptosis, migration and invasion of lung cancer cells through activation of phosphatidylinositol-3-kinase (PI3K)/Akt, NF-κB and c-Src/PKCι/FAK signaling cascades [35, 36]. In addition, this nitrosamine is also reported to induce the survival of lung cancer cells through promotion of thromboxane A2 and its receptor [37]. Further, NNN, another important tobacco specific nitrosamine is used as a strong carcinogenic agent in different laboratory animal models [38]. Increasing lines of evidence suggest that binding of NNK and NNN to the nicotinic acetylcholine receptor leads to the induction of tumor growth through modulation of cell proliferation, survival, migration, and invasion [35]. In addition, nicotine is also found to induce lung cancer cell proliferation via β-arrestin–regulated activation of the Src and Rb–Raf-1 pathways [39, 40]. Further BaP; a polycyclic aromatic hydrocarbon with strong carcinogenic and mutagenic properties, induces lung tumors via local administration or through inhalation [41]. It activates AhR/Src/ERK cascade in lung cells which facilitates CYP1A1 induction and DNA adduct formation [42]. BaP was found to promote cell cycle progression through activation of Chk1 pathway mediated via ERK in lung cancer cells [43]. Therefore, to determine the role of TIPE1 in tobacco induced lung cancer, we treated TIPE1 knockout NCIH460 cells with four different tobacco components; NNK, NNN, nicotine and BaP and their effect on the proliferation, survival and migration were observed. Our findings showed that treatment with these four tobacco components resulted in significant increase in the proliferation, survival and migration of TIPE1 knockout human lung cancer cells as demonstrated by MTT, colony formation and wound healing assay. In case of NNK treated TIPE1 knockout cells, highest increase in the proliferation was observed. In case of NNN, nicotine and BaP treated cells as well, significant increase in the proliferation of TIPE1 knockout cells compared to scrambled control cells were observed. Further, NNN treated TIPE1 knockout cells exerted highest enhancement in the survival fraction. Additionally, in nicotine, NNK, NNN and BaP treated TIPE1 knockout cells, wound created was found to be completely healed at 6 h, whereas in scrambled control cells treated with the respective compounds, around 40–45% wound areas were only found to be healed suggesting the involvement of TIPE1 in the modulation of the migration of human lung cancer cells.

Loss of TIPE1 upregulated the expression of proteins involved in cell growth, survival, apoptosis regulation, migration, metastasis and angiogenesis such as survivin,


A
0 h
6 h
CRISPR/Cas9 Scramble+nicotine
CRISPR/Cas9 TIPE1+nicotine
% wound area remaining
70
60
50
40
30
20
10
0
*
CRISPR/Cas9 Scramble+Nico
CRISPR/Cas9 TIPE1+nicotine
B
0 h
6 h
CRISPR/Cas9 Scramble+NNK
CRISPR/Cas9 TIPE1+NNK
% wound area remaining
60
50
40
30
20
10
0
*
CRISPR/Cas9 Scramble+NNK
CRISPR/Cas9 TIPE1+NNK
C
0 h
6 h
CRISPR/Cas9 Scramble+NNN
CRISPR/Cas9 TIPE1+NNN
% wound area remaining
70
60
50
40
30
20
10
0
*
CRISPR/Cas9 Scramble+NNN
CRISPR/Cas9 TIPE1+NNN
D
0 h
6 h
CRISPR/Cas9 Scramble+BaP
CRISPR/Cas9 TIPE1+BaP
% wound area remaining
70
60
50
40
30
20
10
0
*
CRISPR/Cas9 Scramble+BaP
CRISPR/Cas9 TIPE1+BaP

◂**Fig. 5** Effect of TIPE1 knockout on tobacco induced migration of lung cancer cells. Representative images showing the effect on the migration of (**A**) (left panel) nicotine, (**B**) (left panel) NNK, (**C**) (left panel) NNN and (**D**) (left panel) BaP treated TIPE1 knockout cells along with scrambled control cells treated with the respective compounds. Graphical representation of percent wound area remaining in (**A**) (right panel) nicotine, (**B**) (right panel) NNK, (**C**) (right panel) NNN and (**D**) (right panel) BaP treated TIPE1 knockout cells compared to tobacco components treated scrambled control cells. Data are represented as mean ± SE, * denotes p value <0.05

XIAP, Cyclin D1, c-Myc, MMP-9 and VEGF-A. Survivin and XIAP are members of the inhibitor of apoptosis family which are involved in cellular apoptosis inhibition [44]. Further, Cyclin D1 which can function as a transcriptional co-regulator is involved in the regulation of cell cycle progression [45]. MMP-9 plays a vital role in cancer cell invasion and tumor metastasis whereas VEGF-A is involved in the modulation of angiogenesis and vascular permeability [46, 47]. Interestingly, Liu and co-workers also reported TIPE1 to be involved in the regulation of MMP-2 and MMP-9, which play important roles in tumor progression and EMT in gastric cancer cells [16]. In addition, a study conducted by Wu and co-workers on the role of TIPE1 in H292 and A549 lung cancer cells supports our findings. They found that TIPE1 when overexpressed, exerted anti-tumorigenic characteristics via modulation of TIPE1-mediated expression of Cyclin D1, Cyclin B1, Caspase-3, -8, and MMP-2, -9 [19]. Besides these proteins, Caspase 9, a key player in the intrinsic or mitochondrial apoptosis pathway was found to be downregulated in TIPE1 knockout lung cancer cells whereas LC-3B, a universal marker of autophagy has shown upregulation in NCHI460 cells upon knockout of TIPE1, which in turn might promote lung cancer cell proliferation and survival [48, 49]. In addition, decreased expression of tumor suppressors such as p53 and p21 were also observed in TIPE1 knockout cells. p53 is a prime sensor of cellular stresses which upon stabilization modulates many genes involved in different cellular functions, and its transcriptional target p21 is responsible for the induction of cell-cycle arrest [50]. We found that knockout of TIPE1 upregulated the expression of Akt1, p-Akt^{S473}, p-Akt^{T308}, mTOR, p-mTOR^{S2448}, S6, p-S6$^{S235/236}$, STAT-3 and p-STAT-3^{S727} remarkably. The PI3K/Akt/mTOR pathway is responsible for the regulation of an array of cellular processes which include proliferation, survival, migration and invasion. In case of many cancers this pathway is overactive, which results in decreased apoptosis, enhanced proliferation and angiogenesis [51–53]. Thus, overactivation of PI3K/Akt/mTOR serves as a potent molecular therapeutic target in the treatment of various cancers [54]. Further, mTOR plays its role through its distinct multiprotein complexes namely mTORC1, and mTORC2 and its dysregulation has been found to have notable implication in different pathological conditions in cancer [55]. In addition, constitutive activation of STAT3 is frequently observed in various cancer cells and has been found to have significant association with the proliferation, survival, invasion, metastasis and angiogenesis in different human tumors [56, 57]. Besides, downregulation of PTEN and Redd1 was also observed in TIPE1 knockout lung cancer cells compared to the scrambled control cells., PTEN is the negative regulator of the PI3-kinase pathway which regulates cancer cell growth, proliferation and survival [58], whereas Redd1 is a negative regulator of mTOR which has an inhibitory role on lung cancer cells' invasion [59].

Collectively, this study reports that loss of TIPE1 increases the proliferation, survival, invasion and migration of lung cancer cells through activation of Akt/mTOR/STAT-3 signaling axis. A study carried out by Ha and co-workers showed that oxidative stress-induced TIPE1 caused reduction in mTOR phosphorylation and an increase in autophagy [60]. Further, TIPE1 was shown to promote the proliferation of NPC cells via modulation of AMPK/mTOR cascade [20]. However to the best of our knowledge, no study till date reports the involvement of mTOR in TIPE1 regulated lung cancer. Noteworthy, this is the first report which shows the involvement of Akt/mTOR/STAT-3 signaling axis in TIPE1 mediated lung tumorigenesis. In addition, this study also suggests TIPE1 to be involved in different tobacco components' such as nicotine, NNK, NNN and BaP mediated proliferation, survival, and migration of human lung cancer cells.

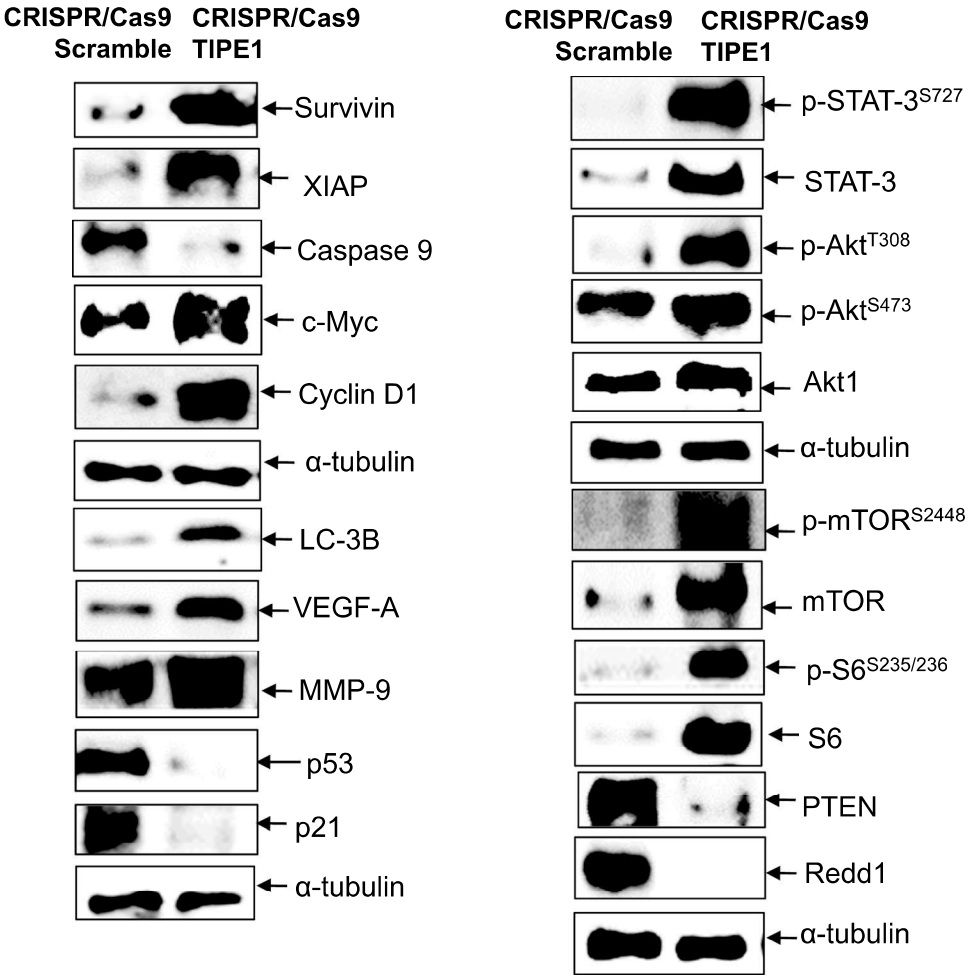


Fig. 6 Effect of TIPE1 knockout on different signaling molecules/pathways. Expression of different proteins involved in the regulation of cancer hallmarks in CRISPR/Cas9 Scramble and CRISPR/Cas9 TIPE1 cells (left panel). Effect of TIPE1 knockout on the modulation of vital constitutes of Akt/mTOR/STAT-3 signaling (right panel). The house keeping gene α-tubulin served as loading control (representative images are shown)

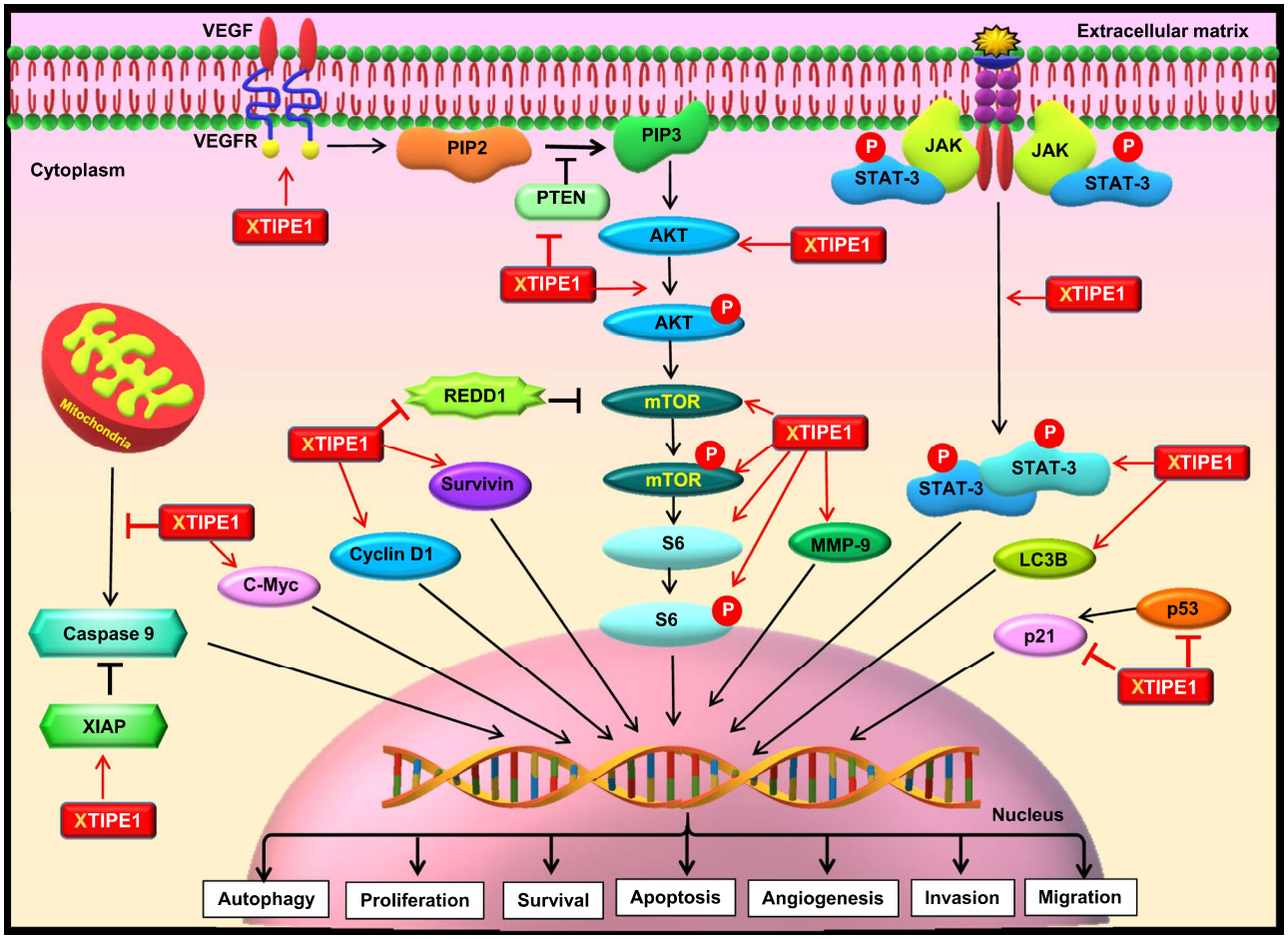


Fig. 7 Representation of the involvement of TIPE1 in the modulation of the Akt/mTOR/STAT-3 signaling axis, CRISPR/Cas9 TIPE1 is indicated by 'XTIPE1' (Figure adapted and modified from Bordoloi et al. [10])

Conclusion

The findings of this study suggest that TIPE1 is responsible for the negative regulation of human lung cancer. Knockout of TIPE1 led to the increased proliferation, survival, migration and invasion of NCIH460 human lung cancer cells. Further, TIPE1 was found to mediate tobacco induced proliferation, survival and migration of lung cancer cells effectively. Mechanistically, TIPE1 exhibited its anti-tumorigenic effect via modulation of the Akt/mTOR/STAT-3 signaling axis. Collectively, this protein plays an important role in the pathogenesis of human lung tumor and thus can be considered as a novel candidate for targeted lung cancer therapies. However, further in vivo and clinical validation are critical to establish the therapeutic significance of this protein in lung cancer.

Supplementary Information The online version of this article (doi:https://doi.org/10.1007/s11010-021-04060-1) contains supplementary material, which is available to authorized users.

Acknowledgement This work is supported by BT/556/NE/U-EXCEL/2016 and BT/PR16998/NER/95/449/2015, Department of Biotechnology (DBT), Government of India. The author Kishore Banik acknowledges UGC, New Delhi, India for providing him the fellowship.

Compliance with ethical standards

Conflict of interest The authors express no conflict of interest.

References

1. Lemjabbar-Alaoui H, Hassan OU, Yang YW, Buchanan P (2015) Lung cancer: biology and treatment options. Biochim Biophys Acta 1856:189–210. https://doi.org/10.1016/j.bbcan.2015.08.002
2. Larsen JE, Minna JD (2011) Molecular biology of lung cancer: clinical implications. Clin Chest Med 32:703–740. https://doi.org/10.1016/j.ccm.2011.08.003
3. Gazdar AF, Brambilla E (2010) Preneoplasia of lung cancer. Cancer Biomarkers 9:385–396. https://doi.org/10.3233/CBM-2011-0166
4. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A (2015) Global cancer statistics, 2012. CA Cancer J Clin 65:87–108. https://doi.org/10.3322/caac.21262
5. Islami F, Torre LA, Jemal A (2015) Global trends of lung cancer mortality and smoking prevalence. Transl Lung Cancer Res 4:327–338. https://doi.org/10.3978/j.issn.2218-6751.2015.08.04
6. Khan N, Mukhtar H (2015) Dietary agents for prevention and treatment of lung cancer. Cancer Lett 359:155–164. https://doi.org/10.1016/j.canlet.2015.01.038
7. Behera D, Balamugesh T (2004) Lung cancer in India. Indian J Chest Dis Allied Sci 46:269–281
8. Bunn PA Jr (2002) Molecular biology and early diagnosis in lung cancer. Lung Cancer 38:S5–S8. https://doi.org/10.1016/s0169-5002(02)00244-1
9. Bordoloi D, Banik K, Vikkurthi R, Thakur KK, Padmavathi G, Sailo BL, Girisa S, Chinnathambi A, Alahmadi TA, Alharbi SA, Buhrmann C, Shakibaei M, Kunnumakkara AB (2020) Inflection of Akt/mTOR/STAT-3 cascade in TIPE mediated human lung carcinogenesis. Life Sci 262:118475. https://doi.org/10.1016/j.lfs.2020.118475
10. Bordoloi D, Banik K, Padmavathi G, Vikkurthi R, Harsha C, Roy NK, Singh AK, Monisha J, Wang H, Kumar AP, Kunnumakkara AB (2019) TIPE2 induced the proliferation, survival, and migration of lung cancer cells through modulation of Akt/mTOR/NF-kappaB signaling cascade. Biomolecules 9:836. https://doi.org/10.3390/biom9120836
11. Bordoloi D, Banik K, Shabnam B, Padmavathi G, Monisha J, Arfuso F, Dharmarajan A, Mao X, Lim LHK, Wang L, Fan L, Hui KM, Kumar AP, Sethi G, Kunnumakkara AB (2018) TIPE family of proteins and its implications in different chronic diseases. Int J Mol Sci 19:2974. https://doi.org/10.3390/ijms19102974
12. Padmavathi G, Banik K, Monisha J, Bordoloi D, Shabnam B, Arfuso F, Sethi G, Fan L, Kunnumakkara AB (2018) Novel tumor necrosis factor-alpha induced protein eight (TNFAIP8/TIPE) family: functions and downstream targets involved in cancer progression. Cancer Lett 432:260–271. https://doi.org/10.1016/j.canlet.2018.06.017
13. Shen P, Zhang H, Su Z, Wang S, Xu H (2015) In silico analysis of tumor necrosis factor alpha-induced protein 8-like-1 (TIPE1) protein. PLoS One 10:e0134114. https://doi.org/10.1371/journal.pone.0134114
14. Hu W, Feng CM, Liu LY, Li N, Tian F, Du JX, Zhao Y, Xiang XX, Liu K, Zhao PQ (2019) TIPE1 inhibits breast cancer proliferation by downregulating ERK phosphorylation and predicts a favorable prognosis. Front Oncol 9:400. https://doi.org/10.3389/fonc.2019.00400
15. Zhao P, Pang X, Jiang J, Wang L, Zhu X, Yin Y, Zhai Q, Xiang X, Feng F, Xu W (2019) TIPE1 promotes cervical cancer progression by repression of p53 acetylation and is associated with poor cervical cancer outcome. Carcinogenesis 40:592–599. https://doi.org/10.1093/carcin/bgy163
16. Liu W, Chen Y, Xie H, Guo Y, Ren D, Li Y, Jing X, Li D, Wang X, Zhao M, Zhu T, Wang Z, Wei X, Gao F, Wang X, Liu S, Zhang Y, Yi F (2018) TIPE1 suppresses invasion and migration through down-regulating Wnt/beta-catenin pathway in gastric cancer. J Cell Mol Med 22:1103–1117. https://doi.org/10.1111/jcmm.13362
17. Siddiqui MA, Gollavilli PN, Ramesh V, Parma B, Schwab A, Vazakidou ME, Natesan R, Saatci O, Rapa I, Bironzo P, Schuhwerk H, Asangani IA, Sahin O, Volante M, Ceppi P (2020) Thymidylate synthase drives the phenotypes of epithelial-to-mesenchymal transition in non-small cell lung cancer. Br J Cancer 124:281–289. https://doi.org/10.1038/s41416-020-01095-x
18. Zhang Z, Liang X, Gao L, Ma H, Liu X, Pan Y, Yan W, Shan H, Wang Z, Chen YH, Ma C (2015) TIPE1 induces apoptosis by negatively regulating Rac1 activation in hepatocellular carcinoma cells. Oncogene 34:2566–2574. https://doi.org/10.1038/onc.2014.208
19. Wu X, Ma Y, Cheng J, Li X, Zheng H, Jiang L, Zhou R (2017) TIPE1 function as a prognosis predictor and negative regulator of lung cancer. Oncotarget 8:78496–78506. https://doi.org/10.18632/oncotarget.19655
20. Liu Y, Qi X, Zhao Z, Song D, Wang L, Zhai Q, Zhang X, Zhao P, Xiang X (2020) TIPE1-mediated autophagy suppression promotes nasopharyngeal carcinoma cell proliferation via the AMPK/mTOR signalling pathway. J Cell Mol Med 24:9135–9144. https://doi.org/10.1111/jcmm.15550
21. McDonald JW, Pilgram TK (1999) Nuclear expression of p53, p21 and cyclin D1 is increased in bronchioloalveolar carcinoma.

Histopathology 34:439–446. https://doi.org/10.1046/j.1365-2559.1999.00632.x
22. Charafe-Jauffret E, Tarpin C, Bardou VJ, Bertucci F, Ginestier C, Braud AC, Puig B, Geneix J, Hassoun J, Birnbaum D, Jacquemier J, Viens P (2004) Immunophenotypic analysis of inflammatory breast cancers: identification of an 'inflammatory signature'. J Pathol 202:265–273. https://doi.org/10.1002/path.1515
23. Monisha J, Roy NK, Padmavathi G, Banik K, Bordoloi D, Khwairakpam AD, Arfuso F, Chinnathambi A, Alahmadi TA, Alharbi SA, Sethi G, Kumar AP, Kunnumakkara AB (2018) NGAL is downregulated in oral squamous cell carcinoma and leads to increased survival, proliferation, migration and chemoresistance. Cancers (Basel) 10:228. https://doi.org/10.3390/cancers10070228
24. Feitelson MA, Arzumanyan A, Kulathinal RJ, Blain SW, Holcombe RF, Mahajna J, Marino M, Martinez-Chantar ML, Nawroth R, Sanchez-Garcia I, Sharma D, Saxena NK, Singh N, Vlachostergios PJ, Guo S, Honoki K, Fujii H, Georgakilas AG, Bilsland A, Amedei A, Niccolai E, Amin A, Ashraf SS, Boosani CS, Guha G, Ciriolo MR, Aquilano K, Chen S, Mohammed SI, Azmi AS, Bhakta D, Halicka D, Keith WN, Nowsheen S (2015) Sustained proliferation in cancer: mechanisms and novel therapeutic targets. Semin Cancer Biol 35(Suppl):S25–S54. https://doi.org/10.1016/j.semcancer.2015.02.006
25. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C (2006) Clonogenic assay of cells in vitro. Nat Protoc 1:2315–2319. https://doi.org/10.1038/nprot.2006.339
26. Lechuga S, Amin PH, Wolen AR, Ivanov AI (2019) Adducins inhibit lung cancer cell migration through mechanisms involving regulation of cell-matrix adhesion and cadherin-11 expression. Biochim Biophys Acta, Mol Cell Res 1866:395–408. https://doi.org/10.1016/j.bbamcr.2018.10.001
27. Xu L, Deng X (2006) Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. J Biol Chem 281:35567–35575. https://doi.org/10.1074/jbc.M607702200
28. Rao G, Pierobon M, Kim IK, Hsu WH, Deng J, Moon YW, Petricoin EF, Zhang YW, Wang Y, Giaccone G (2017) Inhibition of AKT1 signaling promotes invasion and metastasis of non-small cell lung cancer cells with K-RAS or EGFR mutations. Sci Rep 7:7066. https://doi.org/10.1038/s41598-017-06128-9
29. Haney S, Konen J, Marcus AI, Bazhenov M (2018) The complex ecosystem in non small cell lung cancer invasion. PLoS Comput Biol 14:e1006131. https://doi.org/10.1371/journal.pcbi.1006131
30. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674. https://doi.org/10.1016/j.cell.2011.02.013
31. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68:394–424. https://doi.org/10.3322/caac.21492
32. Hirsch FR, Merrick DT, Franklin WA (2002) Role of biomarkers for early detection of lung cancer and chemoprevention. Eur Respir J 19:1151–1158. https://doi.org/10.1183/09031936.02.00294102
33. Pesch B, Kendzia B, Gustavsson P, Jockel KH, Johnen G, Pohlabeln H, Olsson A, Ahrens W, Gross IM, Bruske I, Wichmann HE, Merletti F, Richiardi L, Simonato L, Fortes C, Siemiatycki J, Parent ME, Consonni D, Landi MT, Caporaso N, Zaridze D, Cassidy A, Szeszenia-Dabrowska N, Rudnai P, Lissowska J, Stucker I, Fabianova E, Dumitru RS, Bencko V, Foretova L, Janout V, Rudin CM, Brennan P, Boffetta P, Straif K, Bruning T (2012) Cigarette smoking and lung cancer—relative risk estimates for the major histological types from a pooled analysis of case-control studies. Int J Cancer 131:1210–1219. https://doi.org/10.1002/ijc.27339
34. Chen ZB, Liu C, Chen FQ, Li SY, Liang Q, Liu LY (2006) Effects of tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on the activation of ERK1/2 MAP kinases and the proliferation of human mammary epithelial cells. Environ Toxicol Pharmacol 22:283–291. https://doi.org/10.1016/j.etap.2006.04.001
35. Xue J, Yang S, Seng S (2014) Mechanisms of cancer induction by tobacco-specific NNK and NNN. Cancers (Basel) 6:1138–1156. https://doi.org/10.3390/cancers6021138
36. Shen J, Xu L, Owonikoko TK, Sun SY, Khuri FR, Curran WJ, Deng X (2012) NNK promotes migration and invasion of lung cancer cells through activation of c-Src/PKCiota/FAK loop. Cancer Lett 318:106–113. https://doi.org/10.1016/j.canlet.2011.12.008
37. Huang RY, Li MY, Hsin MK, Underwood MJ, Ma LT, Mok TS, Warner TD, Chen GG (2011) 4-Methylnitrosamino-1-3-pyridyl-1-butanone (NNK) promotes lung cancer cell survival by stimulating thromboxane A2 and its receptor. Oncogene 30:106–116. https://doi.org/10.1038/onc.2010.390
38. Hecht SS, Hoffmann D (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 9:875–884. https://doi.org/10.1093/carcin/9.6.875
39. Grozio A, Catassi A, Cavalieri Z, Paleari L, Cesario A, Russo P (2007) Nicotine, lung and cancer. Anti Cancer Agents Med Chem 7:461–466. https://doi.org/10.2174/187152007781058587
40. Dasgupta P, Rastogi S, Pillai S, Ordonez-Ercan D, Morris M, Haura E, Chellappan S (2006) Nicotine induces cell proliferation by beta-arrestin-mediated activation of Src and Rb-Raf-1 pathways. J Clin Invest 116:2208–2217. https://doi.org/10.1172/JCI28164
41. Vu AT, Taylor KM, Holman MR, Ding YS, Hearn B, Watson CH (2015) Polycyclic aromatic hydrocarbons in the mainstream smoke of popular U.S. cigarettes. Chem Res Toxicol 28:1616–1626. https://doi.org/10.1021/acs.chemrestox.5b00190
42. Vazquez-Gomez G, Rocha-Zavaleta L, Rodriguez-Sosa M, Petrosyan P, Rubio-Lightbourn J (2018) Benzo[a]pyrene activates an AhR/Src/ERK axis that contributes to CYP1A1 induction and stable DNA adducts formation in lung cells. Toxicol Lett 289:54–62. https://doi.org/10.1016/j.toxlet.2018.03.012
43. Wang BY, Wu SY, Tang SC, Lai CH, Ou CC, Wu MF, Hsiao YM, Ko JL (2015) Benzo[a]pyrene-induced cell cycle progression occurs via ERK-induced Chk1 pathway activation in human lung cancer cells. Mutat Res 773:1–8. https://doi.org/10.1016/j.mrfmmm.2015.01.009
44. Li Y, Gao W, Ma Y, Zhu G, Chen F, Qu H (2018) Dual targeting of survivin and X-linked inhibitor of apoptosis protein suppresses the growth and promotes the apoptosis of gastric cancer HGC-27 cells. Oncol Lett 16:3489–3498. https://doi.org/10.3892/ol.2018.9081
45. Alao JP (2007) The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol Cancer 6:24. https://doi.org/10.1186/1476-4598-6-24
46. Huang H (2018) Matrix metalloproteinase-9 (MMP-9) as a cancer biomarker and MMP-9 biosensors: recent advances. Sensors (Basel) 18:3249. https://doi.org/10.3390/s18103249
47. Shibuya M (2011) Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) signaling in angiogenesis: a crucial target for anti- and pro-angiogenic therapies. Genes Cancer 2:1097–1105. https://doi.org/10.1177/1947601911423031
48. Li P, Zhou L, Zhao T, Liu X, Zhang P, Liu Y, Zheng X, Li Q (2017) Caspase-9: structure, mechanisms and clinical application. Oncotarget 8:23996–24008. https://doi.org/10.18632/oncotarget.15098
49. Satyavarapu EM, Das R, Mandal C, Mukhopadhyay A, Mandal C (2018) Autophagy-independent induction of LC3B through oxidative stress reveals its non-canonical role in anoikis of

ovarian cancer cells. Cell Death Dis 9:934. https://doi.org/10.1038/s41419-018-0989-8
50. Benson EK, Mungamuri SK, Attie O, Kracikova M, Sachidanandam R, Manfredi JJ, Aaronson SA (2014) p53-dependent gene repression through p21 is mediated by recruitment of E2F4 repression complexes. Oncogene 33:3959–3969. https://doi.org/10.1038/onc.2013.378
51. Siveen KS, Ahn KS, Ong TH, Shanmugam MK, Li F, Yap WN, Kumar AP, Fong CW, Tergaonkar V, Hui KM, Sethi G (2014) Y-tocotrienol inhibits angiogenesis-dependent growth of human hepatocellular carcinoma through abrogation of AKT/mTOR pathway in an orthotopic mouse model. Oncotarget 5:1897–1911. https://doi.org/10.18632/oncotarget.1876
52. Roy NK, Parama D, Banik K, Bordoloi D, Devi AK, Thakur KK, Padmavathi G, Shakibaei M, Fan L, Sethi G, Kunnumakkara AB (2019) An update on pharmacological potential of boswellic acids against chronic diseases. Int J Mol Sci 20:4101. https://doi.org/10.3390/ijms20174101
53. Kunnumakkara AB, Banik K, Bordoloi D, Harsha C, Sailo BL, Padmavathi G, Roy NK, Gupta SC, Aggarwal BB (2018) Googling the guggul (commiphora and boswellia) for prevention of chronic diseases. Front Pharmacol 9:686. https://doi.org/10.3389/fphar.2018.00686
54. Mohan CD, Srinivasa V, Rangappa S, Mervin L, Mohan S, Paricharak S, Baday S, Li F, Shanmugam MK, Chinnathambi A, Zayed ME, Alharbi SA, Bender A, Sethi G, Basappa, Rangappa KS (2016) Trisubstituted-imidazoles induce apoptosis in human breast cancer cells by targeting the oncogenic PI3K/Akt/mTOR signaling pathway. PLoS One 11:e0153155. https://doi.org/10.1371/journal.pone.0153155
55. Ong PS, Wang LZ, Dai X, Tseng SH, Loo SJ, Sethi G (2016) Judicious toggling of mTOR activity to combat insulin resistance and cancer: current evidence and perspectives. Front Pharmacol 7:395. https://doi.org/10.3389/fphar.2016.00395
56. Tan SM, Li F, Rajendran P, Kumar AP, Hui KM, Sethi G (2010) Identification of beta-escin as a novel inhibitor of signal transducer and activator of transcription 3/Janus-activated kinase 2 signaling pathway that suppresses proliferation and induces apoptosis in human hepatocellular carcinoma cells. J Pharmacol Exp Ther 334:285–293. https://doi.org/10.1124/jpet.110.165498
57. Kim C, Cho SK, Kapoor S, Kumar A, Vali S, Abbasi T, Kim SH, Sethi G, Ahn KS (2014) Beta-caryophyllene oxide inhibits constitutive and inducible STAT3 signaling pathway through induction of the SHP-1 protein tyrosine phosphatase. Mol Carcinog 53:793–806. https://doi.org/10.1002/mc.22035
58. Chalhoub N, Baker SJ (2009) PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol 4:127–150. https://doi.org/10.1146/annurev.pathol.4.110807.092311
59. Jin HO, Seo SK, Woo SH, Kim YS, Hong SE, Yi JY, Noh WC, Kim EK, Lee JK, Hong SI, Choe TB, Park IC (2011) Redd1 inhibits the invasiveness of non-small cell lung cancer cells. Biochem Biophys Res Commun 407:507–511. https://doi.org/10.1016/j.bbrc.2011.03.047
60. Ha JY, Kim JS, Kang YH, Bok E, Kim YS, Son JH (2014) Tnfaip8 l1/Oxi-beta binds to FBXW5, increasing autophagy through activation of TSC2 in a Parkinson's disease model. J Neurochem 129:527–538. https://doi.org/10.1111/jnc.12643