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Devivasha Bordoloi[a,**], Kishore Banik[a], Rajesh Vikkurthi[a], Krishan Kumar Thakur[a], Ganesan Padmavathi[a], Bethsebie Lalduhsaki Sailo[a], Sosmitha Girisa[a], Arunachalam Chinnathambi[b], Tahani Awad Alahmadi[c], Sulaiman Ali Alharbi[b], Constanze Buhrmann[d], Mehdi Shakibaei[d], Ajaikumar B. Kunnumakkara[a,*]

[a] *Cancer Biology Laboratory, DBT-AIST International Center for Translational and Environmental Research (DAICENTER), Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam 781039, India*
[b] *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*
[c] *Department of Pediatrics, College of Medicine, King Saud University [Medical City], King Khalid University Hospital, PO Box-2925, Riyadh 11461, Saudi Arabia*
[d] *Department of Anatomy, Ludwig-Maximilians-University Munich, Munich, Germany*

1. Introduction

Lung cancer is the foremost cause of cancer-related mortality across the world. It presents a group of histologically as well as molecularly heterogeneous diseases and is classified into two broad histological types such as small cell lung carcinoma (SCLC) and non-SCLC (NSCLC). NSCLCs constitute around 85% of all lung cancer cases and consists of three different subtypes namely adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [1–9]. On the other hand, SCLC; a highly aggressive form which spreads into submucosal lymphatic vessels and regional lymph nodes constitutes around 10–15% of all lung cancers [10]. The standard treatment strategy for patients with advanced NSCLC primarily includes platinum-based therapy followed by second-line cytotoxic chemotherapy with around 1-year median survival [11]. Although cigarette smoking was identified as the single most predominant cause of the lung cancer epidemic, various other factors

* Correspondence to: A.B. Kunnumakkara, Cancer Biology Laboratory and DBT-AIST International Center for Translational and Environmental Research (DAICENTER), Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Room # 005, O-Block, Guwahati, Assam 781039, India.
** Correspondence to: D. Bordoloi, 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.
E-mail addresses: devivasha@iitg.ac.in (D. Bordoloi), kunnumakkara@iitg.ac.in (A.B. Kunnumakkara).

such as exposure to workplace agents, environmental pollution etc. are also found to be associated with lung cancer occurrence [12,13]. Recent advances in early detection and improvements in adjuvant therapies have upstretched hopes for the improved patient survival in the coming days [14,15]. However, management of lung cancer patients still presents a major challenge to the oncologists [16]. This can be primarily attributed to factors such as late stage diagnosis, tumor recurrence and chemoresistance [16–18]. In order to overthrow these major drawbacks, there arises an urgent need to develop effective biomarkers for early diagnosis as well as prognosis which in turn can facilitate successful management of this aggressive cancer type. Notably, TIPE (TNF-alpha induced protein 8), a transcription factor nuclear factor-kappa B (NF-κB) inducible, oncogenic molecule and cytoplasmic protein of 21 kDa which was first identified in human head and neck squamous cell carcinoma holds prospect in the management of lung cancer [19,20]. It is reported to be expressed in different normal tissues in human with a much higher expression in lymphoid tissues and placenta [21]. A sequence in the amino terminus of the open reading frame of this protein shares a notable homology to the death effector domain II of Fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein (FLIP), which is involved in the regulation of cell death [21]. In addition, mTIPE; the crystal structure of TIPE from *Mus musculus*, revealed that it resembles a water dipper. It has a cylindrical domain which is connected to an N-terminal grip-like domain, consisting of 20 residues and two long electron densities. Further, it possesses a hydrophobic cavity lined with highly conserved hydrophobic residues, which provides help in substrate binding inside the cavity or hydrophobic cofactors [22]. Notably, TIPE on its own or alone, is reported not to exert oncogenic properties. Its interaction with Gαi; the inhibitory G-protein, was found to be critical for dopamine D2 receptor short form (D2S receptor) transformation of non-transformed, Gi/Go-coupled D2S receptor transfected mesenchymal Balb/c-3T3 fibroblast cells [20]. Further, deletion of TIPE in intestinal epithelial cells resulted in enhanced cell death as well as decreased cell proliferation implying its vital role in the maintenance of colon homeostasis. TIPE also plays an important role in genetic susceptibility as it was reported to contribute to *Staphylococcus aureus* sepsis in A/J mice [20].

The expression and function of TIPE have been studied in various human cancers. Different studies reveal it to have a strong impact on several clinicopathological characteristics of tumor such as TNM stage, lymph node involvement, distant metastasis etc. Growing evidence suggests that TIPE may serve as a potential therapeutic target against a range of neoplasms. Further, it is also reported to possess potential as a prognostic marker for the patients with different cancer types [23–50] (Table 1).

In the present study, we evaluated the expression of TIPE in lung cancer tissues and its role in different processes involved in the development and progression of lung cancer. We found that TIPE plays a pivotal role in the survival, proliferation, invasion, and migration of lung cancer cells through modulation of Akt/mTOR/STAT-3 signaling. Signal transducer and activator of transcription-3 (STAT-3) plays a vital role in the pathogenesis of diverse cancers through regulation of different tumorigenic proteins. Further, alterations in Akt and dysregulation of upstream activators and downstream effectors of mammalian target of rapamycin (mTOR) are observed in case of malignancy [51–58]. As STAT-3 and Akt/mTOR pathways are often reported to be deregulated in case of lung cancer, therefore their negative regulation seems to form a strong basis for the identification of novel biomarkers as well as targeted therapy against this cancer type [4,59]. Furthermore, tobacco components are well known to modulate the growth, survival, migration and invasion of lung cancer cells [60]. Therefore, we also evaluated the effect of four different tobacco components such as nicotine, NNK, NNN, and BaP on NCIH460 human lung cancer cells after knockout of TIPE. The results showed TIPE to be involved in the effective mediation of tobacco induced proliferation, survival and migration of human lung cancer cells.

Table 1
Involvement of TIPE in different cancers.
(Table adapted and modified from Bordoloi et al. [19].)

Status of TIPE; model	Targets/outcome	References
Breast cancer		
↑TIPE; tissue samples	Tumor progression	[23]
↓TIPE; MCF-7 cells	↑p21	[48]
↓TIPE; MDA-231, LM2-4175 cells	↑SNX1, ↑NR4A1, ↑AP2A1, ↓IL5, ↓SRC, ↓MAPT, ↓NEK2, ↓TRAF4, ↓PDCL, ↓GTF2F2, ↓GRAP2, ↓ABL1, ↓AKAP2, ↓GAP43, ↓PIK3CA, ↓EGFR	[45]
TIPE, HS578T, MCF-7 cells	–	[50]
↓TIPE, MDA-MB-435 cells, athymic mice	↓VEGFR-2, ↓MMP-1, ↓MMP-9`	[24]
↑TIPE, MDAMB-231 cells	–	[26]
Cervical cancer		
↑TIPE, tissue samples	Platinum resistance	[25]
Colon cancer		
↑TIPE, CACO2, HCT116	↑Cyclin D1, ↑phospho-Rb	[27]
↓TIPE, HCT116	↑p21	[48]
TIPE; HCT116	–	[49]
Endometrial cancer		
↑TIPE, tumor specimens	↑Ki-67, ↑MMP-9	[28]
Esophageal squamous cell carcinoma		
↑TIPE, TE-1, TE-8, TE-15 cells	–	[30]
↓TIPE, Eca109 cells	↑Apoptosis	[29]
Gastric cancer		
↓TIPE; BGC823 cells	Modulation of caspase-3, -8, -9	[32]
↓TIPE; athymic mice	↓tumor growth	
↑TIPE; tissue samples	↑metastasis, ↓prognosis	[33]
↑TIPE; MKN-28, SGC-7901, MGC-803 cells	–	[31]
↑TIPE; tissue samples	–	[34]
Hepatocellular carcinoma		
↑TIPE; Bel7402, SK-Hep-1, HepG2, SMMC7721, Huh7 cells	↓YAP phosphorylation	[35]
Lung cancer		
↑TIPE; H460, H1299 cells	↓Phosphorylated LATS1	[37]
↓TIPE; A549 cells	↑p21	[48]
TIPE; H1299 cells	–	[49]
↑TIPE; tissue samples	–	[38]
↑TIPE; tissue samples	–	[39]
Melanoma		
↓TIPE; MDA-MB435 cells	↑NR4A1, ↑AP2A1, ↓TOP2A, ↓EGFR, ↓PDCL, ↓IL5, ↓GRAP2, ↓GTF2F2, ↓AKAP2, ↓GAP43, ↓ABL1	[45]
Non-Hodgkin lymphoma		
TIPE; 514 NHL patients, 557 cancer-free controls	Polymorphism rs1045241C > T	[36]
Osteosarcoma		
↑TIPE; 143b, LM7, HOS, SaOS-2, U2OS, MG-63 cells	Modulation of miR-138	[43]
↑TIPE; KHOS, 143b, LM7, U2OS, MG-63 cells	Modulation of miR-99a	[42]
↓TIPE; U2OS cells	↑p21	[48]
Ovarian cancer		
↑TIPE; tissue samples	↓Survival	[40]
↑TIPE; tissue samples	Platinum resistance	[41]
Pancreatic cancer		
↑TIPE; tissue samples`	↑EGFR	[44]
Prostate cancer		
↓TIPE; PC-3 cells	↑IGFBP3, ↑NR4A1, ↑AP2A1, ↓IL5, ↓MAPT, ↓TOP2A, ↓TRAF4, ↓EGFR, ↓PDCL, ↓GTF2F2, ↓GRAP2, ↓ABL1, ↓GAP43, ↓AKAP2, ↓GRIP1	[45]
↑TIPE; LNCaP, PC-3, DU-145 cells	↑integrin, ↑MMP, ↑VEGFR-2	[46]

(*continued on next page*)

Table 1 (*continued*)

Status of TIPE; model	Targets/outcome	References
Renal cancer		
↑TIPE; RCC-RS cells	–	[26]
Thyroid cancer		
↑TIPE; tissue samples	–	[47]

2. Materials and methods

2.1. Tissue microarray

Immunohistochemical analysis was done to analyze the expression of TIPE in different stages, grades and pathological conditions of lung cancer tissues compared to normal lung tissues. For this analysis, tissue microarray (TMA) which contained paraffin-embedded tissues (US Biomax, Inc., Cat. No. LC1503) from different individuals were used. The slide comprised of a total of 75 tissues (duplicated cores from the same patient in all cases) 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 (Suppl. Table 1).

2.2. Immunohistochemistry

Immunohistochemical analysis was done using Histostain-Plus IHC Kit, HRP, broad spectrum (Invitrogen, Cat. No. 859043; CA, USA), Metal enhanced DAB Substrate Kit (Cat No. 34065; Invitrogen, CA, USA) and anti-TIPE primary antibody (Cat. No. ab64988; abcam®, Cambridge, USA; 1:50 dilution) following the manufacturer's protocol. The immunostained slide was analyzed under Olympus light microscope. Brown color stained tissues are taken as positive for the presence of antigen of interest and scored as per the staining intensity (scaled from 1 to 3) and number of positive cells (scaled from 0 to 4 +) [61,62]. The scoring was done twice, independently (blinded fashion) and then average was taken into consideration.

2.3. The Cancer Genome Atlas (TCGA) dataset analysis

Open data portal of the TCGA and cbioportal were used for procuring information regarding the genetic alteration of TIPE in the samples of non-small cell lung cancer (NSCLC) patients (http://www.cbioportal.org). Evaluation of NSCLC patients survival linked with alteration in TIPE was done with the help of Kaplan-Meier survival curve [56,63–65].

2.4. Cell culture

NCIH460 lung cancer cells were obtained from 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 in a CO_2 incubator.

2.5. CRISPR/Cas9 mediated gene knockout

For performing gene knockout studies, CRISPR/Cas9 mediated gene editing tool was used. NCIH460 cells were seeded in a 24 well plate at a density of 25,000 cells/well and allowed to attain confluency by 70–80%. The cells were then transfected with CRISPR/Cas9 All-in-One Lentivector sets (Human) expressing human Cas9 and scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (Cat. No. K010; Applied Biological Materials, Richmond, BC, Canada) and TNFAIP8 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No K2414505; Applied Biological Materials, Richmond, BC, Canada) with the help of Lentifectin™ transfection reagent (Cat. No. G074, Applied Biological Materials, Richmond, BC, Canada) in incomplete opti-MEM media (Suppl. Table 2). Then, 10% FBS (Gibco®, NY, USA) was added to the transfected cells after 5–8 h and post 24 h incubation, fresh DMEM medium (with 10% FBS and 1× Penstrep) was added to the cells after removing the previous plasmid containing media. Upon recovery for 24 h, positive selection of cells was carried out by adding puromycin (Cat. No. P8833, Sigma-Aldrich, Missouri, USA) at the concentration of 2.5 μg/ml to the cells. As the CRISPR/Cas9 plasmids have the puromycin resistance gene, therefore the transfected cells should only exhibit puromycin resistance. Subsequently, the puromycin resistant cells were allowed to grow followed by single cell selection which was carried out by plating single cell per 100 μl in 96 well plates. Finally, selected single cell clones were confirmed for TIPE knockout with the help of Western blot analysis. The clones exhibiting complete inhibition of the expression of TIPE protein was regarded as the one with successful TIPE knockout and was grown further for post-knockout studies.

2.6. Cell proliferation assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed to evaluate the effect of TIPE knockout on the proliferation of lung cancer cells. Further, effect on the proliferation of tobacco components' such as nicotine-derived nitrosamine ketone (NNK) (Cat No. 78013; Sigma-Aldrich, Missouri, USA), N-nitrosonornicotine (NNN) (Cat No. 75285; Sigma-Aldrich, Missouri, USA), nicotine (Cat No. N3876; Sigma-Aldrich, Missouri, USA) and Benzo(*a*)pyrene (BaP) (Cat No. B1760; Sigma-Aldrich, Missouri, USA) treated TIPE knockout cells was also determined using this assay. Firstly, 2×10^3 cells/well of scrambled control and TIPE knockout cells were seeded in 96 well plates and incubated for 24 h in a CO_2 incubator. After 0 and 72 h, MTT (Cat. No. M2128, Sigma-Aldrich, Missouri, USA) was added to the wells. After incubation for 2 h, 100 μl of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added upon removing the culture medium followed by 1 h incubation at room temperature and then absorbance was measured at 570 nm with a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland). In case of evaluation of the effect on the proliferation of tobacco treated TIPE knockout cells, after 24 h incubation of the seeded cells, NNK (0.05 μM), NNN (0.05 μM), nicotine (1 μM) and BaP (0.25 μg/ml) were added to the scrambled control as well as TIPE knockout cells. MTT was added at 0 and 24 h and the same procedure was followed henceforth. % proliferation was determined by normalizing with the 0 h absorbance value and considering the absorbance of scrambled control cells as 100%. For evaluating the proliferation in NNK, NNN, nicotine and BaP treated TIPE knockout cells, scrambled control cells treated with the respective components was considered as 100%.

2.7. Colony formation assay

The colony forming ability of TIPE knockout lung cancer cells was evaluated using this assay. In addition, this assay was also used to evaluate the colony formation ability of tobacco components-treated TIPE knockout cells. Briefly, Scrambled control and TIPE knockout cells were seeded at a low density in 6 well plates and allowed to grow for 2 weeks with frequent replacement of media. In case of evaluation of the clonogenic potential of tobacco components-treated TIPE knockout cells, NNK (0.05 μM), NNN (0.05 μM), nicotine (1 μM) and BaP (0.25 μg/ml) were added to the scrambled control and TIPE knockout cells after 24 h incubation of the seeded cells and allowed to grow for a week. The colonies formed in both the cases were then fixed with 70% ethanol followed by crystal violet (Cat No: 548-6209; SRL Pvt. Ltd., India) staining. The images of the colonies formed were captured and the survival fraction was determined.

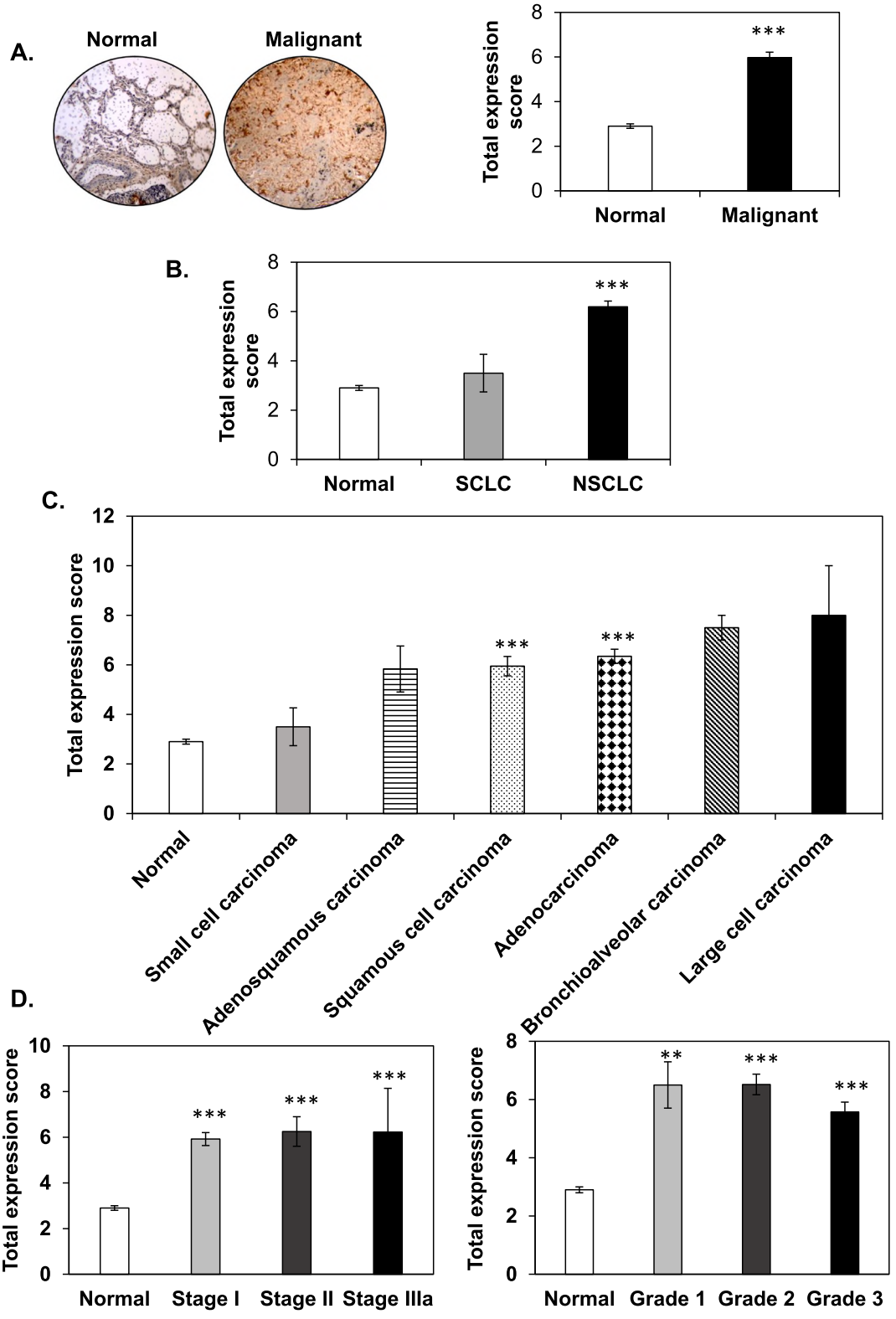


Fig. 1. Immunohistochemical analysis of TIPE expression in lung cancer tissues. A. Representative images of TIPE expression in lung cancer tissues (left panel). Graphical representation of TIPE expression in lung cancer tissues compared to non-malignant lung tissues (right panel); B. TIPE expression in NSCLC and SCLC; C. TIPE expression in small cell carcinoma, adenosquamous carcinoma, squamous cell carcinoma, adenocarcinoma, bronchioalveolar carcinoma and large cell carcinoma; D. TIPE expression in different stages of lung cancer i.e. stage I, II and III (left panel). TIPE expression in different grades of lung tumor i.e. grade 1, 2, and 3 (right panel); data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

2.8. Migration and Invasion assays

Migration assay was performed to assess the effect of TIPE knockout on the migration of human lung cancer cells. Further, the effect of tobacco components on the migration potential of TIPE knockout cells was also determined with this assay. Initially, 6×10^5 cells/well of scrambled control and TIPE knockout cells were seeded and waited until the formation of a monolayer. Subsequently, serum free DMEM medium was added to the cells, incubated for 6–8 h and then a wound was created in the culture well. The migration of the cells was determined by monitoring the wound area at different time intervals using an inverted microscope (Nikon T1-SM, Japan) and images were captured and analyzed using ImageJ software. For analyzing the effect of treatment with tobacco components, after serum starvation followed by scratching of wound, NNK (0.05 μM), NNN (0.05 μM), nicotine (1 μM), and BaP (0.25 μg/ml) were added to the cells and the same procedure was adopted thereafter. Further, the effect on the invasion of NCIH460 lung cancer cells upon TIPE knockout was evaluated using a Boyden chamber assay, for which serum starved (for 18 h) cells were seeded (5×10^4 cells/500 μl serum free DMEM medium) in the upper chamber of the transwell insert pre-coated with matrigel (24-well, 8 mm; Cat No. 354480; Corning, New york, USA). Subsequently, DMEM medium with 10% FBS was added to the lower chamber followed by incubation in a CO_2 regulated incubator for 24 h. The cells on the upper surface of the membrane were then scraped off using cotton swabs and those at the bottom of the insert were fixed using 70% ethanol. They were then stained using 0.01 % (w/v) crystal violet, eluted and finally absorbance was measured at 590 nm uisng a plate reader (TECAN Infinite 200 PRO multimode reader, Switzerland) and % invaded cells were determined.

2.9. Western blot

Western blot analysis was performed to determine the different targets of TIPE and also to evaluate effect on different proteins in tobacco components (NNK; 0.05 μM, NNN; 0.05 μM, nicotine; 1 μM and BaP; 0.25 μg/ml) treated TIPE knockout cells. Briefly, cells were lysed using whole cell lysis buffer (20 mM HEPES, 2 mM EDTA, 250 mM NaCl, 0.1% (v/v) Triton-X100, 2 μg/ml leupeptin hemisulfate, 2 μg/ml aprotinin, 1 mM PMSF, 1 mM DTT) and the protein concentration was determined using Bradford reagent (Cat. No. 500-0205; Bio-Rad, California, USA). 50 μg of proteins were mixed with 5× Laemmli Buffer (250 mM Tris HCl, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) and then resolved in a SDS-Polyacrylamide gel (12% or 8%). The proteins were then transferred to nitrocellulose membrane (Bio-Rad, California, USA), blocked and then probed with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Suppl. Table 3). Clarity Western ECL Substrate (Cat. No. 1705061; Bio-Rad, California, USA) in a ChemiDoc™ XRS System (Bio-Rad, California, USA) was used for visualizing the bands. The house keeping gene α-tubulin was used as the loading control.

2.10. Statistical analysis

Student's t-test was done for the statistical analysis. The data are represented as mean ± SE. p-Value < 0.05 indicates statistically significant; p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

3. Results

In this study, firstly we determined the expression of TIPE in lung cancer tissues of different histological types, pathological conditions, stages and grades of lung tumor. In addition, this study unravels the role of TIPE in the regulation of different process in lung cancer cells along with underlined mechanism. It is well established that tobacco is the major risk factor of lung cancer. Therefore, in this study we also deciphered the role of this important protein in the modulation of tobacco mediated proliferation, survival and migration of lung cancer cells and the mechanism involved.

3.1. Expression analysis of TIPE in human lung cancer tissues

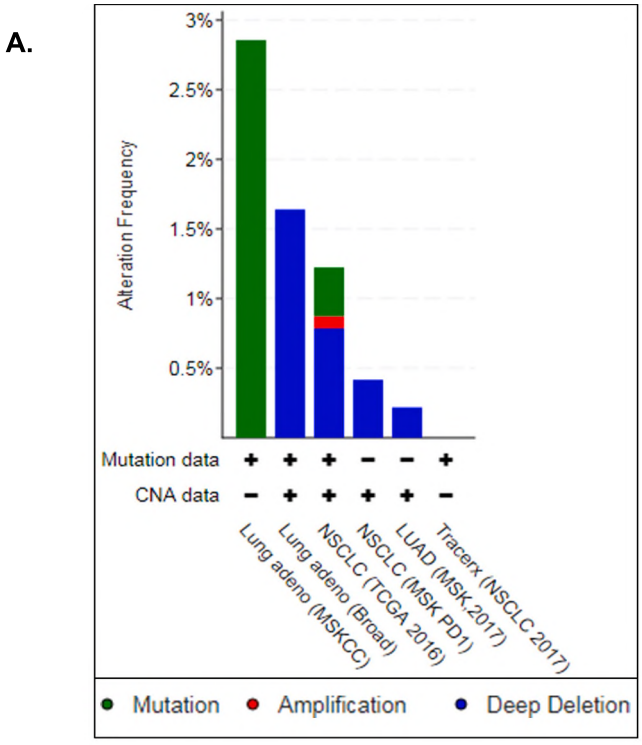
In the present study, we first analyzed the expression of the TIPE protein in lung cancer tissues. Our analysis revealed TIPE to be significantly upregulated in lung cancer tissues compared to non-malignant lung tissues (Fig. 1A). Notably, TIPE exerted around 2-fold increase in its expression in the malignant lung tissues when compared to the normal lung tissues. In addition, the expression of TIPE was analyzed in both SCLC and NSCLC, two major histological types of lung cancer and it was found to be upregulated in both SCLC and NSCLC tissues compared to the normal tissues. Interestingly, TIPE exerted more than 2-fold increase in its expression in NSCLC type whereas in case of SCLC, the increase was not found to be significant (Fig. 1B). Besides, upon analyzing the expression of TIPE with respect to disease pathology, it was found to be significantly upregulated in squamous cell carcinoma and adenocarcinoma tissues compared to the normal tissues. Furthermore, upregulation of TIPE was also observed in other disease pathologies such as adenosquamous cell carcinoma, small cell carcinoma, large cell carcinoma and bronchioalveolar carcinoma compared to the normal tissues (Fig. 1C). In addition, analysis of the stage as well as grade wise difference in the expression of TIPE, it was observed that TIPE exerted significant upregulation in different stages of lung cancer such as stage I, II and IIIa and different grades of lung tumor which include grade 1, 2 and 3 in comparison with normal tissues (Fig. 1D).

3.2. Association between genetic alteration of TIPE and overall survival of NSCLC patients

The genetic alterations in TIPE in the tissues of different NSCLC patients were studied. 3% genetic alteration was found to be present in TIPE as analyzed in a total of 1144 NSCLC cases in TCGA datasets. Different alterations present in TIPE include mutations such as missense mutation, truncating mutation, amplification and deep deletion (Fig. 2A). Upon consideration of the univariate analysis for NSCLC patients' survival data from the TCGA portal, decreased overall survival (OS) of NSCLC patients was observed with the increased copiousness of alterations in TIPE. The median survival of a total 11 cases with TIPE alteration was reported to be 26.3 months. On the other hand, in 943 cases without TIPE alteration, median survival was reported to be 43.91 months (Fig. 2B). However, the number of TIPE mutant patients is low and the survival differences are not statistically significant, hence analysis with greater number of NSCLC patients would be more substantial.

3.3. Effect of TIPE knockout on the proliferation of lung cancer cells

The effect of knockout of TIPE on the proliferation of human lung cancer cells was determined with the help of MTT assay. Our results showed that knockout of TIPE caused significantly decreased proliferation of NCIH460 cells. Further, we determined the effect of NNK, NNN, nicotine and BaP on the proliferation of NCIH460 human lung cancer cells after knockout of TIPE. After treating with all the four tobacco components separately, a significant decrease in the proliferation of TIPE knockout lung cancer cells was observed in comparison with scrambled control cells treated with the respective components. The highest inhibition in the proliferation was observed in nicotine treated TIPE knockout cells followed by BaP, NNK and NNN treated TIPE knockout cells respectively. Thus, these results suggest that TIPE is involved in the positive regulation of the proliferation of lung cancer cells. In addition, it is also found to modulate tobacco induced proliferation of lung cancer cells effectively (Fig. 3).



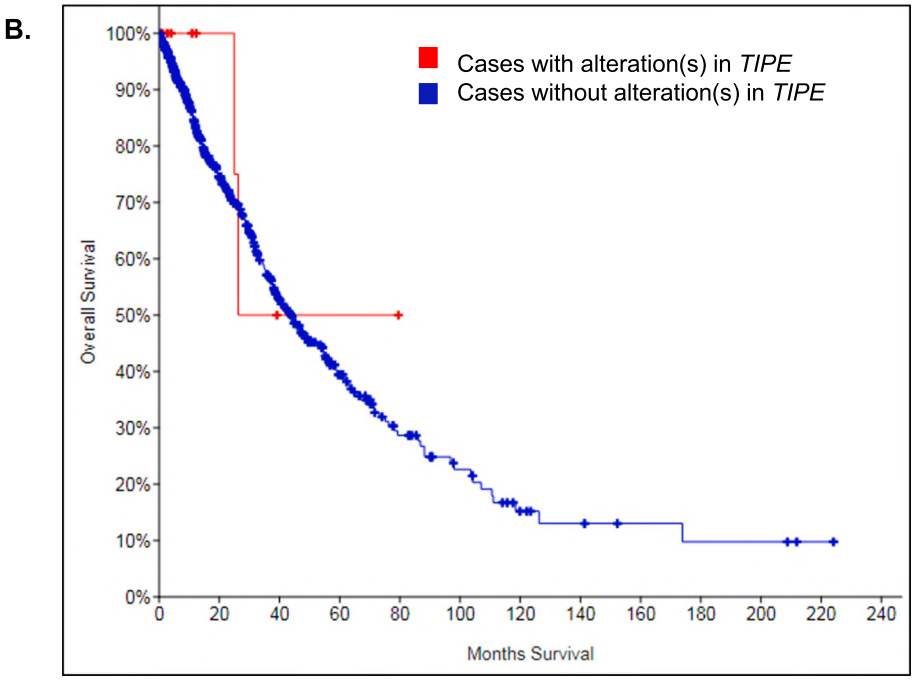


Fig. 2. Genetic alterations in TIPE in NSCLC tissue samples and its relationship with patients' overall survival (OS) in case of NSCLC. A. Genetic alterations such as mutation, amplification and deep deletion in TIPE of NSCLC patients as per data procured from TCGA portal. B. Association between genetic alterations in TIPE with OS of patients with NSCLC.

3.4. Effect of TIPE knockout on the clonogenic potential of lung cancer cells

Colony formation assay was performed to measure the survival fraction of human lung cancer cells after knockout of TIPE. The results of our findings showed that knockout of TIPE led to significantly reduced clonogenic potential of NCIH460 cells compared to scrambled control cells implying the involvement of TIPE in enhancing the survival of lung cancer cells. In addition, similar to the proliferation assay, in this assay as well, decreased survival fraction of TIPE knockout cells treated with different tobacco constituents such as NNK, NNN, nicotine and BaP were observed than the scrambled control cells treated with the respective components. The highest reduction in survival fraction was observed in case of nicotine treated TIPE knockout cells followed by NNK, NNN and Bap treated TIPE knockout lung cancer cells

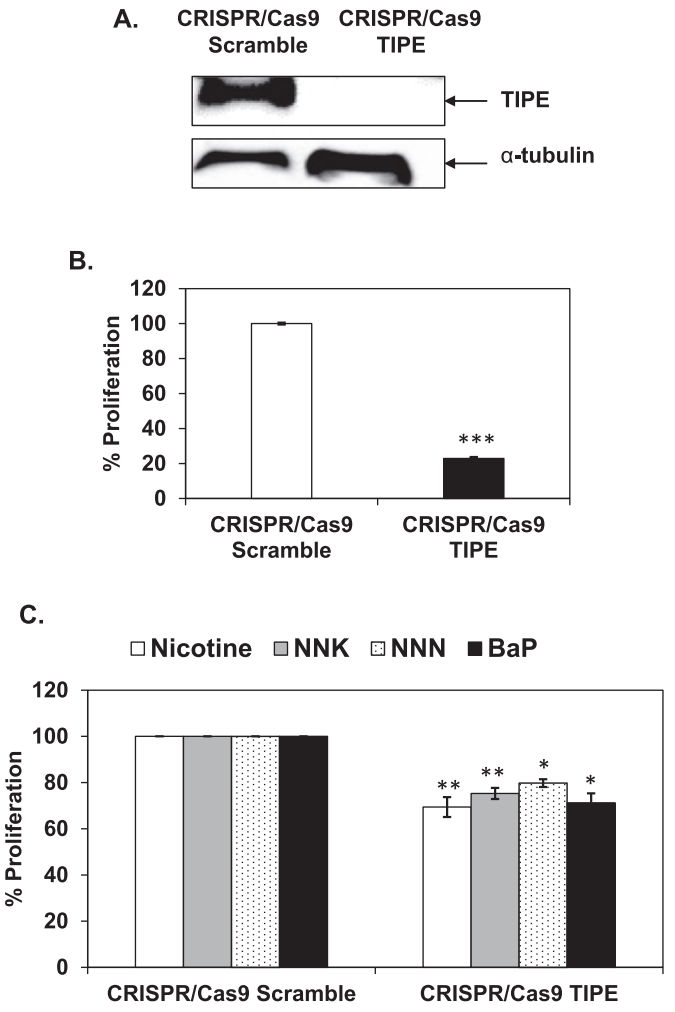


Fig. 3. Role of TIPE on the regulation of the lung cancer cells' proliferation. A. TIPE expression after CRISPR/Cas9 mediated knockout in NCIH460 lung cancer cells as shown by Western blot analysis; B. effect of TIPE knockout on NCIH460 lung cancer cells' proliferation shown using MTT assay; C. effect of TIPE knockout on nicotine, NNK, NNN and BaP mediated proliferation of NCIH460 lung cancer cells observed from MTT assay compared to scrambled control treated with the respective components. Data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

(Fig. 4). Thus, these results clearly imply that TIPE is responsible for the positive regulation of the lung cancer cell survival. Further, it is also strongly associated with the regulation of tobacco induced survival of lung cancer cells.

3.5. Effect of TIPE knockout on the migration of lung cancer cells

In order to decipher whether TIPE has any role in the migration of lung cancer cells, migration assay was carried out. The results showed that loss of TIPE inhibited the migration potential of lung cancer cells effectively. In scrambled control cells, complete healing of wound was observed at 24 h. However, in case of TIPE knockout lung cancer cells, 92% wound area was found to remain. In case of NNK, NNN, nicotine and BaP treated scrambled control cells, complete healing of the wound was observed at 12 h. Increasing lines of evidence suggest that tobacco and its components have significant involvement in cancer cell migration [66–68]. Therefore, in tobacco treated cells, complete healing of the wound was observed 12 h prior to the untreated scrambled control cells. However, in case tobacco components treated TIPE knockout cells, significantly more wound area was observed to remain at 12 h. Notably, in all the four tobacco components treated TIPE knockout cells, more than 60% wound area was found to remain (Fig. 5A–C). Therefore, TIPE can be considered to have important role in the regulation of the migration potential of lung cancer cells. Further, it is also involved in the regulation of tobacco mediated migration of NCIH460 cells.

3.6. Effect of TIPE knockout on the invasion of lung cancer cells

Boyden chamber assay was carried out to determine whether knockout of TIPE has any effect on the invasive potential of human lung cancer cells. We observed that the % invaded cells to the lower part of the transwell insert were significantly less in TIPE knockout NCIH460 cells compared to scrambled control cells (Fig. 5D). Thus, TIPE is involved in the regulation of invasive potential of lung cancer cells as loss of TIPE caused decreased invasion of NCIH460 lung cancer cells.

3.7. Effect of TIPE knockout on signaling molecules/pathways in lung cancer cells

The findings obtained thus far in this study indicate TIPE to have vital role not only in the proliferation but also in the survival, migration and invasion of human lung cancer cells. Notably, there are various signaling molecules/pathways associated with diverse cancer hallmarks [69]. Thus, modulation of these pathways inflects cancer cell growth, proliferation, survival, migration, invasion etc. [69,70]. Therefore, it is critical to know the involvement of these signaling molecules/pathways and hence expression analysis of different proteins with the help of Western blot was performed. Notably, loss of TIPE downregulated the expression of apoptosis regulatory proteins such as survivin, XIAP, Bcl-2 and upregulated the expression of caspase 9. Further, loss of TIPE resulted in the downregulation of Cox-2, c-Myc and Cyclin D1 effectively. Notably, in TIPE knockout cells, downregulation in the expression of LC-3B was also observed. In addition, knockout of TIPE resulted in the downregulation of CXCR-4, MMP-9 and VEGF-A which are involved in cancer cell invasion, migration, metastasis and angiogenesis [71,72]. Importantly, loss of TIPE expression led to the upregulation of two tumor suppressors namely p53 and p21 effectively (Fig. 6A). Emerging evidence suggests that PI3K/Akt/mTOR pathway plays a vital role in oncogenesis and is often found to be activated in lung cancer [73]. Aberrations in different messenger molecules of this pathway lead to proliferation, inhibition of apoptosis, angiogenesis and metastasis of tumor cells [74,75]. Therefore, we determined whether TIPE mediated lung cancer has any involvement with this signaling axis. The results suggested that knockout of TIPE affected the important components of Akt/mTOR pathway. Loss of TIPE downregulated the expression of p-Akt^{S473}, p-mTOR^{S2448}, and p-S6$^{S235/236}$. Further, an upregulation in the expression of PTEN was observed. The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a phosphatase which antagonizes phosphoinositol-3-kinase/Akt pathway resulting in reduced cell proliferation and survival [62,76–81]. Thus, TIPE plays role in the positive regulation of lung cancer through activation of Akt/mTOR signaling pathway. Further, reports suggest that interaction between STAT-3 and Akt signaling pathway plays an important role in tumor development and progression in various cancers [77–91]. Constitutive activation of PI3K/Akt leads to the aberrant activity of STAT-3 [5,92–98]. Our results showed that knockout of TIPE downregulated the expression of p-STAT-3^{S727} notably (Fig. 6B). Altogether, loss of TIPE can be suggested to reduce the proliferation, survival, invasion and migration of lung cancer cells through inactivation of Akt/mTOR/STAT-3 signaling. Noteworthy, this is the first report which indicates the involvement of Akt/mTOR/STAT-3 signaling axis in TIPE mediated lung tumorigenesis.

In this study, we further determined the mechanism of action of TIPE in NNK, NNN, nicotine and BaP treated TIPE knockout cells. NNK treated TIPE knockout cells showed downregulation in the expression

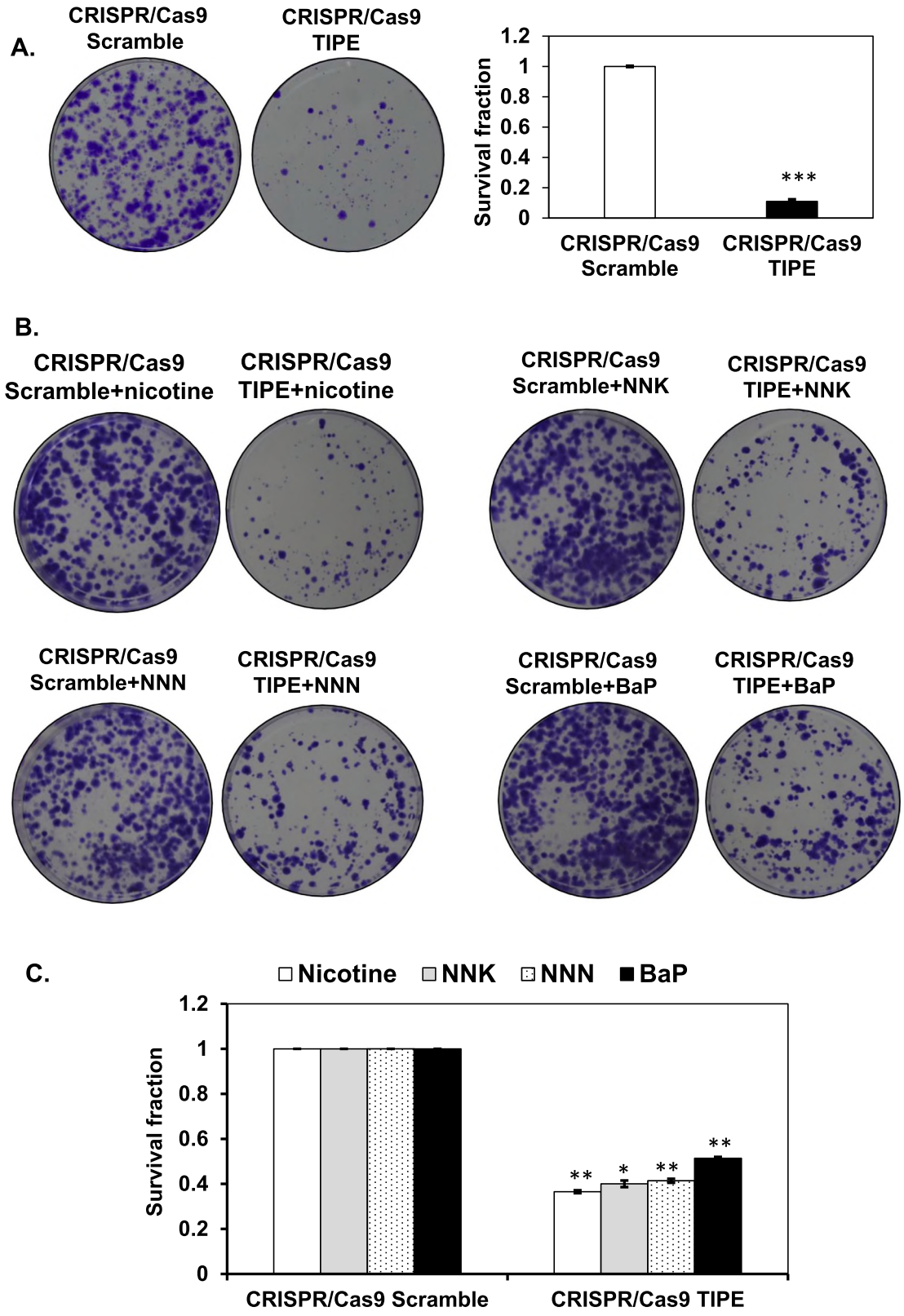


Fig. 4. Role of TIPE on the regulation of the lung cancer cells' survival. A. Representative images of clonogenic potential of TIPE knockout NCIH460 lung cancer cells compared to scrambled control cells shown using colony formation assay (left panel). Graphical representation of the effect of TIPE knockout on the survival fraction of NCIH460 lung cancer cells observed from colony formation assay (right panel); B. representative images showing the colony formation ability of TIPE knockout NCIH460 lung cancer cells treated with nicotine, NNK, NNN and BaP in comparison with scrambled control cells treated with the respective components; C. graphical representation of the survival fraction of tobacco components treated TIPE knockout lung cancer cells in comparison with scrambled control cells treated with the same. Data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

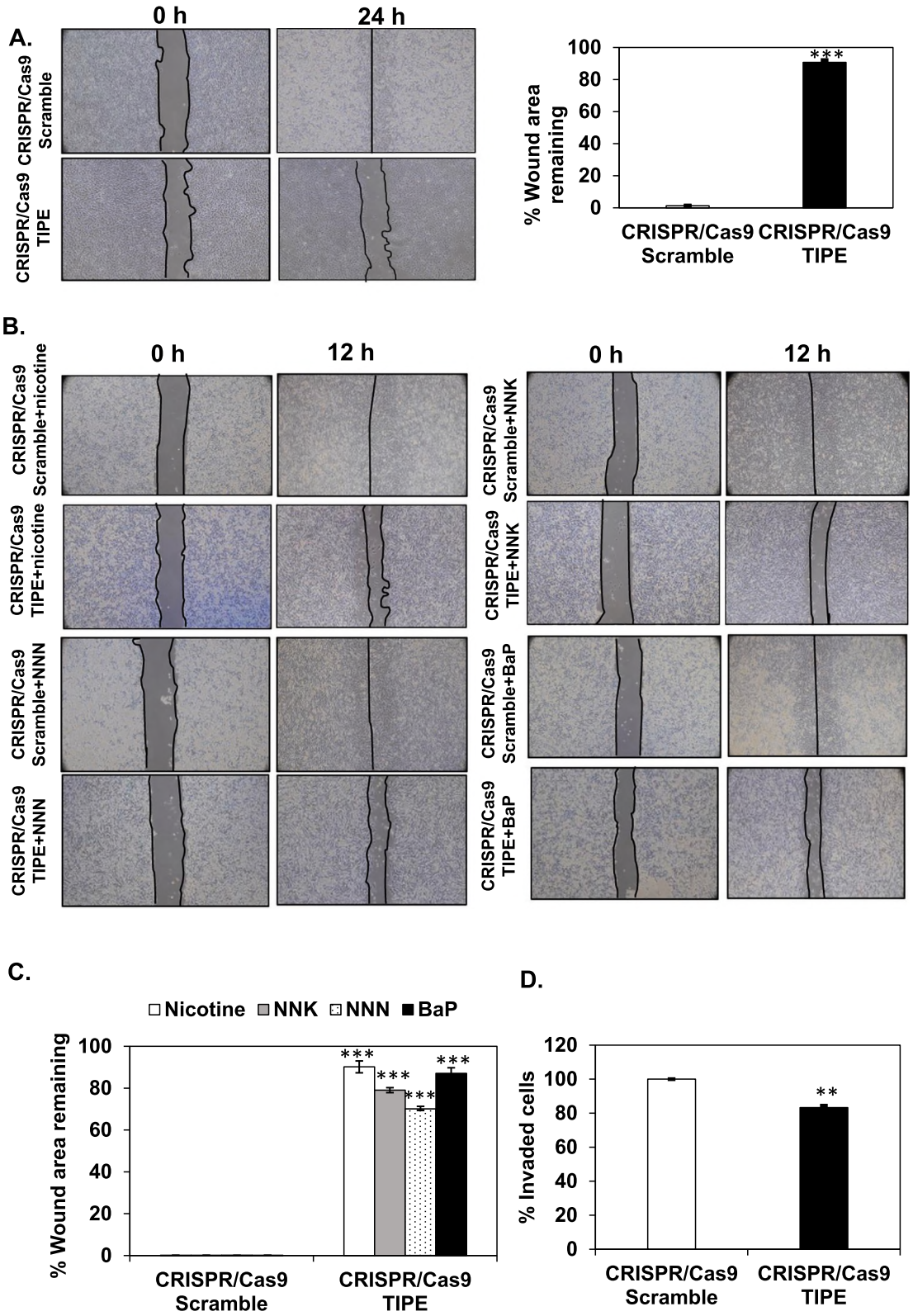


Fig. 5. Role of TIPE on the regulation of the lung cancer cells' migration and invasion. A. Effect of TIPE knockout on NCIH460 cells' migration determined using wound healing assay. Images were captured at 0 and 24 h (10× magnification) (left panel). Graphical representation of the percent wound area remaining in TIPE knockout lung cancer cells in comparison with scrambled control cells (right panel); B. representative images of the effect of TIPE knockout on the migration of nicotine, NNK, NNN and BaP treated TIPE knockout cells along with scrambled control cells treated with the respective components; C. graphical representation of percent wound area remaining in different tobacco components treated TIPE knockout cells in comparison with scrambled control cells treated with the same; D. effect of TIPE knockout on the invasion potential of NCIH460 lung cancer cells shown using Boyden chamber assay (right panel). Data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

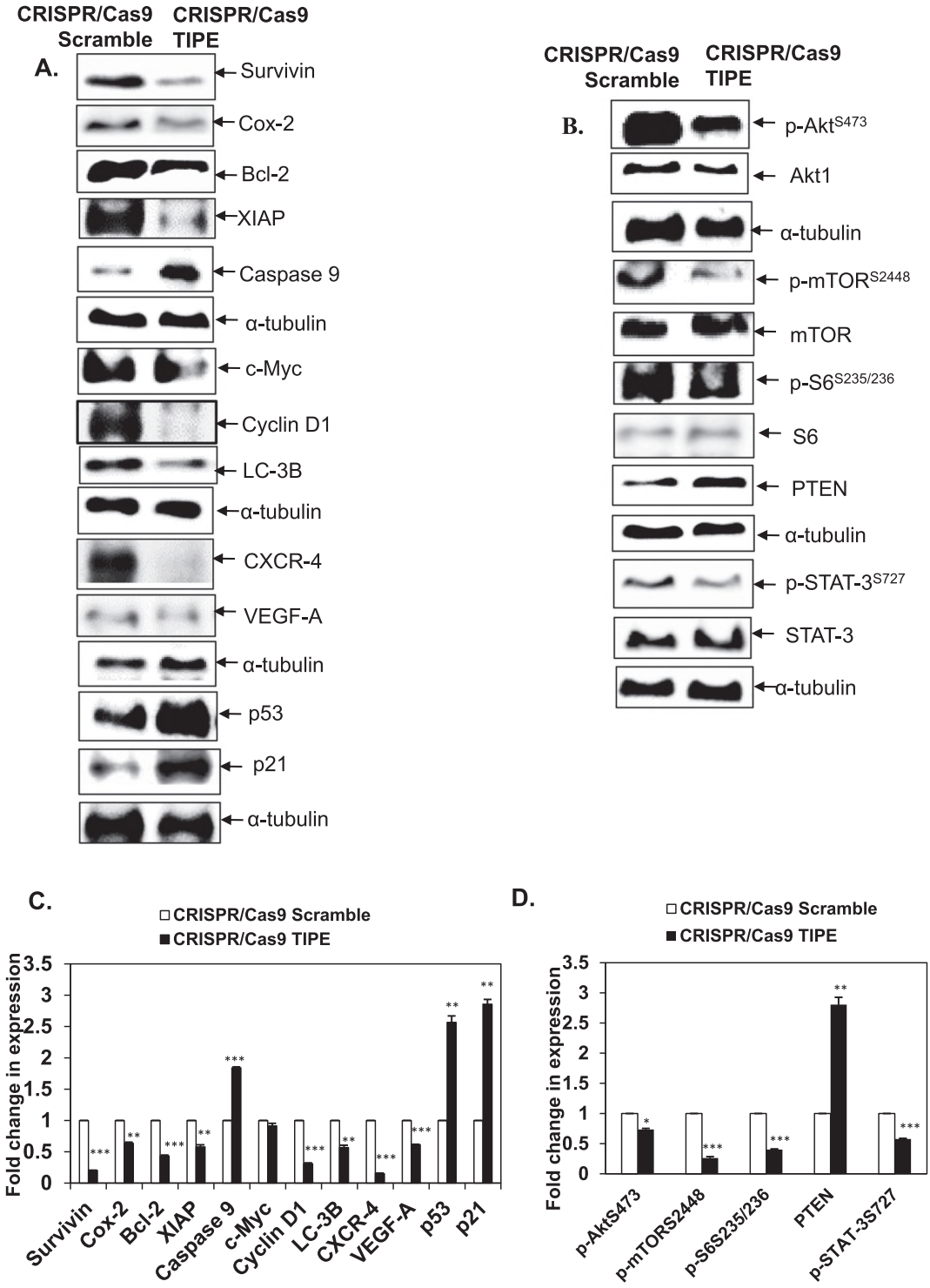


Fig. 6. Role of TIPE in the regulation of different signaling molecules/pathways in lung cancer cells. A. Modulation of the expression of different proteins involved in diverse cellular processes in cancer cells such as proliferation, survival, regulation of apoptosis and cell cycle progression, autophagy, invasion, migration, angiogenesis in TIPE knockout NCIH460 lung cancer cells (left panel) B. modulation of Akt/mTOR/STAT-3 signaling proteins in TIPE knockout NCIH460 lung cancer cells. α-tubulin served as the loading control; C. densitometric analysis of proliferation, survival, apoptosis, cell cycle progression, autophagy, invasion, migration and angiogenesis regulatory proteins in TIPE knockout lung cancer cells; D. densitometric analysis of Akt/mTOR/STAT-3 signaling proteins in TIPE knockout lung cancer cells. Densitometry was done using Image Lab software. α-Tubulin served as the loading control. Data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

of proteins involved in survival and angiogenesis such as survivin and VEGF-A compared to scrambled control cells treated with NNK. Further, upregulation in the expression of p53 and p21 tumor suppressor proteins were also observed. In addition, NNK treated TIPE knockout cells showed downregulation of p-Akt^{S473}. Thus, these results suggest that TIPE is involved in the positive regulation of tobacco mediated proliferation, survival and migration of lung cancer cells through Akt signaling axis. Akt is involved in the regulation of different cellular processes which include glucose metabolism, cell cycle progression, apoptosis etc. In 90% of the NSCLC cells, constitutive

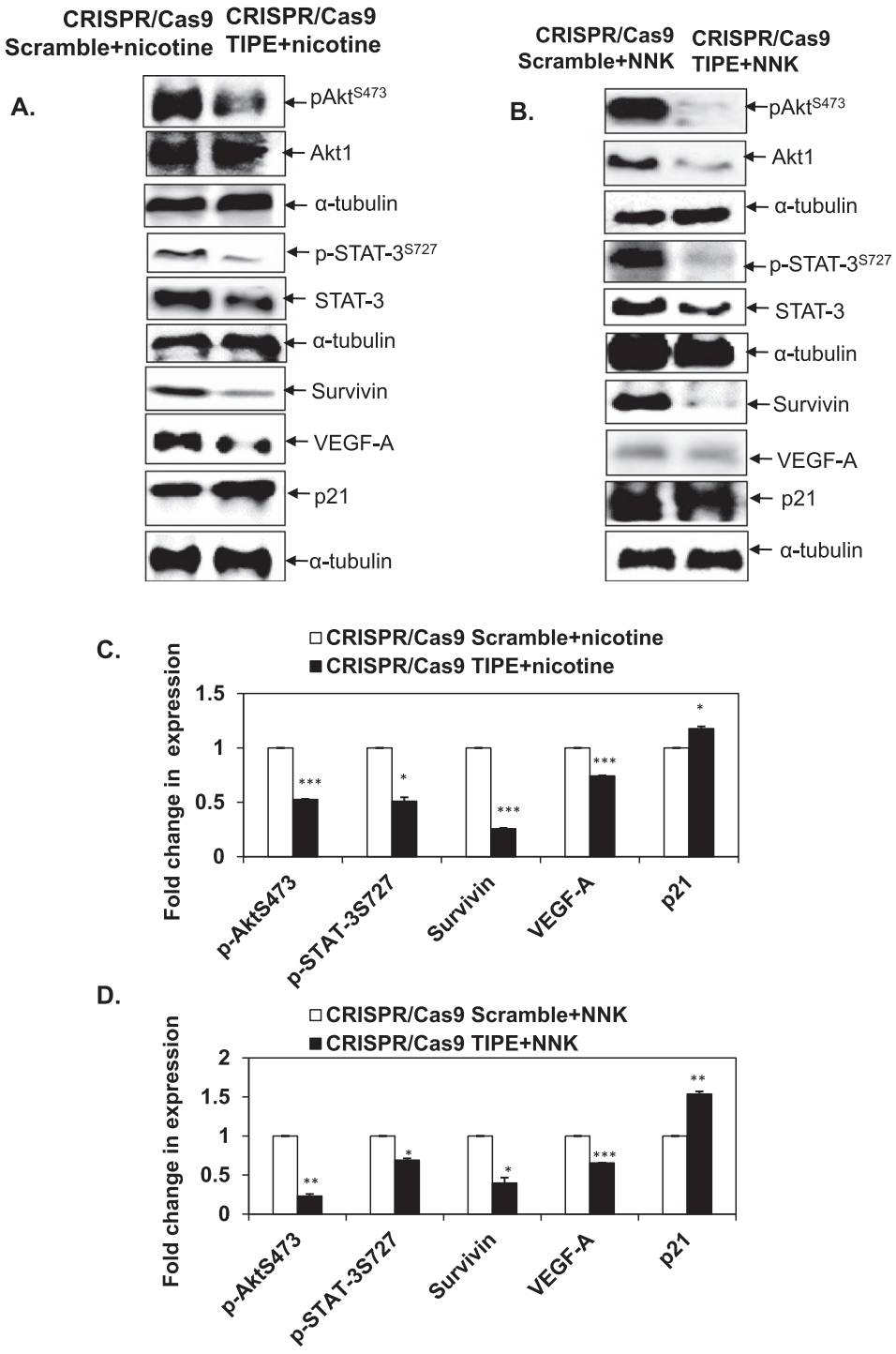


Fig. 7. Role of TIPE in the regulation of different signaling molecules/pathways in nicotine and NNK mediated lung cancer. Effect on the expression of various proteins involved in the regulation of different cancer hallmarks and signaling pathways in A. nicotine and B. NNK treated TIPE knockout lung cancer cells. Densitometric analysis of different cancer hallmark regulatory proteins and signaling molecules in C. nicotine and D. NNK treated TIPE knockout lung cancer cells compared with nicotine and NNK treated scrambled control cells. Densitometry was done using Image Lab software. α-tubulin served as the loading control. Data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

activation of PI3K/Akt pathway has been noticed which promoted cellular survival as well as resistance to γ-irradiation and chemotherapy. In addition, nicotine and NNK induced activation of Akt is also reported to cause tobacco-related carcinogenesis through regulation of growth and apoptosis in tumor cells [99]. Further, downregulation in p-STAT-3^{S727} was also observed in NNK treated TIPE knockout cells compared to NNK treated scrambled control. It is well established that constitutive activation of STAT-3 occurs in different tumor cells. STAT-3 activation is also considered as an early event in oral carcinogenesis induced by tobacco chewing [100]. In addition, nicotine treated TIPE knockout cells also showed downregulation in the expression of survivin and VEGF-A together with upregulation of p21. Additionally, NNN and BaP treated TIPE knockout cells were found to exhibit downregulation in the expression of proteins involved in the growth, invasion, migration and angiogenesis such as VEGF-A, p53 and p21. Further, NNN, nicotine and BaP treated TIPE knockout cells showed upregulation of the tumor suppressors such as p53 and p21. Further, in all the three cases, downregulation in p-Akt^{S473}, p-$S6^{S235/236}$, and p-STAT-3^{S727} was observed compared to respective treated scrambled control cells (Figs. 7 & 8). Taken together, TIPE can be suggested to be involved in the positive regulation of tobacco induced lung carcinogenesis via Akt/STAT-3 signaling axis and its downstream targets.

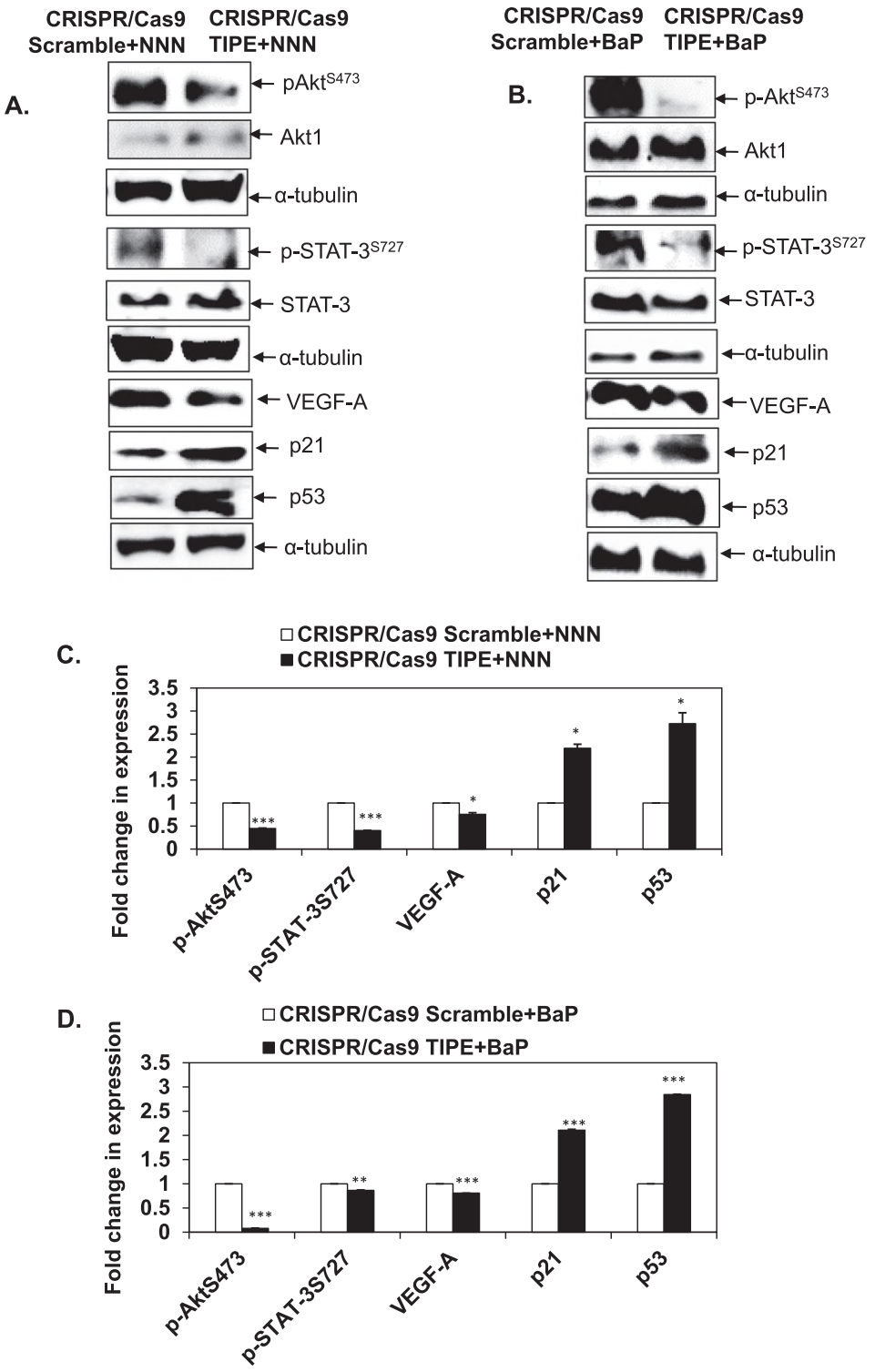


Fig. 8. Role of TIPE in the regulation of different signaling molecules/pathways in NNN and BaP mediated lung cancer. Effect on the expression of various proteins involved in the regulation of different cancer hallmarks and signaling pathways in A. NNN and B. BaP treated TIPE knockout lung cancer cells. Densitometric analysis of different cancer hallmark regulatory proteins and signaling molecules in C. NNN and D. BaP treated TIPE knockout lung cancer cells compared with NNN and BaP treated scrambled control cells. Densitometry was done using Image Lab software. α-Tubulin served as the loading control. Data are presented as mean ± SE, p-value < 0.05, < 0.005, < 0.001 are denoted as *, ** and *** respectively.

4. Discussion

Despite the advances made in the field of therapy against lung cancer, its prognosis still remains very poor [10,15–18,101]. The poor survival rate of lung cancer is not only due to the ineffectiveness of existing lung cancer therapies, but also due to lack of effective biomarkers for its early diagnosis as well as prognosis [102]. Hence, for the effective management of this aggressive cancer type, there is an urge to develop novel biomarkers. Notably, TIPE2, a protein belonging to TIPE family of proteins, was found to have profound role in the pathogenesis of lung cancer and also in tobacco promoted lung cancer as shown in our recent study [60]. Increasing lines of evidence suggest TIPE also to play important role in the prognosis of different cancers and thus possess enormous prospect as a clinical biomarker in lung cancer.

Our findings revealed TIPE to exhibit higher expression in lung cancer tissues than the non-malignant lung tissues indicating it as a positive regulator of lung carcinogenesis. It was found to be upregulated in both SCLC and NSCLC tissues, whereas upregulation in NSCLC tissues was found to be more pronounced. Additionally, it showed upregulation in different lung cancer pathologies such as squamous cell

carcinoma, adenocarcinoma, adenosquamous cell carcinoma, small cell carcinoma, large cell carcinoma and bronchioalveolar carcinoma tissues compared to the normal tissues. Notably, TIPE showed upregulation in different stages (stage I, II and IIIa) and grades (grade 1, 2 and 3) of lung tumor compared to the normal lung tissues. Interestingly, in line with our study, TIPE was reported to be overexpressed in different cancers including breast cancer, cervical cancer, colon cancer, endometrial cancer and ESCC [23,25,27–29]. Further, its expression was reported to be strongly associated with advanced pathological T stage, p-TNM stage, lymph node metastasis and poor survival in NSCLC patients [103]. A study carried out by Hadisaputri and group also reported TIPE to possess a strong correlation with different clinicopathological features including TNM stage, lymph node involvement, tumor depth, lymphatic and venous invasion, and distant metastasis in patients with ESCC [30]. In case of gastric, colon and lung cancers as well, TIPE expression was found to have strong association with TNM stage and lymph node metastasis [27,31,32,39]. Additionally, similar to our findings, overexpression of TIPE was found to be associated with higher histologic grade and lymph node metastasis in EC [28]. In ovarian cancer, TIPE overexpression was linked with large residual tumor size as well as high histologic grade [40]. Further, Zhang and group showed both nuclear and cytoplasmic overexpression of TIPE to be linked with high grade prostatic adenocarcinomas [46]. Altogether, these results indicate TIPE to be involved in the positive regulation of lung cancer.

The role of TIPE in the regulation of different cancer hallmarks was studied after CRISPR/Cas9 knockout of TIPE in NCIH460 cells. The evaluation of inadvertent CRISPR-Cas9-mediated off-target analysis would have increased the rigor of the study but could not be done. However, our results showed that knockout of TIPE caused significantly decreased proliferation (77% inhibition) of lung cancer cells. Along with proliferation, enhanced survival also presents a major feature of cancer cells [104]. Knockout of TIPE also reduced the survival fraction of lung cancer cells compared to scrambled control cells. Notably, metastasis which initiates with the tumor cells invading the stroma and migrating to the blood stream, presents one of the most predominant cause for the extremely low survival rate of lung cancer patients [105,106]. Therefore, we evaluated the effect of TIPE on the migration and invasion of lung cancer cells and observed that loss of TIPE led to the significantly inhibited migration as well as invasion of lung cancer cells. Similar to our findings, Miao and group also showed knockdown of TIPE to result in reduced proliferation and clonogenic potential of colon cancer cells [27]. TIPE overexpression resulted in increased proliferation and expression of Cyclin and connective tissue growth factor proteins in liver cancer cells and its depletion led to decreased proliferation and motility in esophageal and gastric cancer cells [29,30,32,35]. Further, in line with our findings, downregulation of TIPE inhibited both migration and invasion of gastric cancer cells [31]. It promoted lung cancer cells' invasion via Hippo pathway [37]. Additionally, its expression showed strong correlation with increased invasion as well as frequency of pulmonary colonization in breast cancer cells [24].

It is well evinced that various signaling molecules or pathways are involved in the regulation of different hallmarks of cancer [69]. Hence, to determine the mechanism of action of TIPE mediated lung cancer, we analyzed the expression of different target proteins through Western blot analysis. The results showed that loss of TIPE downregulated the expressions of survivin, XIAP, Bcl-2 and upregulated the expression of Caspase 9. Survivin, XIAP and Bcl-2 plays important role in inhibiting apoptosis [107–109]. Further, we observed that TIPE knockout led to the downregulation of Cox-2, Cyclin D1, and c-Myc in TIPE knockout cells. Cox-2, Cyclin D1 and c-Myc are involved in regulating cellular growth and metabolism [107,110,111]. Moreover, downregulation of LC-3B, the marker of autophagosomes' formation was also observed in TIPE knockout cells [112,113]. A recent report showed knockdown of TIPE to downregulate the expression of autophagy markers such as LC3β I/II, 4EBP1, Beclin-1, and ATG3 in hepatocellular carcinoma (HCC) cells implying the novel role of TIPE in regulating autophagy [109]. In addition, downregulation of CXCR-4 and VEGF-A, which play crucial role in tumor growth, invasion, metastasis, and angiogenesis was observed in TIPE knockout cells [71,114]. Further, knockout of TIPE led to the upregulation of p53 and its target p21, which regulate cell growth, migration, invasion, apoptosis and senescence [115]. Mounting evidence suggests that PI3K/Akt/mTOR pathway plays a vital role in oncogenesis [73,116,117]. Notably, in 90% of the NSCLC cells, PI3K/Akt pathway is reported to be constitutively activated which promotes cell survival [118]. Further, Akt/mTOR pathway is responsible for regulating cellular autophagy [117]. Therefore, we examined the effect of TIPE knockout on Akt/mTOR pathway in NCIH460 cells. Our findings suggested that loss of TIPE inflected the critical constituents of this pathway which include downregulation of p-Akt^{S473}, p-mTOR^{S2448}, and p-S6$^{S235/236}$ and upregulation of PTEN, a negative regulator of Akt [75]. In line with our findings, Day and group also showed that knockdown of TIPE led to the downregulation of IGF-1-mediated pIGF1R and pAkt, and upregulation of IGF-1-binding protein 3 (IGFBP3), which is involved in the negative regulation of IGF-1/IGF1R cascade [45]. In a very recent report, Niture et al. found that transient or stable expression of TIPE resulted in reduced phosphorylation of Serine-473 of Akt and Serine-2448 of mTOR in HCC cells. The study depicted TIPE induction to inhibit the Akt/mTOR pathway and cause HCC cells' autophagy [117]. Porturas and their group showed that shRNA mediated knockdown of TIPE led to the increased Akt phosphorylation at Serine 473 residue in Hepa1–6 murine liver cells [119]. Notably, constitutive activation of PI3K/Akt is reported to cause aberrant activity of STAT-3 which is responsible for the regulation of different cancer hallmarks [91,120–127]. This study also showed that knockout of TIPE led to the reduced expression of p-STAT-3^{S727}. Altogether, TIPE mediated lung carcinogenesis can be suggested to be plausibly modulated through Akt/mTOR/STAT-3 signaling axis (Fig. 9).

Aforementioned, tobacco components are strongly involved in modulating different hallmarks such as growth, survival, migration and invasion of lung cancer cells [60]. Therefore, to determine the association between TIPE and tobacco induced lung cancer, TIPE knockout lung cancer cells were treated with four different tobacco components which include nicotine, NNK, NNN, and BaP and their effects on cell proliferation, survival and migration were evaluated. Nicotine, the principal addictive constituent of cigarette is reported to induce proliferation, angiogenesis, migration, invasion of cancer cells in vitro and tumor growth and metastasis in vivo. It promoted the survival and proliferation of lung cancer cells through activation of PKC/Raf/MEK/ERKs cascade [128–133]. Puliyappadamba and group reported that nicotine induced clonogenic potential in lung cancer cells via up-regulation of p53 and p21 [130]. Further, NNK; which is considered to be the strongest carcinogen among all the tobacco-specific nitrosamines was reported to induce NF-κB-dependent survival of tumor cells [129,132–135]. In addition, NNN, a well-known pulmonary carcinogen is another tobacco constituent responsible for lung cancer [136–139]. The binding of NNK and NNN to the nAChR induces proliferation, survival, migration, and invasion of tumor cells. Further, BaP was also found to promote the proliferation, migration and invasion of lung cancer cells effectively [140–142]. Our findings showed that, individual treatment with all the four tobacco components resulted in significant decrease in the proliferation and survival of TIPE knockout lung cancer cells than the scrambled control cells treated with the respective compounds. It is well evinced that tobacco and its components possess significant association with cancer cell migration [24,66,67,142]. Our study showed that treatment with NNK, NNN, nicotine and BaP resulted in significant inhibition in the wound healing in TIPE knockout lung cancer cells implying its involvement in tobacco induced migration of lung cancer cells. Further, nicotine, NNK, NNN, and BaP treated TIPE knockout cells exhibited downregulation of survivin, VEGF-A and upregulation of p53, p21. Notably, nicotine and NNK mediated activation of Akt is reported to result in tobacco-related carcinogenesis via

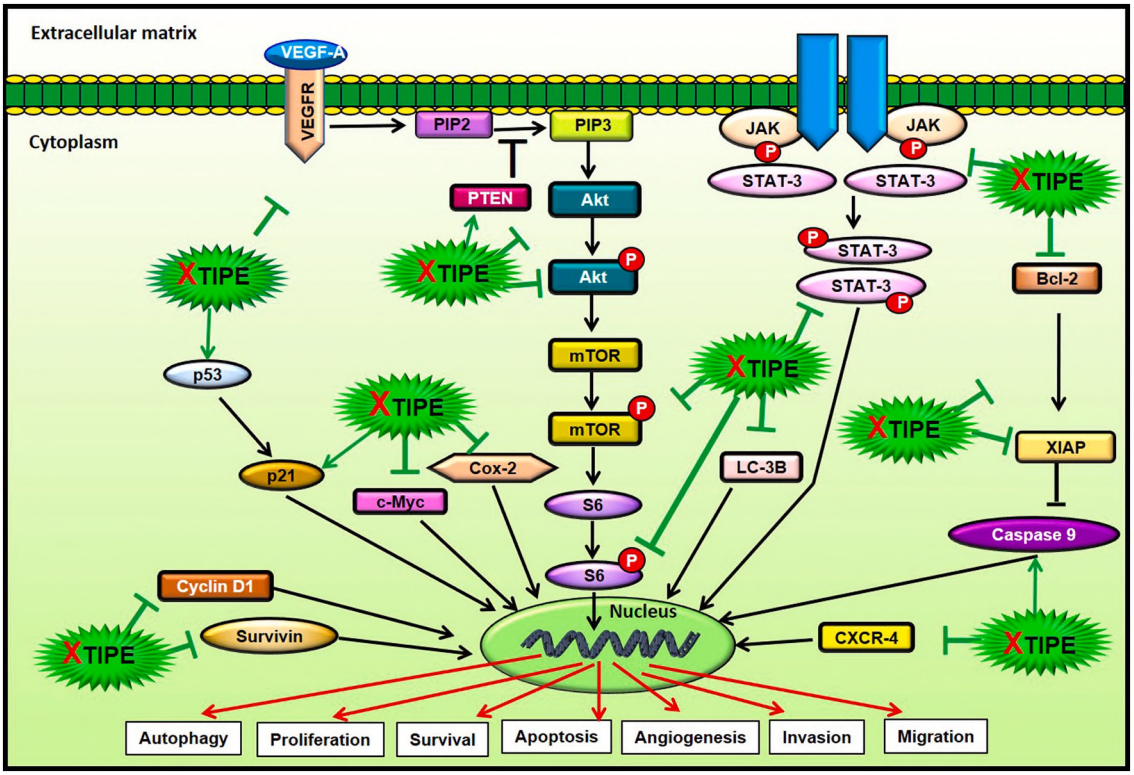


Fig. 9. Representation of the effect of TIPE knockout on Akt/mTOR/STAT-3 signaling axis, knockout of TIPE is denoted by 'XTIPE'. (Figure adapted and modified from Bordoloi et al. [60].)

regulation of tumor cell growth and apoptosis [118]. Activation of STAT-3 is also considered as an early event in oral carcinogenesis induced by tobacco chewing [100]. In the present study, tobacco components-treated TIPE knockout cells exhibited downregulation of p-Akt^{S473} and p-STAT-3^{S727}. Altogether, TIPE can be suggested to play an important role in the positive regulation of tobacco mediated lung carcinogenesis through Akt/STAT-3 and different proteins involved in regulating cancer cell growth, survival, migration, invasion and angiogenesis.

5. Conclusion

The present study evaluated the role of TIPE in the regulation of different cancer hallmarks. Our results showed that knockout of TIPE reduced the proliferation, survival, invasion and migration of lung cancer cells plausibly through Akt/mTOR/STAT-3 signaling axis. Notably, this study also showed for the first time that TIPE might be involved in the positive regulation of nicotine, NNK, NNN, and BaP mediated proliferation, survival and migration of lung cancer cells through modulation of Akt/STAT-3 signaling. As Akt/mTOR/STAT-3 pathways are found to be mostly deregulated in lung cancer cases, hence their negative regulation might hold a strong basis for the development of effective biomarkers and targeted therapies. Besides, TIPE modulated the expression of different proteins involved in growth, proliferation, survival, apoptosis regulation, invasion, angiogenesis, migration and metastasis of lung cancer cells. Taken together, TIPE can be implied to play a crucial role in lung cancer and also in tobacco-induced lung carcinogenesis and hence, targeting this protein seems to hold prospect in therapeutic interventions for lung cancer. Nevertheless, these findings need to be further validated through extensive in vitro, in vivo and clinical studies to establish its clinical importance wholly.

CRediT authorship contribution statement

Conceptualization, A.B.K.; methodology, D.B., G.P. and A.B.K.; software, D.B., K.B., R.V., K.K.T., B.L.S. and S.G.; validation, K.B., R.V., K.K.T., G.P., B.L.S., S.G. and A.B.K.; formal analysis, D.B., K.B., and R.V. investigation, D.B. and A.B.K.; resources, A.B.K.; data curation, D.B., R.V. and K.B.; writing—original draft preparation, D.B., A.C. and T.A.A.; writing—review and editing, D.B., K.B., R.V., G.P., C.B., M.S., S.A.A. and A.B.K.; visualization, D.B. and K.B.; supervision, A.B.K.; project administration, D.B. and A.B.K.; funding acquisition, A.B.K.

Declaration of competing interest

The authors express no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118475.

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