STUDIES ON
THE ROLE OF TIPE FAMILY OF PROTEINS IN THE DEVELOPMENT AND PROGRESSION OF LUNG CANCER

A thesis submitted for the degree of

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To

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

By

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March, 2019

Dedicated to

My dear parents and loving sister

For the untiring love, unflinching support and constant encouragement

DEPARTMENT OF BIOSCIEENCES AND BIOENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
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DECLARATION

I hereby declare that the contents of the research work described in this thesis titled **"Studies on the role of TIPE family of proteins in the development and progression of lung cancer"**, is a presentation of my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Ajaikumar B. Kunnumakkara.

Sincere efforts have been made to duly acknowledge the contributions from others for their ideas, technical help, references or any other help which may be involved in the completion of this thesis work.

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CERTIFICATE

This is to certify that the work described in the thesis titled **"Studies on the role of TIPE family of proteins in the development and progression of lung cancer"**, submitted by Devivasha Bordoloi (Roll no: 146106028) to Indian Institute of Technology Guwahati, India, for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere for award of any other degree or diploma.

March, 2019

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Devivasha Bordoloi

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Chapter 1

Introduction and Review of literature

1.1. Introduction

Lung cancer, an extremely invasive, highly metastasizing and one of the most prevalent neoplasm; develops in a multi-stage process through a series of genetic and epigenetic alterations in the lung epithelial cells (Lemjabbar-Alaoui *et al.,* 2015; Larsen and Minna, 2011; Gazdar and Brambilla, 2010). It is the leading cause of mortality due to cancer in men and also one of the most deadly cancers in women (Arcaro, 2015; Jemal *et al.,* 2011), constituting around 11.6% of all new cancer cases and 18.4% of total cancer related deaths (GLOBOCAN, 2018) (Figure 1.1). The risk of developing lung cancer for men is 1:13 whereas it is 1:16 in case of women (Gitlitz *et al.,* 2010). Notably, in lung cancer mortality rates as well, there exist a large variation (30-fold) worldwide among males and females which can be primarily attributed to the trend of smoking, as tobacco smoking is responsible for almost 90% of all lung cancer cases. 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; whereas the lowest mortality rates were reported in sub-Saharan Africa in both the genders. 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 (Torre *et al.,* 2015; Islami *et al.,* 2015; Khan and Mukhtar, 2015). Apart from smoking, exposure to varied occupational and environmental carcinogens and diverse dietary factors are also strongly implicated in the development of lung cancer (Behera and Balamugesh, 2004). In India, lung cancer constitutes 6.9 % of all new cancer cases and 9.3 % of all cancer related deaths in both males and females. It is also

the most common cancer as well as the cause of cancer related death in men, with the highest reported incidences from Mizoram in both the sexes. The time trends of lung

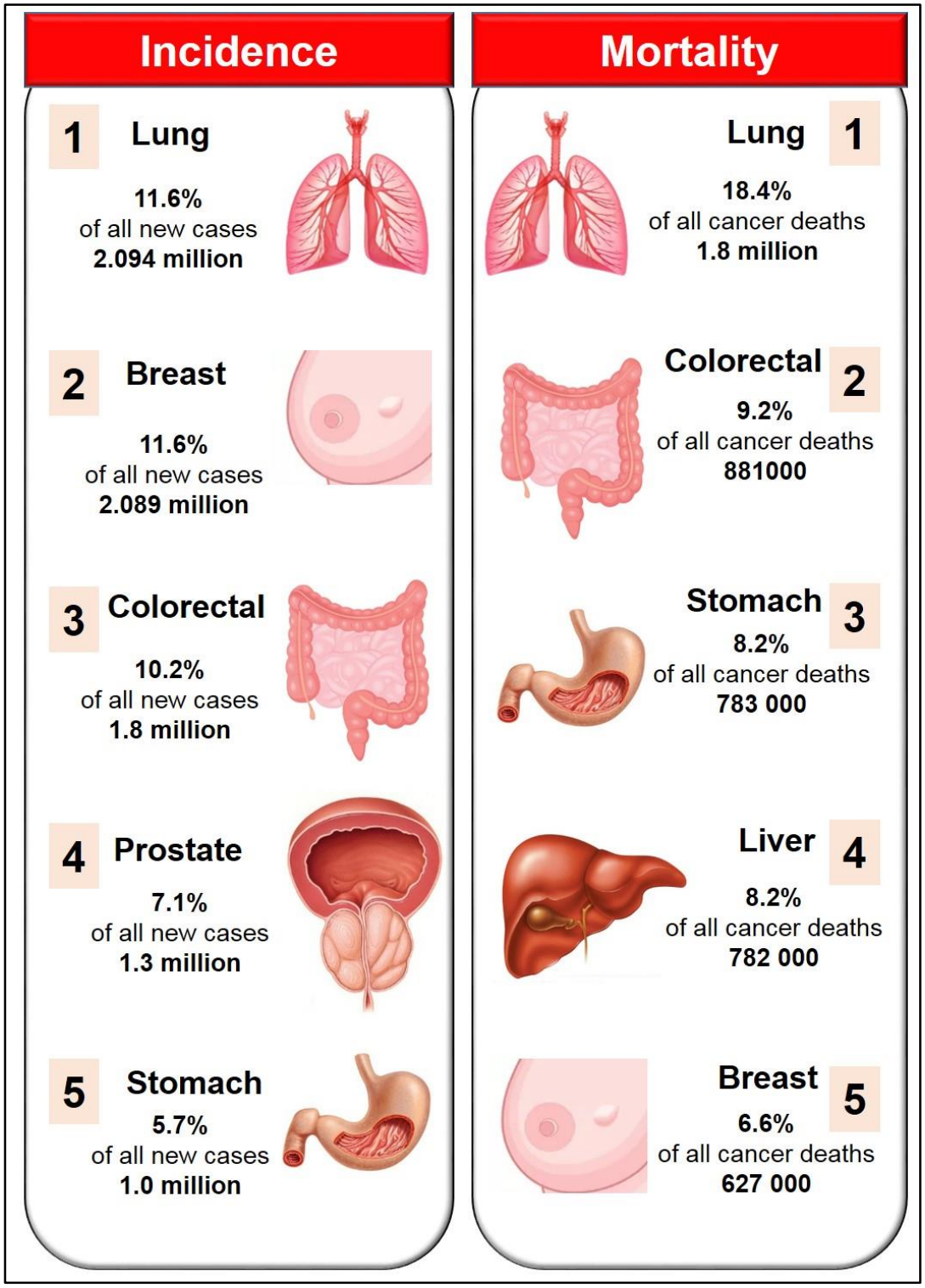

Figure 1.1. Percentages of new cancer cases and cancer deaths worldwide in 2018 (GLOBOCAN 2018)

cancer exhibit a marked rise in Delhi, Chennai and Bengaluru in both the genders (Malik and Raina, 2015). With increasing smoking habits, lung cancer has reached epidemic proportions in India. It has outdone the previous most common cancer type, that of oropharynx, and now has become the commonest malignancy in males. Contrary to western countries, squamous cell carcinoma is the most common histological type in India, although adenocarcinoma is becoming quite common than before (Behera and Balamugesh, 2004). 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. Propensity for early spread, lack of suitable biomarkers and effective therapeutic strategies contribute enormously to the poor survival of the lung cancer patients (Bunn, 2002).

Increasing lines of evidence suggest tumor necrosis factor-α-induced protein eight (TIPE/TNFAIP8/Oxi-α) family of proteins, a novel group of proteins which was discovered just a decade ago to play vital role in the modulation of tumorigenesis, inflammation, cell death and diverse other cellular activities. It has been found to be strongly associated with cancers of breast, bone, brain, cervix, colon, esophagus, endometrium, liver, lung, stomach, and thyroid (Bordoloi *et al.,* 2018; Padmavathi *et al.,* 2018*).* However, the potential crosstalk of all the four different TIPE proteins with diverse signaling molecules involved in lung tumorigenesis is not understood wholly. Therefore, the present study focuses on elucidating the role of this newly identified family of proteins in the pathogenesis of lung cancer which would certainly help us to develop effective biomarkers and targets for the management of this cancer.

1.2. Types of lung cancer

Lung cancer is categorized into two broad classes namely non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), based on histological, clinical and neuroendocrine characteristics. NSCLC represents around 80-85% of total lung cancer cases whereas SCLC constitutes around 15-20% of all lung cancers. These two lung cancer types also differ at the molecular level with different genetic alterations and exert subtype specificity (Larsen and Minna, 2011; Caulo *et al.*, 2012).

1.2.1. Non-small cell lung cancer

NSCLC represents one of the most genomically diverse lung cancer type which offers challenges in both prevention and treatment strategies (Li *et al.*, 2013). It is reported to exert strong primary resistance against anticancer drugs (Indovina *et al.*, 2011). NSCLC is again of three different subtypes such as squamous-cell carcinoma, large-cell carcinoma, and adenocarcinoma (Pao and Girard, 2011). These categories are used for prognosis and also in deciding the treatment strategies. Signs and symptoms of the disease may vary among these subtypes based on tumor type and degree of metastases (Collins *et al.*, 2007*).* Squamous cell carcinoma arises in the main bronchi and advance to the carina region. It constitutes around 25–30% of all lung cancer cases (Lemjabbar-Alaoui *et al.*, 2015). Large cell carcinomas comprise of poorly differentiated forms of the other types and, plausibly, some truly undifferentiated "stem cell like" tumors (Gazdar and Brambilla, 2010). Adenocarcinomas consist of tumors arising in peripheral bronchi and account for approximately 40% of all lung cancers. Progression of adenocarcinomas occur through production of lobar atelectasis and pneumonitis. In addition, bronchioloalveolar cancers which arise in alveoli and spread through the interalveolar connections are also reclassified into adenocarcinoma *in situ* and minimally invasive adenocarcinoma. Further, large cell anaplastic carcinomas, also

termed NSCLC not otherwise specified comprise about 10% of NSCLC cases and they behave same as small cell cancers with a rapid fatal spread (Lemjabbar-Alaoui *et al.*, 2015).

1.2.2. Small cell lung cancer

SCLC, derived from the hormonal cells of the lung, are the most dedifferentiated cancers and tend to be central mediastinal tumors. SCLC comprises 10–15% of all lung cancer cases and represents an extremely aggressive form which spreads rapidly into submucosal lymphatic vessels and regional lymph nodes, and almost always present without a bronchial invasion (Lemjabbar-Alaoui *et al.*, 2015). SCLC is typically associated with cigarette smoking and possess utterly poor prognosis (Arcaro, 2015). The overall 5-year survival rate of SCLC patients remain very low (<7%), and majority of the patients survive only for a year or even less after being diagnosed (Byers and Rudin, 2015). Despite the fact that a considerably huge amount of research has been done to understand the complexity of SCLC and to develop targeted therapies for its management, the treatment of SCLC still remains a major challenge. This definitely necessitates the utter need to develop novel agents or therapeutic regimens as well as biomarkers by understanding the underlined molecular mechanisms and involved signaling cascades for the prevention and treatment of SCLC (Joshi *et al.*, 2013). However, in contrast to NSCLC, SCLC is highly responsive to chemo and radiation therapy, but it is often diagnosed at an extremely advanced stage where cure is scarcely possible (Indovina *et al.*, 2011).

1.3. Etiology of lung cancer

Extensive research carried out over the last several years identified diverse factors to be associated with the risk of lung cancer. Although cigarette smoking was identified as the single most predominant cause of the lung cancer epidemic, various other factors such as exposure to workplace agents, environmental pollution etc. are also found to be associated with lung cancer occurrence (Alberg *et al.,* 2005) (Figure 1.2) and are described below in details.

1.3.1. Cigarette smoking

The most predominant risk factor for the development of lung cancer is smoking which increases the risk of lung cancer by 10 fold on average compared to the non-smokers (Indovina *et al.,* 2011). Cigarette smoke is a composite mixture of chemicals which include different genotoxic lung carcinogens as well (Hecht, 2012). There are more than 5000 compounds identified in cigarette smoke and among them more than 20 compounds were identified to be lung carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), nicotine-derived nitrosaminoketone etc. (Wang *et al.,* 2015b). Of the carcinogens associated with lung cancer, tobacco-specific nitrosamine namely 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most potent one (Ge *et al.,* 2015; Larsen and Minna, 2011). The general mechanisms of the carcinogen are hypothesized to be the formation of DNA adducts, their conversion into water soluble forms through catalyzing activity of drug metabolizing enzymes like cytochrome P450 (CYP), glutathione S-transferases, and UDP-glucuronosyl transferases etc. In addition, tobacco smoke constituents such as nicotine and tobacco-specific nitrosamines bind directly to the cellular receptors which in turn results in the activation of Akt and other pathways, which are involved in the development and progression of cancer (Hecht, 2012). Apart from active smoking, secondhand smoking was also reported to cause

around 21,400 lung cancer deaths annually (Islami *et al.*, 2015). The analysis of International Agency for Research on Cancer (IARC) report suggests that the risk of lung cancer increases by 35% in men and 25% in women who are exposed to second hand smoking than the non-exposed men and women (Pallis and Syrigos, 2013).

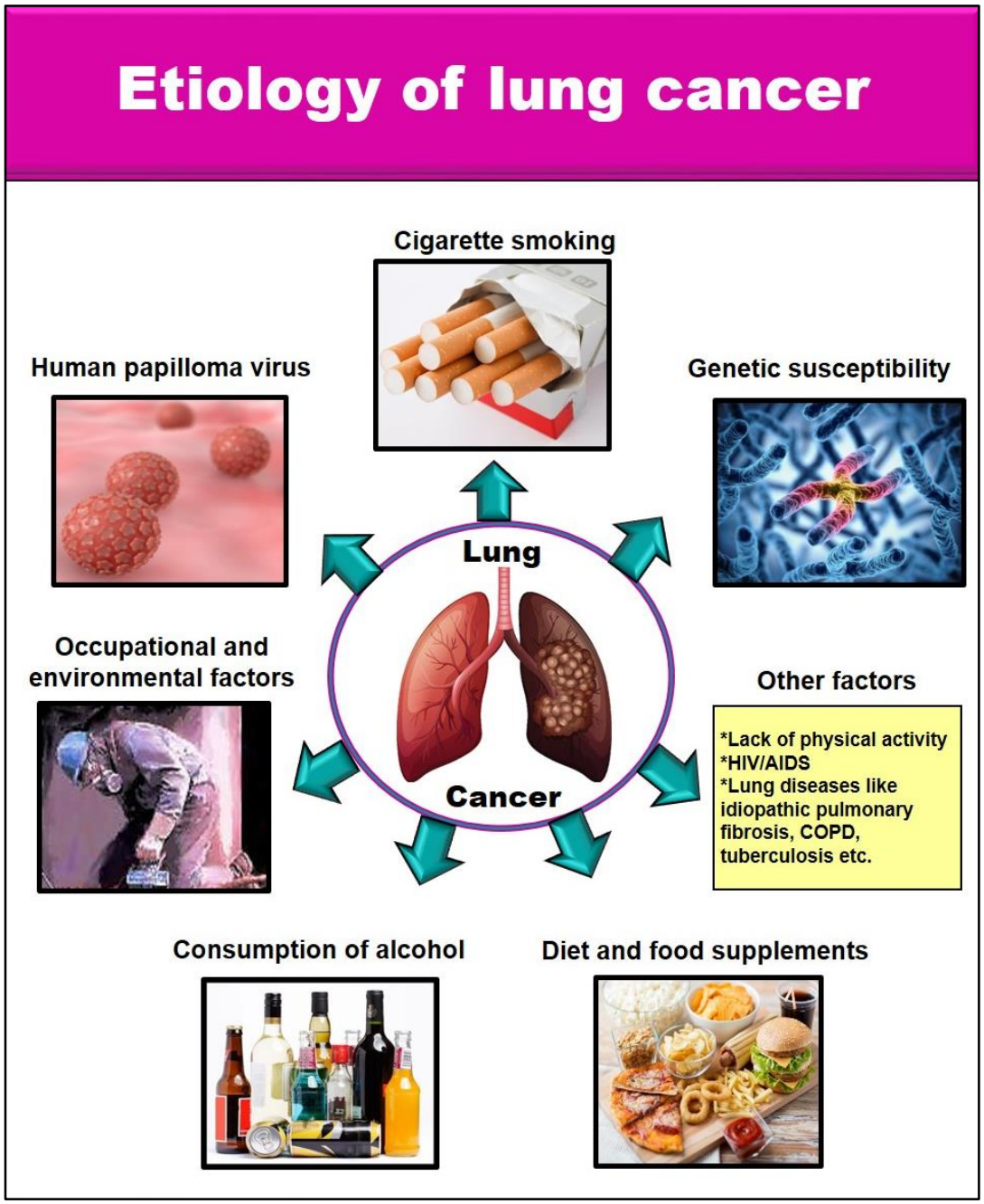

Figure 1.2. Different risk factors associated with lung cancer

Besides, studies reported that passive smoking during childhood also increases lung cancer risk in adulthood by around 3.6 fold (Molina *et al.*, 2008).

1.3.2. Genetic susceptibility

Numerous studies have been performed on inherited predisposition to lung cancer such as familial linkage studies and polymorphism studies linked with lung cancer risk. Three independent genome-wide association studies (GWASs) identified single nucleotide polymorphism (SNP) variations at 15q24-q25.1 to be associated with an increased risk of lung cancer. Besides, recent reports suggest that variation at 15q25.1, 5p15.33, and 6p21.33 also increases the risk of lung cancer (Larsen and Minna, 2011). In addition, a familial susceptibility locus to 6q23-25 was mapped through genome-wide linkage study of pedigrees bearing several generations of lung cancer. Further, *RGS17* which regulates G-protein signaling was also identified as a potent causal gene associated with familial lung cancer (Larsen and Minna, 2011).

1.3.3. Human papilloma virus

Human papilloma virus (HPV) has also been reported to play an important role in pathogenesis of lung cancer. E6 and E7, which are HPV oncoproteins cause inactivation of tumor suppressors such as p53 and Rb respectively. A meta-analysis of 4,508 cases reported a mean incidence of 25% HPV positive lung cancer cases of all subtypes (Larsen and Minna, 2011).

1.3.4. Occupational and environmental risk factors

Various occupational and environmental factors are also associated with the risk of lung cancer. The most common occupational risk factor for lung cancer is exposure to asbestos. Asbestos and all its commercial forms are declared as human carcinogens by

the IARC (Hashim and Boffetta, 2014). Various other common occupational and environmental risk factors of lung cancer include exposure to radon, chromium, vinyl chloride, nickel, arsenic and ionizing radiation (Collins *et al.,* 2007; Alberg *et al.,* 2005). Further, indoor emissions from household combustion is also reported to be a potent environmental lung carcinogen (Field and Withers, 2012). Besides, outdoor air pollution; exposure to hazardous chemicals in some occupations, such as aluminum production and coal gasification; and exposure to silica dust etc. predominantly increase the risk of lung cancer in human (Islami *et al.,* 2015). Further, nuclear plant workers and uranium miners who are exposed to radioactive particulate also possess increased risk of lung cancer (Molina *et al.,* 2008).

1.3.5. Consumption of alcohol

Alcohol is also considered as a risk factor of lung cancer. It was reported that people who consume at least 30 g/d of alcohol are at higher risk of lung cancer than those who abstained from it (Molina *et al.,* 2008). Although the mechanism through which alcohol increases cancer risk is not completely known; different hypotheses have been postulated. It does not initiate cancer on its own but may potentiate the effect of carcinogens by allowing the cellular entry of carcinogens, affecting their metabolism, tumor promotion and DNA repair inhibition (Bandera *et al.,* 2001).

1.3.6. Diet and food supplements

The relation between diet and lung cancer risk is also well evidenced, in particularly among smokers. Individuals with high dietary intake of fruits or vegetables possess a lesser risk of lung cancer. Further, increasing lines of evidence suggest that phytoestrogens, flavonoids, and glucosinoids have a strong association with lung

cancer. Intake of flavonoids, which possess strong anti-oxidant effect is also reported to be linked with decreased lung cancer risk. Further, isothiocyanates exert anticancer effects through phase II detoxification enzymes mediated inhibition of carcinogens. In addition, high intake of cruciferous vegetables also reduces the risk of lung cancer as they contain high amount of glucosinolates, resulting in higher endogenous isothiocyanate concentrations (Alberg *et al.,* 2005). On the other hand, cured meat like cured pork, sausage, pressed duck, deep-fried cooking etc. have been reported to be linked with enhanced lung cancer risk (Molina *et al.,* 2008).

1.3.7. Other factors

Apart from the above mentioned, there are a few other factors associated with lung cancer risk. For instance, physically active individuals possess a reduced risk of lung cancer. It is reported that a moderate to high levels of leisure-time physical activity is strongly linked with decreased lung cancer risk by around 13- 30% (Molina *et al.,* 2008). Furthermore, hormone estrogen receptor-α (ER-α) expression and lack of estrogen receptor -β (ER-β) expression are involved in poor prognosis of NSCLC in female (Kawai *et al.,* 2005). In addition, patients with HIV/AIDS are known to possess high risk of lung cancer (McErlean *et al.,* 2011). Besides, past history of other nonmalignant lung diseases which include idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and tuberculosis also causes enhanced risk of lung cancer (Collins *et al.,* 2007).

1.4. TNM staging of lung cancer

The International Association for the Study of Lung Cancer (IASLC) has developed a classification system for lung cancer based on a refined statistical analysis of an

Table 1.1. TNM classification for lung cancer.

T (PRIMARY TUMOR)	
T0	No primary tumor
Tis	Carcinoma in situ (squamous or adenocarcinoma)
T1	Tumor <3 cm
T1mi	Minimally invasive adenocarcinoma
T1a	Superficial spreading tumor in central airways*
T1a	Tumor ≤ 1cm
T1b	Tumor>1 but ≤ 2 cm
T1c	Tumor>2 but ≤ 3 cm
T2	Tumor>3 but < 5 cm or tumor involving: visceral pleura,y main bronchus (not carina), atelectasis to hilum +
T2a	Tumor >3 but ≤ 4 cm
T2b	Tumor>4 but ≤ 5 cm
T3	Tumor>5 but ≤ 7 cm or invading chest wall, pericardium, phrenic nerve; or separate tumor nodule(s) in the same lobe
T4	Tumor>7 cm or tumor invading: mediastinum, diaphragm, heart, great vessels, recurrent laryngeal nerve, carina, trachea, esophagus, spine; or tumor nodule(s) in a different ipsilateral lobe
N (REGIONAL LYMPH NODES)	
N0	No regional node metastasis
N1	Metastasis in ipsilateral pulmonary or hilar nodes
N2	Metastasis in ipsilateral mediastinal or subcarinal nodes
N3	Metastasis in contralateral mediastinal, hilar, or supraclavicular nodes
M (DISTANT METASTASIS)	
M0	No distant metastasis
M1a	Malignant pleural or pericardial effusion # or pleural or pericardial nodules or separate tumor nodule(s) in a contralateral lobe
M1b	Single extrathoracic metastasis
M1c	Multiple extrathoracic metastases (1 or>1 organ)

*Superficial spreading tumor of any size but confined to the tracheal or bronchial wall.+Atelectasis or obstructive pneumonitis extending to hilum; such tumors are classified as T2a if>3 and ≤ 4 cm, T2b if>4 and ≤ 5 cm. # Pleural effusions are excluded that are cytologically negative, nonbloody, transudative, and clinically judged not to be due to cancer.

international database of more than 100,000 patients and published in a series of articles in the Journal of Thoracic Oncology particularly. Tumor node metastasis (TNM) clearly addresses T, N and M components; the stage groups, the methodology adapted and validation used for both NSCLC and SCLC (Detterbeck, 2018) (Table 1.1).

1.5. Premalignant and early invasive bronchial lesions of lung cancer

From biological, histopathologic, and clinical standpoints, lung cancer is an utterly complex neoplasm which involves multiple pre-neoplastic pathways (Wistuba, 2007). Studies carried out by different groups provided ample information on the histological as well as molecular characteristics of premalignant changes in bronchial mucosa. The earliest lesions include reserve cell hyperplasia and squamous metaplasia without any association of cellular atypia/dysplasia. With smoking cessation, as these lesions spontaneously retrogress, they are supposed to display reactive changes rather than true premalignant lesions. Contrariwise, dysplasia (low and high grade) and carcinoma *in situ* (CIS) are believed as true premalignant lesions responsible for increased lung cancer risk. The extent of association of low grade dysplasia with lung cancer occurrence is ambiguous. However, high-grade dysplasia and CIS have been reported to have a strong association with increased risk of lung cancer (Hirsch *et al.,* 2002*)* (Figure 1.3).

1.6. Molecular alterations in lung cancer

Diverse molecular alterations and driver mutations have been identified in lung cancer which are known to play critical role in lung carcinogenesis. The most common early genetic alterations in NSCLC involve loss of genomic regions of chromosomes 3p and 9p, deletions of chromosomal arm on 5p and mutations in K-Ras and p53. Loss of

chromosomal regions on 3p and 9p chromosomes is considered to be an early event which is mainly detected in pre-invasive lesions and epithelium of smokers. *p53* and *K-Ras* mutations were reported mostly in later stages of pre-neoplasia.

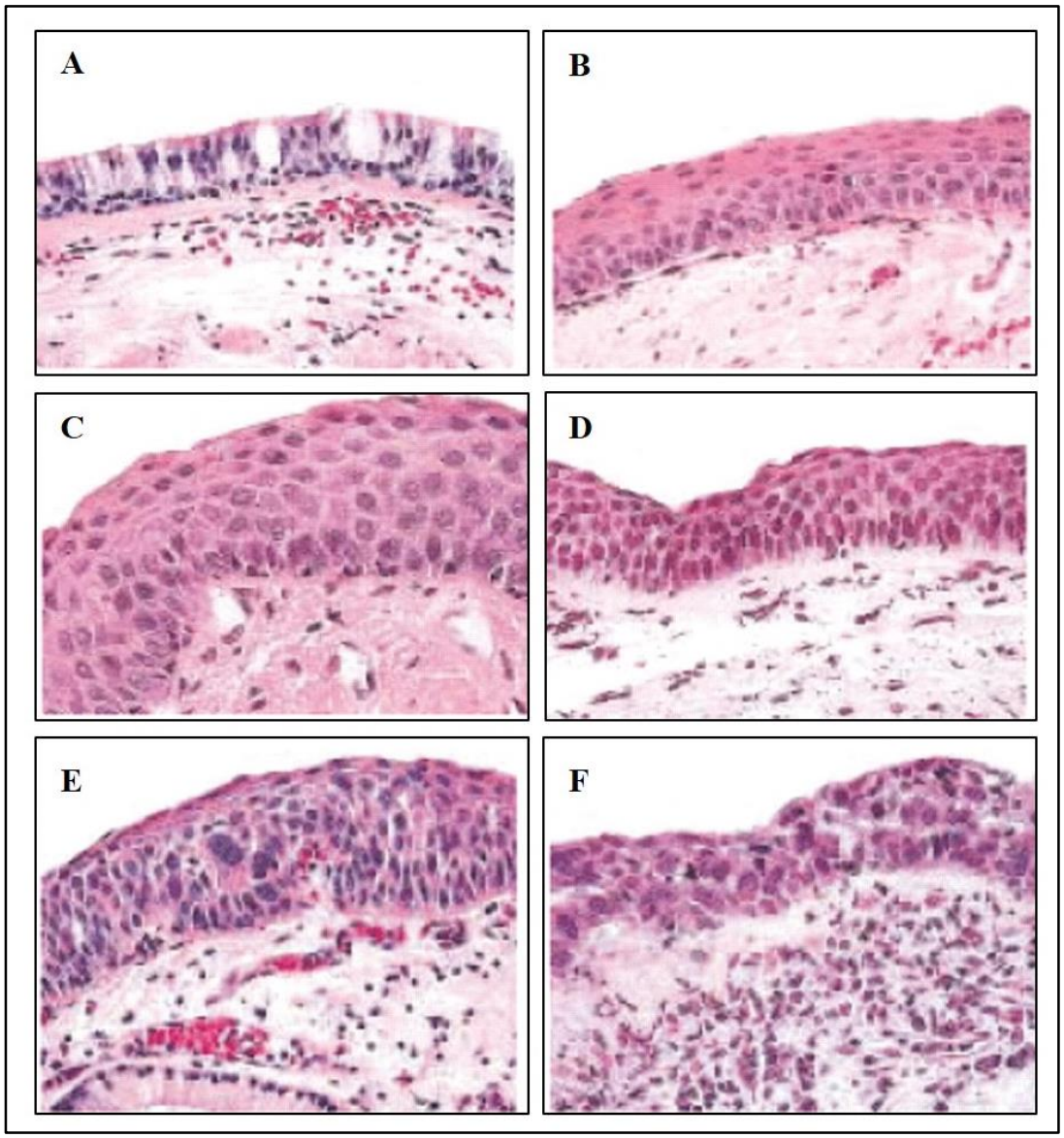

Figure 1.3. Grading of morphological changes in bronchial epithelium used for classification scheme applied in chemoprevention and other studies at the University of Colorado Cancer Centre. A. Normal (grade 1), B. Squamous metaplasia (grade 2), C. Mild dysplasia (grade 4), D. Moderate dysplasia (grade 5), E. Severe dysplasia (grade 6) and F. Carcinoma *in situ* (grade 7) (Hirsch *et al.*, 2002).

Further, amplification in chromosomal region of q arm of chromosome 3 has been identified in invasive carcinomas (Massion and Carbone, 2003). Interestingly, these mutations vary among different ethnic and geographic populations. For instance, the frequency of *EGFR* mutation is around 10% among Caucasian population, whereas it is prevalent in 60% Asian population. On the other hand, in India its mutation is prevalent in around 25-50 % population as per different studies (Malik and Raina, 2015). This mutation mainly targets adenocarcinoma subtype than other histologies of NSCLC (Shigematsu and Gazdar, 2006). Various *EGFR* mutations have been detected which include frame deletion in exon 19, G719C point mutation in exon 18, L858R point mutation in exon 21 etc. (Noronha *et al.*, 2013). Besides, various other driver mutations have been found to be associated with lung cancer. For example, driver mutations in *ALK*, *HER2*, *BRAF*, *PIK3CA*, *Akt1*, *MAP2K1*, *MET* etc. (Pao and Girard, 2011). *EML4-ALK* fusions which arise from different small inversions within the short arm of chromosome 2 were also found in NSCLC. In addition, HER2 protein was reported to be overexpressed in about 20% of NSCLC. Mutations in *BRAF* and *MAP2K1* are reported mostly in adenocarcinomas. Further, genes encoding for different receptor tyrosine kinase (RTK) families were also found to be mutated in lung cancer which include *ERBB3*, *ERBB4*, *EPHA3*, *EPHA5*, *EPHA7*, *EPHB1*, *EPHB6*, *FGFR1*, *FGFR2*, *FGFR4*, *NTRK1*, *NTRK2*, *NTRK3*, *KDR* etc. (Pao and Girard, 2011). Weir and group identified a lineage-specific transcription factor encoding gene namely *NKX2-1*, to have strong involvement in lung adenocarcinomas (Weir *et al.*, 2007). The tumor suppressor genes *TP53* and *Rb1* are also frequently reported to be mutated in SCLC. Further, *JAK2* and *Myc* were found to have copy number gains. Besides, diverse somatic mutations were reported in different cancer genes such as enzymes involved in chromatin modification, downstream signaling components of RTKs etc. (Arcaro,

2015). In addition, varied targetable genetic alterations were also reported in lung cancer which include oncogenic gene overexpression by microRNAs, enhanced telomerase activity etc. Notably, in 57% of NSCLC cases, the *hTERT* gene was found to be amplified (Lemjabbar-Alaoui *et al.*, 2015).

1.7. Treatment approaches available for lung cancer

Although lung cancer remains the leading cause of cancer death worldwide, recent advances in early detection and improvements in adjuvant therapies have upstretched hopes for the improved patient survival in the coming days (Hamaji *et al.*, 2013; Horeweg and Koning, 2014).

1.7.1. Treatment approaches for NSCLC

With the recent advances in the field of oncotherapy, several approaches have emerged for the management of NSCLC. However, surgical resection remains the primary and preferred approach for the treatment of stage I and II NSCLC. Lobectomy or greater resection is performed in case of T1b and larger tumors. Although adjuvant radiation or chemotherapy did not exert any significant benefit for stage I NSCLC, the use of adjuvant chemotherapy for stage II NSCLC has been found to be beneficial. For patients refusing surgical resection, primary radiation therapy remains the chief curative approach (Howington *et al*., 2013). However, more than 70 % of NSCLC patients are diagnosed in highly advanced or metastatic stage. For patients with resectable stage IIIA NSCLC, surgery followed by chemotherapy is performed whereas for unresectable stage IIIA patients, either a sequential or concurrent chemo-radiation therapy is done. In case of stage IIIB, a successive combination of chemotherapy or external radiation therapy is given to the patients. For stage IV NSCLC patients, palliative external

radiation therapy, combination chemotherapy and targeted therapy, laser therapy or internal endoscopic radiation therapy are considered as suitable treatment strategies (Lemjabbar-Alaoui *et al.,* 2015).

Docetaxel (Taxotere), pemetrexed, erlotinib and gefitinib are approved as second-line therapy for advanced NSCLC patients with an acceptable performance status and who have failed first-line platinum therapy (Lemjabbar-Alaoui *et al.,* 2015). The first approved targeted drugs for NSCLC patients are agents with ability of specifically blocking EGFR which include tyrosine kinase inhibitor (TKI); erlotinib (Tarceva) and gefitinib (Iressa). It is well evinced that angiogenic pathways aid in tumor growth through development of new blood vessels and thus they provide an important target in NSCLC treatment. The monoclonal antibody named bevacizumab which targets circulating vascular endothelial growth factor (VEGF) is approved as the first-line therapy for advanced NSCLC patients in combination with chemotherapy. Various other anti-angiogenic agents such as sorafenib and sunitinib are still under investigation (Lemjabbar-Alaoui *et al.,* 2015). Apart from these, histone deacetylase (HDAC) inhibitors, insulin-like growth factor inhibitors and talactoferrin (human recombinant lactoferrin) are also underway (Lemjabbar-Alaoui *et al.,* 2015). Besides, a few platinum analogs such as picoplatin and satraplatin are also in early clinical development against NSCLC. Additionally, the epothilones, a novel class of drugs which include ixabepilone, sagopilone and patupilone, are also used for the treatment of lung cancer (Chang, 2011). Recently, immunotherapeutics were also found to have profound potential to increase the overall survival of lung cancer patients (Hall *et al.,* 2013). Two monoclonal antibodies, namely ipilimumab and tremelimumab have been used in NSCLC against the cytotoxic T-lymphocyte-associated antigen (CTLA-4), an inhibitory T-cell co-receptor found on activated T-cells as well as regulatory T-cell

subsets, effectively. In addition, different clinical trials are presently ongoing to check the efficacy of other immune checkpoint inhibitors such as anti-PD-1 as monotherapy or in combination with chemotherapy in NSCLC. Besides, various vaccines have also been developed for the treatment of NSCLC which include Mucin-1 (MUC1), Melanoma-associated antigen A3 vaccine (MAGE-A3), CimaVax EGF, PRAME vaccine etc. (Lemjabbar-Alaoui *et al.*, 2015).

1.7.2. Treatment approaches for SCLC

Similar to other lung cancers, the treatment strategies for SCLC patients are also determined on the basis of histology, stage, general health and comorbidities of the patient. 30% of SCLC patients are diagnosed with limited-stage disease (LS-SCLC), whereas 70% of SCLC patients are diagnosed with extensive-stage disease (ES-SCLC). In case of LS-SCLC, the cancer is confined to the hemithorax, the mediastinum, or the supraclavicular lymph. However, in case of ES-SCLC, the tumors spread beyond the supraclavicular areas. For LS-SCLC, platinum based chemotherapy and radiation therapy, combination chemotherapy alone, surgery followed by chemotherapy or chemoradiation therapy and prophylactic cranial irradiation are the standard treatment strategies adopted. In case of ES-SCLC patients, combination chemotherapy, radiation therapy and prophylactic cranial irradiation is performed. Further, for recurrent SCLC, the standard treatment options include chemotherapy and palliative therapy (Lemjabbar-Alaoui *et al.*, 2015).

In the past several decades, different attempts have been made to design targeted therapies against RTK and their downstream signaling mediators such as Ras and PI3K/Akt/mTOR etc. for the treatment of SCLC (Arcaro, 2015). Some of these targeted therapies developed against SCLC include VEGF inhibitors bevacizumab, sorafenib,

sunitinib, and cediranib; EGFR inhibitors, bendamustine (an alkylating agent which causes DNA breaks through its alkylating activity) etc. Besides, several studies were performed to evaluate the efficacy of immunotherapy such as BEC2/BCG and INGN-225 vaccines for the treatment of SCLC (Lemjabbar-Alaoui *et al.,* 2015).

1.8. Problems associated with lung cancer

Management of lung cancer patients still presents a major challenge to the oncologists (Chang,2011). This can be primarily attributed to factors such as late stage diagnosis, tumor recurrence and chemoresistance.

1.8.1. Late stage diagnosis

This can be primarily attributed to the poor prognosis associated with this disease due to which most of the lung cancer cases are not identified until it reaches a highly advanced stage (Hirsch *et al.,* 2002). As a matter of fact, approximately 55% of patients have been reported to diagnose at metastatic (stage IV) disease stage (Chang A, 2011). Consequently, majority of the patients face an intensive and invasive treatment regimen comprising surgery, radiotherapy, or chemotherapy, or their combinations depending on disease stage or patient's performance status. Moreover, most of patients were reported to undergo multiple lines of therapy as they develop chemoresistance to the particular chemotherapeutic agents they are exposed to (Chang, 2011).

1.8.2. Tumor recurrence

NSCLC is associated with high recurrence rate which is approximately 30-75%. It has been reported that in more than 80% of the cases, recurrence occur within the first two years of presentation with an added annual recurrence rate of 3-6% (Caulo *et al.,* 2012). Patients in stages I and II of the TNM system suffer recurrence quite frequently,

which end fatally (Chudacek *et al.,* 2014). This recurrence can be either loco-regional where recurrence is confined within the treated hemithorax or distant metastases in which multiple organ metastases are often noticed (Caulo *et al.,* 2012). Further, reports suggest that up to 24% of the patients recur locally after lung cancer surgery and its risk further increases with the stage of the primary cancer. Overall survival time after local recurrence differs broadly from 7 to 26 months as per different studies (Fedor *et al.,* 2013).

1.8.3. Development of chemoresistance

Resistance to chemotherapeutic agents currently available for the treatment of lung cancer presents a major obstacle in improving the long-term after effects for the susceptible group of patients (Chang, 2011). Resistance to doxorubicin, etoposide, gemcitabine, docetaxel, vinorelbine, paclitaxel, and topotecan was reported in 75, 63, 72, 52, 42, 40 and 31% of samples, respectively. All NSCLC patients were reported to develop chemoresistance against the agents in due course of time to which they are exposed, even after exerting a good initial response. Different traditional resistance mechanisms reported in other solid tumors have also been found in various NSCLC studies (Chang 2011; Yang *et al.,* 2015).

Therefore, 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, the TIPE family of proteins seems to hold enormous prospect in this regard.

1.9. TIPE family of proteins

The TIPE family is a newly known family of proteins which is responsible for the regulation of immunity and tumorigenesis. This family comprises of four members such as, TIPE or TNFAIP8 (tumor necrosis factor-α-induced protein 8), TIPE1 (TNFAIP8-like 1, or TNFAIP8L1), TIPE2 (TNFAIP8-like 2 or TNFAIP8L2), and TIPE3 (TNFAIP8-like 3 or TNFAIP8L3) (Goldsmith *et al.*, 2017). Interestingly, although these four proteins of this family share a considerable sequence homology (~54% sequence identity), but they are involved in diverse cell functions (Bordoloi *et al.*, 2018).

1.9.1 TIPE

TIPE, the first and the most studied member of this family is a transcription factor nuclear factor- kappa B (NF-κB) inducible, oncogenic molecule and cytoplasmic protein of 21 K-Da; initially identified in human head and neck squamous cell carcinoma (Bordoloi *et al.*, 2018). It is expressed in various human normal tissues, however considerably higher expression was found in placenta and lymphoid tissues (Kumar *et al.*, 2000). The open reading frame of this protein possess a sequence in the amino terminus which presents marked homology to the death effector domain II of the cell death regulatory protein, Fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein (FLIP) (Kumar *et al.*, 2000). Further, the crystal structure of TIPE from *Mus musculus* (mTIPE) revealed it to posses similarity to a water dipper. Its cylindrical domain posses a dimension of 48 × 31 × 30 Å linked to an N-terminal grip-like domain, comprising of 20 residues and contains two long electron densities. It has a hydrophobic cavity lined with highly conserved hydrophobic residues, thereby aids in the binding of substrates inside the cavity or hydrophobic cofactors (Kim *et al.*, 2017b).

1.9.2. TIPE1

TIPE1 (tumor necrosis factor-α-induced protein 8-like 1) is a recently found member of the TIPE family which function as a cell death regulator. TIPE1 was predicted to interact with caspase-8 and FBXW5. Besides, different post-translational modifications were also prophesied to exist in case of TIPE1 (Shen *et al.*, 2015; Bordoloi *et al.*, 2018).

1.9.3 TIPE2

TIPE2 (tumor necrosis factor-α-induced protein 8-like 2), a cytoplasmic protein consisting of 184 amino acids and the third member of this family, is a latterly discovered negative regulator of innate and cellular immunity, with considerable sequence homology with the other members of the family (Bordoloi *et al.*, 2018). TIPE2 plays critical role in the maintenance of immune homeostasis. It was found to be highly expressed in inflamed but not normal nervous tissue (Zhang *et al.*, 2009). TIPE2 was found to be expressed in varied cell types such as neurons in the brain and brainstem, hepatocytes, squamous epithelial cells in the cervix and esophagus, glandular epithelial cells in the colon, stomach, and appendix and transitional epithelial cells in the ureter and bladder (Zhang *et al.*, 2011). The crystal structure of human TIPE2 showed that it comprises of a total of six antiparallel α -helices. Among them, α5 helix bears a kink, due to Pro153, leading to its splitting into α5a and α5b, where the later forms the base of helical bundle. TIPE2 possess a cylindrical cavity located centrally which is mostly hydrophobic in nature whereas the outer surface of TIPE2 is found to be highly charged. The central cavity is predicted to be poised for cofactor binding and thus helps in immune homeostasis. Notably, the structure of TIPE2 differs from that of death effector domain (DED); firstly TIPE2 consists of around 150 amino acids which is relatively larger than that of DED and secondly, the topology of both are

different. In fact, the topology of TIPE2 seems to be a mirror image of that of DED (Zhang *et al.*, 2009) (Figure 1.4).

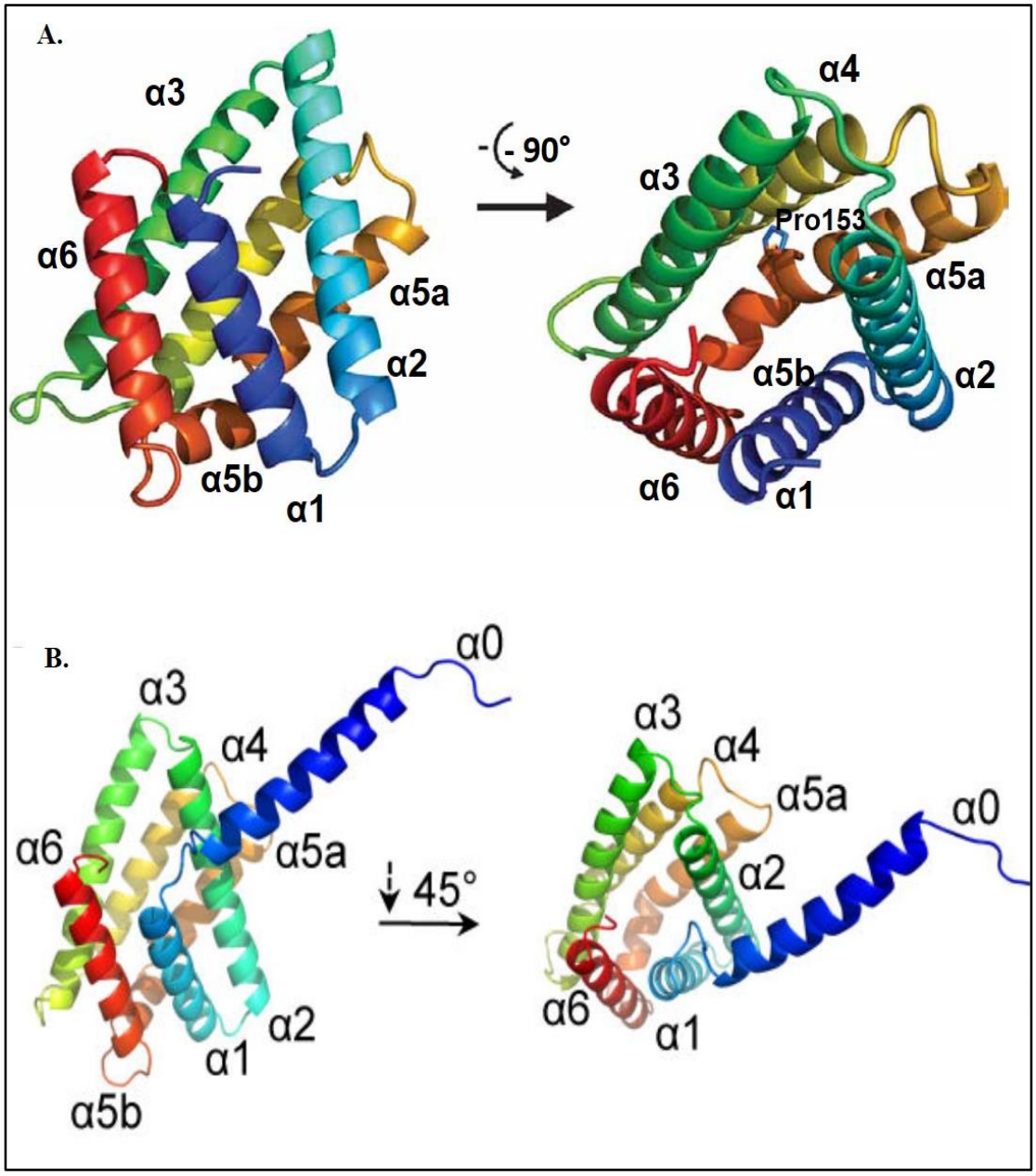

Figure 1.4. The structure of TIPE2 and TIPE3. A. The structure of TIPE2 defines a previously uncharacterized fold that is different from the DED. It is is shown in two perpendicular views. The six α-helices are rainbow colored. Pro153, which breaks α5 into two helices, is indicated at right (Zhang *et al.*, 2008), B. Cartoon presentation of TH-domain of human TIPE3 (trTIPE3, residues 21-204). Helices are rainbow-colored with α0 in blue and α6 in red (Fayngerts *et al.*, 2014).

1.9.4. TIPE3

TIPE3 (tumor necrosis factor-α-induced protein 8-like 3), the latest member of TIPE family acts as a transfer protein for lipid second messengers PIP2 (phosphatidylinositol 4,5-bisphosphate) and PIP3 (phosphatidylinositol 3,4,5-trisphosphate), and increases their level in the plasma membrane (Fayngerts *et al.*, 2014; Bordoloi *et al.*, 2018). Further, TIPE3 is expressed in different human organs and is highly upregulated in certain cancer types such as cancers of the cervix, colon, esophagus and lung (Lian *et al.*, 2017). In addition, the crystal structure of TIPE3 reveals that it consists of an N-terminal α0 helix, and six additional helices (α1-α6) which are similar to those of TIPE2. The most important feature of the TH fold of TIPE3 is the presence of a large centrally located hydrophobic cavity, possessing 20 Å depth and 10 Å diameter, which is speculated to serve as the binding site for a lipophilic molecule. Thus TIPE3 binds to phosphoinositides with the help of its TH domain, which essentially helps in its function as transfer protein for lipid second messengers. Further, the hydrophobic cavity in TIPE3 is occupied by two electron density tubes, which are connected at the surface opening of the cavity (Fayngerts *et al.*, 2014) (Figure 1.4).

1.10. Role of TIPE family of proteins in different cancers

Cancer, which stems from the perturbations of multiple signaling pathways, affects people of all ages and is a major health concern worldwide (Bordoloi *et al.*, 2016). The TIPE family of proteins plays a vital role in carcinogenesis and metastasis through its deregulated expression and function. It has been found to be strongly associated with cancers of breast, bone, brain, cervix, colon, esophagus, endometrium, liver, lung, stomach, and thyroid (Figure 1.5).

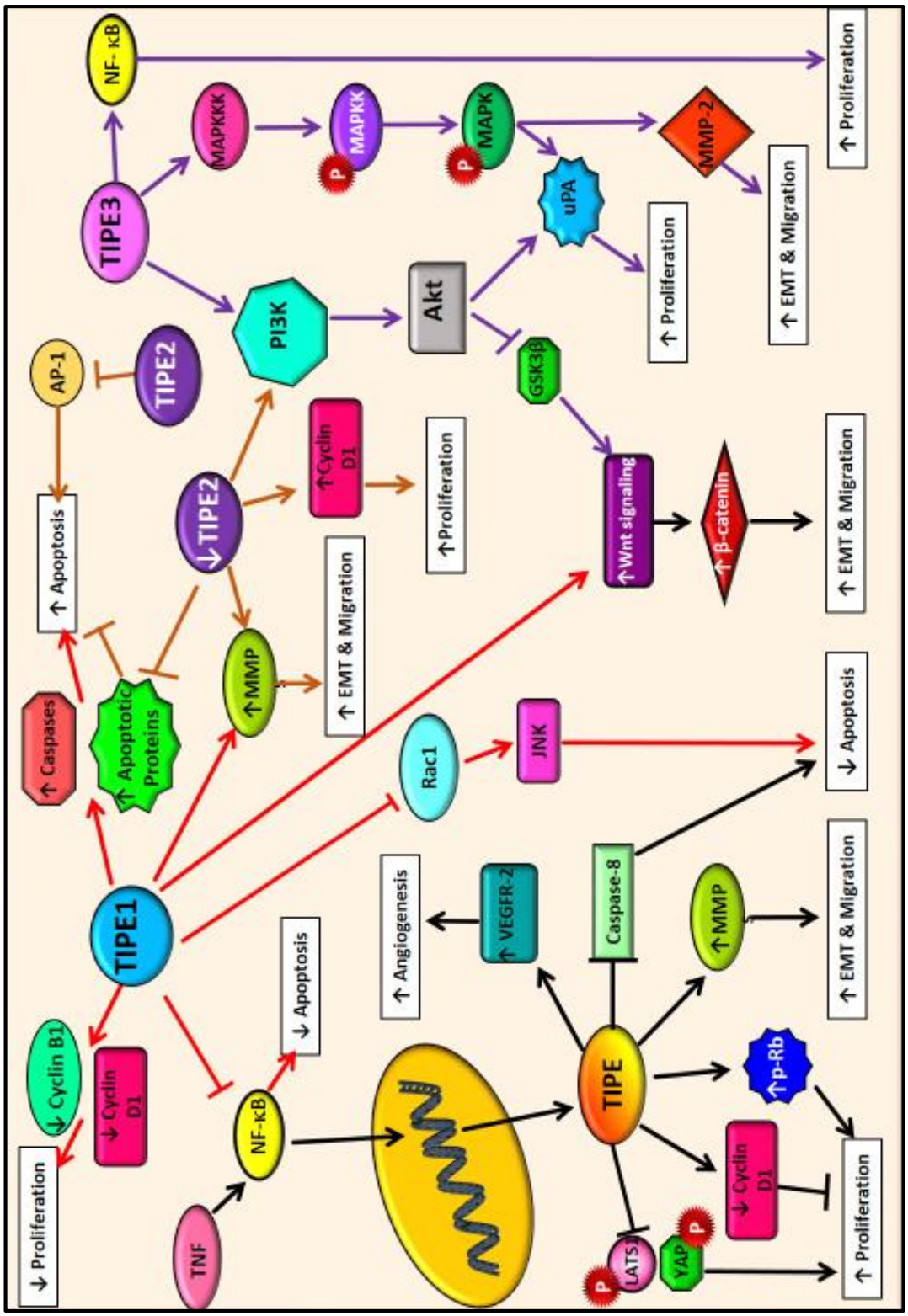

Figure 1.5. Role of TIPE family of proteins and the associated molecular mechanisms in different cancers.

1.10.1. TIPEs and breast cancer

TIPE has been found to be overexpressed in various human cancers. In the case of invasive ductal breast carcinoma (IDC) patients, high expression of TIPE was observed and correlated with shorter survival time and axillary lymph node metastasis. Its overexpression contributes to tumor progression and thus may serve as a novel prognostic biomarker for patients with IDC (Xiao *et al.*, 2017). Another study showed that the expression of TIPE in MDA-MB 435 human breast cancer cells is correlated with enhanced invasion as well as frequency of pulmonary colonization of tumor cells. Downregulation of TIPE in tumor cells was found to be linked with reduced expressionof matrix metalloproteinase (MMP)-1 and MMP-9, and the inhibition of its endogenous expression was strongly associated with decreased expression of vascular endothelial growth factor receptor (VEGFR) -2 in tumor cells (Zhang *et al.*, 2006). Further, FLAG-tagged TIPE transfected MDA-MB 435 cells exhibited an increased growth rate and cell migration in collagen I *in vitro* and remarkably increased tumor growth *in vivo* when compared to the control transfectants (Kumar *et al.*, 2004). Wang and group showed that TIPE2 was significantly downregulated in human breast cancer cells as well as tissues, and its overexpression inhibited the proliferation of tumor cells and tumor xenograft growth. TIPE2 inhibited the migration or invasion of breast cancer cells via inhibition of β-catenin, Cyclin D1, and c-Myc together with epithelial-to-mesenchymal transition (EMT) (Wang *et al.*, 2017). Further, TIPE3 was found to be highly upregulated in human breast cancer tissues than the adjacent normal tissues from the same patients as well as in highly-invasive breast cancer cells compared to low-invasive cells. Further, knockdown of TIPE3 inhibited the proliferation, invasion, and migration of breast cancer cells. Additionally, TIPE3 promoted the metastasis of breast

cancer cells significantly, augmented the level of urokinase-type plasminogen activator (uPA) and MMP2, and also activated the NF-κB and Akt signaling pathways, suggesting its potential role as a breast cancer metastasis biomarker (Lian *et al.*, 2017).

1.10.2. TIPEs and cervical cancer

Cervical cancer is the second most common cancer among women worldwide (Maheshwari *et al.*, 2016). TIPE, the negative mediator of apoptosis, plays an important role in carcinogenesis of the cervix. For instance, HeLa cells were transiently transfected with FLAG epitope-tagged TIPE cDNA or expression vector and, treatment with tumor necrosis factor-alpha (TNF-α), resulted in increased apoptotic cells than the untreated one. Nevertheless, TIPE transfectants showed a remarkable decrease in the number of apoptotic cells compared to the vector transfectants (Kumar *et al.*, 2000). Another study investigated the links between cervical cancer risk, as well as platinum resistance and clinical outcomes in Eastern Chinese women and three functional single nucleotide polymorphisms in TIPE. They found that the TIPE-rs11064 variant GG genotype was linked with an increased risk of cervical cancer compared with AA/AG genotypes. In addition, high expression of TIPE was responsible for cisplatin and nedaplatin resistance, tumor recurrence and death due to cervical cancer (Shi *et al.*, 2013).

1.10.3. TIPEs and colon cancer

Colon cancer positions third among the most commonly occurring malignancies across the globe. Despite the substantial developments in cancer therapy, mortality from colon cancer remains the same, emphasizing the need of improved therapies and effective biomarkers (Nautiyal *et al.*, 2011). Growing evidence suggest that TIPE may serve as

a potential therapeutic target against colon cancer. It was found to be upregulated in 48.9 % of colon cancer specimens and was strongly linked with TNM stage, lymph node metastasis, and proliferation index. Further, knockdown of TIPE in colon cancer cells resulted in the reduction of cell proliferation and colony formation, together with downregulation of Cyclin D1 and phospho-Rb. (Miao *et al.*, 2012).

1.10.4. TIPEs and endometrial cancer

Endometrial carcinoma (EC) is one of the most common form of malignancies in women worldwide. In fact, it has been ranked at sixth position among female cancers (Morice *et al*. 2016). The role of TIPE protein in the pathogenesis of EC was studied by Liu and group in a total of 225 tumor specimens. Overexpression of TIPE strongly correlated with various clinico-pathologic factors such as advanced International Federation of Gynecology and Obstetrics stage, deep myometrial invasion, higher histologic grade, lymphovascular space invasion, lymph node metastasis, and tumor recurrence, together with increased MMP-9 and Ki-67 expression. Patients with a high expression level of TIPE as well as Ki-67 had weak overall and disease free survival rates, whereas overexpression of MMP-9 did not alter the survival rate. Taken together, these results are indicative of the potential of TIPE as a prognostic marker for the patients with EC recurrence (Liu *et al.*, 2014a).

1.10.5. TIPEs and esophageal squamous cell carcinoma

Esophageal squamous cell carcinoma (ESCC) has extremely poor prognosis as a result of lymphatic metastatic recurrence after esophagectomy. Sun and colleagues attempted to decipher the association between TIPE and lymphatic recurrence after enrolling a total 120 pN0 ESCC patients who had an Ivor Lewis esophagectomy performed. 59.8%

of patients exhibited TIPE overexpression and they also showed a significantly high 3-year lymphatic metastatic recurrence rate. Furthermore, transient silencing of TIPE led to reduced cell motility and invasion, and enhanced apoptosis, whereas its stable silencing resulted in decreased proliferation, motility, and invasion, and enhanced apoptosis in ESCC derived cells. Thus, TIPE overexpression may serve as a potent biomarker in identifying pN0 ESCC patients who are at high risk of lymphatic recurrence (Sun *et al.*, 2016). Another study carried out by Hadisaputri and group sought to reveal the functional and clinical significance of TIPE in ESCC, where they reported TIPE to have a strong impact on several clinicopathological characteristics such as TNM stage, lymph node involvement, tumor depth, lymphatic and venous invasion, and distant metastasis of the ESCC patients. High expression of TIPE correlated with poor survival of ESCC patients. In addition, depletion of TIPE resulted in reduction in proliferation and induction of apoptosis after cisplatin administration in ESCC cells (Hadisaputri *et al.*, 2012).

1.10.6. TIPEs and glioma:

Gliomas, also known as glial tumors, are the primary brain tumors presenting a major health burden worldwide (Jobin Christ and Parvathi, 2012). TIPE2 has been involved in the development and progression of glioma. Its expression was found to be remarkably less in glial cells. It is well proven that hypoxia-induced EMT plays a vital role in the progression of tumors. Liu and group showed that overexpression of TIPE2 suppressed the EMT phenomenon and prevented hypoxia-induced migration and invasion, and suppressed the expression of β-catenin, c-Myc, and Cyclin D1 in human glial cells (Liu *et al.*, 2016).

1.10.7. TIPEs and gastric cancer

Gastric cancer constitutes to be the third leading cause of cancer-related deaths globally, following lung and liver cancer (Ferlay *et al.,* 2015). TIPE has been found to be highly upregulated in gastric cancer cells as well as tissues. The expression of this protein was further correlated with TNM stage and lymphatic metastasis of gastric cancer. Furthermore, decreased expression of TIPE led to the inhibition of growth, invasion, and migration of gastric cancer cells, suggesting that TIPE represents a potential biomarker for gastric cancer progression (Li *et al.*, 2015). Another study also showed TIPE to possess strong correlation with TNM stage, tumor depth, and lymph node metastasis as well as distant metastasis. Further, anti-human death receptor 5 single-chain antibody- induced TIPE depletion caused apoptosis and decreased proliferation of gastric cancer cells (Hu *et al.*, 2016). In case of gastric adenocarcinoma, a high expression of level of TIPE was observed to be associated with high metastasis and poor prognosis (Chen *et al.*, 2016). Yang and colleagues showed that enhanced expression of TIPE in intestinal-type gastric adenocarcinoma strongly correlated with invasion, lymph node metastasis, Lauren classification, weak overall survival and disease-free survival of patients (Yang *et al.,* 2014). In the case of TIPE1, it was observed that TIPE1 expression was significantly lower in gastric cancer tissues, which negatively linked with differentiation status and distant metastasis in those tissues. Furthermore, TIPE1 overexpression inhibited 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. Mechanistic evaluation reported that a connection between TIPE1 and EMT is established via Wnt/β-catenin signaling cascade mediated by TIPE1, and interestingly, TIPE1 is involved in the regulation of MMP-2 and MMP-9, which play important roles in tumor progression and EMT (Liu *et al.,* 2018b). Further, Yin and colleagues conducted a study on TIPE2 to reveal its association with

EMT in gastric cancer. They reported that adenovirus-mediated human TIPE2 gene transfer (AdVTIPE2) in gastric cancer cells resulted in upregulation of E-cadherin, while it led to the downregulation of Vimentin, N-cadherin, mesenchymal markers, Snail1, Snail2/Slug, Zeb1 EMT-inducing transcription factors, tripartite motif-containing 29, and phosphatase regenerating liver-3 gastric cancer-specific metastasis markers. Mechanistically, TIPE2 downregulated Snail1 and Snail2/Slug in a GSK-3β- and proteasome-dependent fashion plausibly mediated via Akt (Yin *et al.,* 2017). Consistent with the previous study, Wu and group also reported that TIPE2 suppressed the metastasis of gastric cancer cells through downregulation of the β-catenin signaling pathway via activation of GSK3β and inhibition of Akt (Wu *et al.,* 2016). Another study reported that TIPE2 expression is lost in gastric cancer cells when compared to normal human gastric mucous epithelial cells. Further, adenovirus-mediated TIPE2 overexpression inhibited the growth and induced apoptosis of gastric cancer cells both *in vitro* and *in vivo* through upregulation of Bax, cleaved poly ADP ribose polymerase (PARP), cleaved caspase-3, -9, and downregulation of B-cell lymphoma (Bcl)-XL, phosphorylated-extracellular signal-regulated kinase 1/2 (p-ERK1/2) and phosphorylated-Akt. Thus, taken together, it can be concluded that AdVTIPE2 suppressed the growth of gastric cancer cells plausibly via reduction of Akt and ERK1/2 and activation of the intrinsic apoptotic pathway, indicating TIPE2 as an effective target for therapies against gastric cancer (Zhu *et al.,* 2016).

1.10.7. TIPEs and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) or liver cancer is the fifth most frequently occurring cancer in men and seventh in women (Soldera *et al*., 2016). TIPE has been found to promote HCC progression through modulation of the LATS1-yes-associated protein

(YAP) signaling cascade. Dong and group reported that overexpression of TIPE was strongly linked with TNM stage, recurrence, and poor prognosis in primary HCC samples. Furthermore, its overexpression led to the inhibition of YAP phosphorylation but enhanced its nuclear localization and stabilization, resulting in the enhanced cell proliferation and upregulation of Cyclin and connective tissue growth factor proteins. They also reported TIPE to interact with LATS1 and decrease its phosphorylation, whereas depletion of LATS1 and YAP was shown to block the effects of TIPE (Dong *et al.*, 2017). Another study reported that TIPE2 could suppress HCC metastasis induced by TNF-α through inhibition of NF-κB and Erk1/2, implying that TNF-α as well as TIPE2 could be potential targets against HCC metastasis (Zhang *et al.*, 2015a). Zhang and group showed that TIPE1 diminished the growth and tumor weight of murine liver cancer homografts remarkably and also inhibited the growth and colony forming ability of HCC cells. Mechanistically, interaction of TIPE1 with Rac1 led to the inhibition of Rac1 activation as well as the 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 (Zhang *et al.*, 2015b).

1.10.8. TIPEs and non-Hodgkin's lymphoma

Non-Hodgkin's lymphoma (NHL), a refractory malignant tumor arising from the lymphatic system includes both peripheral T-cell lymphoma (PTCL) and diffuse large B-cell lymphoma (DLBCL). To elucidate the role of TIPE proteins in lymphomagenesis, a few studies were conducted. For instance, Hao and colleagues found enhanced expression of TIPE2 in both PTCL and DLBCL, though its expression in T lymphoma was relatively poor compared to that of DLBCL. Moreover in DLBCL,

TIPE2 exerted remarkably stronger expression in the germinal center B-cell (GCB) compared to the non-GCB type, suggesting TIPE2 as a potent predictive marker for DLBCL (Hao *et al.,* 2016). Furthermore, genetic polymorphisms of candidate genes associated with pro-inflammatory and autoimmune responses may affect the susceptibility towards developing NHL. A study in a Chinese population showed that the polymorphism of TIPE rs1045241C>T might increase the susceptibility to NHL. Interestingly, the risk associated with the T allele was more obvious in patients of age group 40-60 years, light- or non-smokers, and subjects with normal or overweight (Zhang *et al.*, 2012).

1.10.9. TIPEs and lung cancer

A few studies were conducted to unveil the relation between the TIPE family of proteins and lung cancer as well. One study showed that TIPE promoted proliferation and invasion in lung cancer cells via modulation of the Hippo pathway through interaction with LATS1. TIPE also increased Cyclin D1, cyclin-dependent kinase (CDK) 6, total YAP protein, and nuclear localization of YAP, and decreased p27 in lung cancer cells. Further, overexpression of TIPE led to enhanced MMP-7, and depletion of TIPE resulted in reduction of MMP-7 without causing any alteration in the level of N-cadherin, E-cadherin, and vimentin (Han *et al.,* 2018). Another study demonstrated that the level of TIPE in tumor-infiltrating $CD4^+$ and $CD8^+$ T cells was significantly less compared to peripheral $CD4^+$ and CD8+ T cells. In addition, patients with advanced NSCLC displayed lower TIPE expression in tumor-infiltrating $CD8^+$ T cells than those in primary stages of the disease (Wang *et al.,* 2014). Dong and group conducted a study in order to evaluate the expression profile and clinicopathological significance of TIPE in NSCLC patients. TIPE showed marked upregulation in lung cancer tissues compared

to normal tissues, which correlated with p-TNM stage, lymph node metastasis, expression of Ki-67, and poor survival (Dong *et al.*, 2010). Further, the expression and biological function of TIPE1, which acts as a regulator of cell death, 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 MMP-2, -9. Unfailingly, TIPE1 also inhibited the growth and tumor weight of murine lung cancer homografts, clearly revealing the potent anti-tumor role of TIPE1 in lung cancer cells (Wu *et al.*, 2017). Li and group evaluated the role and expression of TIPE2 in human NSCLC where TIPE2 was found to be upregulated in NSCLC tissues, which negatively correlated with the primary tumor size, lymph node metastasis, and advanced clinical stage. The same study again reported that overexpression of TIPE2 exerted an inhibitory effect on the colony formation, invasion, and migration of NSCLC cells and also suppressed proliferation, migration, and tube formation of vascular endothelial cells. Further, TIPE2 was found to repress tumor invasiveness and angiogenesis via blocking of Rac1 activation as well as the expression of its downstream targets such as VEGF and F-actin polymerization (Li *et al.*, 2016). Coming to TIPE3, it was found to be broadly expressed in lung tissues of NSCLC patients. Wang and group showed that TIPE3 induced the proliferation and migration of NSCLC cells based on its localization on plasma membrane, while TIPE3 located in the cytoplasm might exert a negative effect. Knockdown of endogenous TIPE3, which is mostly expressed in the plasma membrane, led to the inhibition of proliferation and migration of NSCLC cells. Further, transient overexpression of TIPE3 with an N-terminal flag, predominantly present in the cytoplasm, prevented the growth and migration of NSCLC cells together with inactivation of ERK and Akt. Contrastingly,

stable overexpression of TIPE3 with a C-terminal flag, which might be localized in the plasma membrane, induced the growth and migration of NSCLC cells mediated via activation of the ERK and Akt pathway (Wang *et al.*, 2018a).

1.10.10. TIPEs and ovarian cancer

Ovarian cancer is the most fatal gynecological cancer worldwide with a 5-year survival rate of 46% (Brasseur *et al.*, 2017). TIPE functions as a potent therapeutic target for epithelial ovarian cancer (EOC) as TIPE expression status can predict both cancer-specific survival as well as disease-free survival of EOC patients. Overexpression of TIPE was associated with high histologic grade, large residual size of tumor, recurrence and poor response to chemotherapy, and the presence of lymph node and intraperitoneal metastasis (Liu *et al.*, 2013). Further, TIPE overexpression was found to be associated with platinum resistance in EOC following optimal cytoreduction, and thus it may serve as a biomarker for targeted therapy (Liu *et al.*, 2014b).

1.10.11. TIPEs and osteosarcoma

Osteosarcoma (OS) is characterized as the most common primary malignant bone tumor in adolescents and young adults all across the globe. Notably, microRNAs (miRNAs) have been found to be involved in the cellular biology and development of cancers including osteosarcoma. Xing *et al.* depicted TIPE as a direct target of miR-99a, which was upregulated in OS. Further, knockdown of TIPE resulted in decreased viability and growth of OS cells via induction of apoptosis and inhibition of cell cycle in pre-clinical settings (Xing *et al.*, 2016). Again, TIPE was recognized as a novel target of miR-138, which directly targets TIPE and acts as a tumor suppressor in OS. Overexpression of miR-138 as well as downregulation of TIPE attenuated OS cell

invasion mediated through blockage of MMP-2 and MMP-9 expression (Zhou *et al.*, 2017).

1.10.12. TIPEs and pancreatic cancer

Pancreatic cancer remains one of the most deadly cancers, with mortality rate almost similar to the incidence rate (Siegel *et al*., 2014; Kamisawa *et al*., 2016). TIPE plays an important role in the progression of pancreatic cancer. The expression of TIPE protein was higher in pancreatic cancer tissues than the normal pancreas tissues. Further, patients with low tumor stage exhibited a higher expression level of TIPE compared to the high tumor stage, and the expression was found to be strongly linked with tumor stage and lymph node metastasis of patients (Liu *et al.*, 2012).

1.10.13. TIPEs and prostate cancer

Prostate cancer is the second most commonly occurring cancer among males, with 1.1 million new cases across the globe (Torre *et al*., 2015). TIPE and TIPE2 proteins have been found to take part in prostate carcinogenesis. Knockdown of TIPE in prostate cancer cells resulted in the enhanced expression of tumor suppressors which include IL24 (mda-7), FAT3, EPHA3, LPHN2, IGFBP3, and reduced expression of tumor progression markers such as FOXA1, KRAS, NFAT5, OSTF1, MALAT1, MAP2K6, S100P, FLRT2, and MET (Day *et al*., 2017). Further, treatment with a liposomal formulation of TIPE antisense oligonucleotide (LE-AS5) in hormone-refractory PC-3 prostate tumor xenografts led to the attenuated expression of TIPE, whereas co-administration of LE-AS5 and radiation or docetaxel caused marked inhibition of PC-3 tumor growth than either agents alone. Moreover, cytoplasmic and nuclear overexpression of TIPE was associated with high grade prostatic adenocarcinomas, and

nuclear overexpression was reported to be a disease recurrence and tumor grade prediction indicator (Zhang *et al.*, 2013). Li and group evaluated how TIPE2 contributes to prostate cancer progression along with mechanisms involved in TIPE2-mediated oncogenesis. The results showed that TIPE2 expression was lower in prostate cancer cells and tissues. Further, overexpression of TIPE2 caused suppression of migration, invasion, and the process of EMT in prostate cancer cells through inhibition of the PI3K/Akt signaling pathway (Lu *et al.*, 2016).

1.10.14. TIPEs and thyroid cancer

Thyroid cancer is a common endocrine malignancy which include both papillary and follicular subtypes (Gupta-Abramson *et al.*, 2008). Duan and group evaluated the expression of TIPE in both immune cells and tumor cells in the thyroid. The results showed that TIPE was expressed at significantly high levels in peripheral $CD4^+$ and $CD8^+$ T cells but not monocytes and natural killer T cells in patients compared to the healthy donors. Moreover in $CD4^+$ T cells, only enhanced TIPE mRNA was observed, whereas tumor-infiltrating $CD4^+$ T cells showed enhanced TIPE expression at both the mRNA and protein level. Again, TIPE expression was remarkably higher in thyroid tissues when compared to the adjacent noncancerous thyroid tissues. Additionally, patients bearing cervical lymph node metastasis exerted higher TIPE expression than those without cervical lymph node metastasis (Duan *et al.*, 2014).

1.11. TIPEs and other chronic diseases

Although, literature mostly reveals about the involvement of TIPE family of proteins with diverse cancer types, they were reported to play role in different chronic diseases as well (Figure 1.6). For instance, TIPE and TIPE2, the regulators of immunity, have

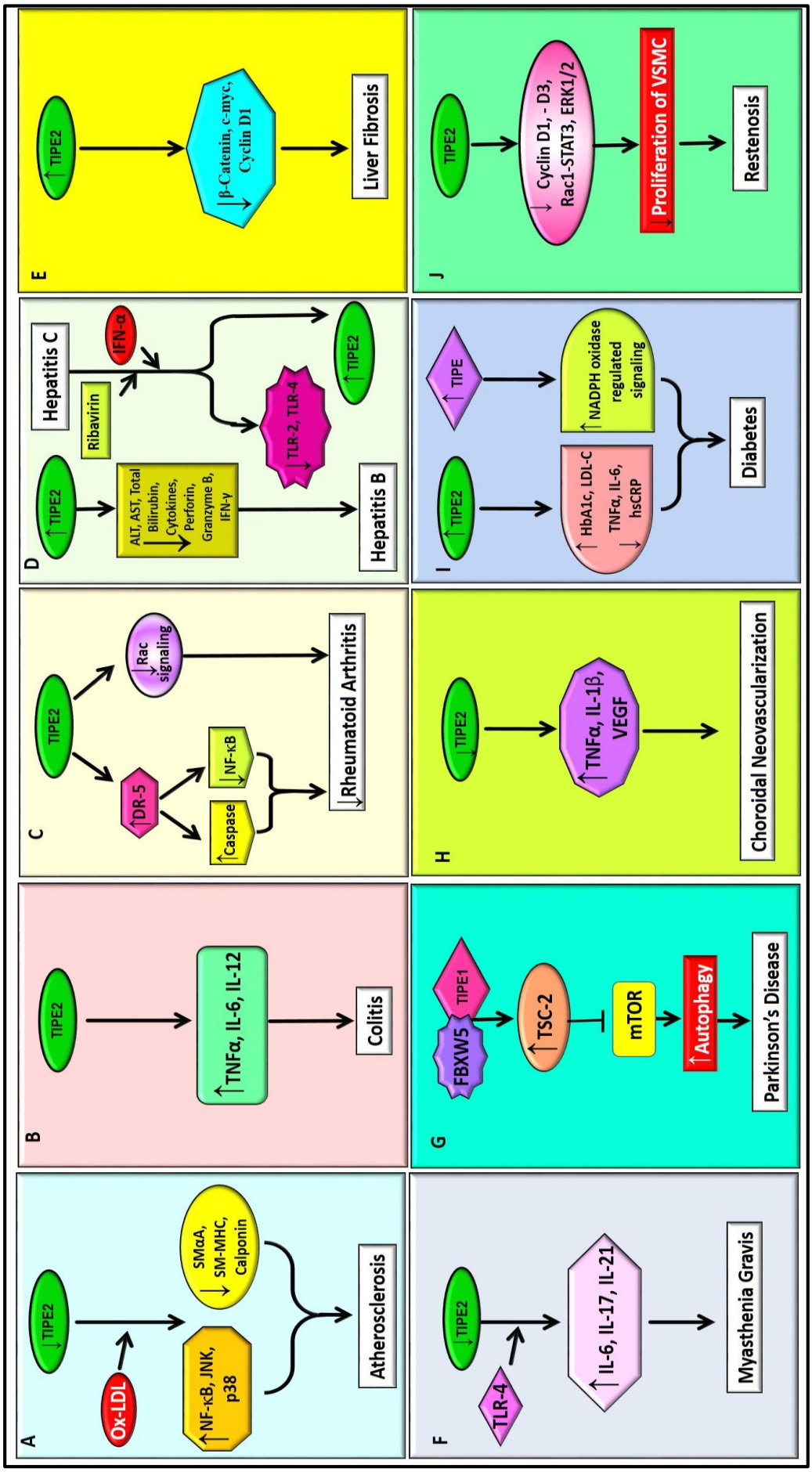

Figure 1.6. Role of TIPE family of proteins and their molecular mechanisms in different chronic diseases. A. Atherosclerosis, B. Colitis, C. Rheumatoid Arthritis, D. Hepatitis B, E. Liver Fibrosis, F. Myasthenia Gravis , G. Parkinson's Disease, H. Choroidal Neovascularization, I. Diabetes, J. Restenosis (Bordoloi *et al.*, 2018).

been demonstrated to show protectin against inflammatory diseases such as atherosclerosis, colitis, and rheumatoid arthritis. Further, these proteins exerted their effect against various infectious diseases such as hepatitis B, hepatitis C, listera infection, and liver fibrosis. In addition, TIPE1 and TIPE2 have been found to exhibit their effect in Parkinson's disease and Myasthenia Gravis respectively and thus can serve as important targets for therapies against these diseases. Apart from the abovementioned, this newly discovered family of proteins is strongly involved in various other diseases such as choroidal neovascularization (CNV), restenosis, and metabolic disease like diabetes (Bordoloi *et al.*, 2018).

1.12. Importance of the study

Lung cancer is the most frequent cause of cancer-related death worldwide. Although several advancements have been made in lung cancer treatment till date, but yielded only modest improvement in survival over the past few decades. Consequently, the survival rate of lung cancer is almost the lowest among all the cancers. Notably, the high mortality due to lung cancer can be attributed to its propensity for early spread, late stage diagnosis and lack of effective biomarkers for early diagnosis and prognosis. Therefore, there is an urgent need to develop novel biomarkers for early diagnosis and prognosis which in turn can provide newer therapeutic avenues for the management of this neoplasm. Notably, a number of biomarkers have been identified till date, however most of them were found to have low specificity, sensitivity and reproducibility and thus exerted utmost minimal use. Increasing lines of evidence suggest that TIPE family of proteins plays immensely important role in inflammation, immunity, tumorigenesis and diverse other cellular process. Most importantly, the members of this family share a significant sequence homology, but are involved in varied biological activities.

Though this family of proteins plays a critical role in carcinogenesis, metastasis, and development of different human chronic diseases, its exact molecular functions, detailed mechanism of action, and the probable crosstalk among the members of this family in lung carcinogenesis, in particular tobacco induced lung carcinogenesis remain unclear. Therefore, a better understanding of the association between this important family of proteins and development of lung cancer, especially tobacco induced lung cancer would definitely provide key insights for development of novel biomarkers and effective treatment approaches for the successful management of this aggressive neoplasm.

1.13. Objectives

The objectives of the current work are framed as follows:

1. To determine the differential expression of TIPE family of proteins in normal human lung tissues and human lung cancer tissues.
2. To examine the effect of tobacco extract and various tobacco components on the expression of TIPE family in human lung epithelial cells.
3. To elucidate the role of TIPE family of proteins in different processes involved in the development and progression of lung cancer.

Chapter 2

Differential expression of TIPE family of proteins in normal human lung tissues and lung cancer tissues

2.1. Introduction

Aforementioned, lung cancer is the commonest cause of cancer deaths in the world. It also represents the most common cancer as well as the most common cause of death due to cancer in men, in India. Despite the significant advances in the field of therapy for management of lung cancer, the prognosis for patients with lung cancer remains utterly poor. The poor prognosis linked with this disease is closely related to the fact that most lung cancer cases are not identified until their malignancy has reached an advanced or metastatic stage. Therefore, a thorough understanding of its pathogenesis and identification of new biomarkers will greatly improve the diagnosis and treatment of this malignancy. As described earlier, TIPE family of proteins plays vital role in the regulation of different processes such as inflammation, immunity, tumorigenesis etc. and exerts differed expression pattern in different tumors and thus they can be employed as a potent biomarker. Despite the fact that all the four proteins of this family share a significant sequence homology, they are involved in the regulation of different cellular activities. Hence, the present study was aimed at evaluating the expression and role of these four proteins in the development and progression of lung cancer. A few studies were found in the literature, where the expression of these proteins were studied individually. However, comparative expression analysis of the four proteins of TIPE family was not performed till date. Therefore, in this study we have performed an extensive comparative expression analysis of these proteins in lung cancer tissues of different subtypes, pathologies, stages, grades, age groups and sexes.

2.2. Materials and Methods:

2.2.1. Tissue Microarray:

Expression of TIPE proteins in normal lung tissues and different stages of lung cancer tissues was determined by immunohistochemical analysis. For this purpose, tissue microarray (TMA) containing paraffin-embedded normal and malignant lung tissues (US Biomax, Inc., Cat. No. LC1503) was used. The TMA slide contains a total of 75 tissues, 150 cores (duplicated cores from the same patient in all cases) from different individuals: 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 (Table 2.1).

2.2.1.1. Tissue Microarray details

Lung cancer tissue array, including TNM, clinical stage and pathology grade, 75 cases/150 cores

Name: LC1503

Description: Lung cancer tissue array, including TNM, clinical stage and pathology grade

Cases: 75

Cores: 150

Row number: 10

Column number: 15

Core Diameter: 1 mm

Thickness: 5 µm

Table 2.1. Lung cancer tissue array details.

Position	Age	Sex	Organ	Pathology diagnosis	TNM	Grade	Stage	Type
A1	68	M	Lung	Adenocarcinoma	T2N0M0	2-3	I	Malignant
A2	37	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
A3	47	M	Lung	Adenocarcinoma	T1N0M0	2-3	I	Malignant
A4	46	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
A5	67	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant

A6	66	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
A7	70	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
A8	59	M	Lung	Adenocarcinoma	T2N1M0	2-3	II	Malignant
A9	60	F	Lung	Adenocarcinoma (sparse)	T2N0M0	-	I	Malignant
A10	58	M	Lung	Adenocarcinoma	T2N1M0	2	II	Malignant
A11	70	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
A12	52	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
A13	70	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
A14	67	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
A15	45	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
B1	68	M	Lung	Adenocarcinoma	T2N0M0	2-3	I	Malignant
B2	37	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
B3	47	M	Lung	Adenocarcinoma	T1N0M0	2-3	I	Malignant
B4	46	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
B5	67	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
B6	66	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
B7	70	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
B8	59	M	Lung	Adenocarcinoma	T2N1M0	2-3	II	Malignant
B9	60	F	Lung	Adenocarcinoma (fibrous tissue and blood vessel)	T2N0M0	-	I	Malignant
B10	58	M	Lung	Adenocarcinoma	T2N1M0	2	II	Malignant
B11	70	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
B12	52	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
B13	70	M	Lung	Adenocarcinoma	T1N0M0	2	I	Malignant
B14	67	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
B15	45	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
C1	62	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
C2	60	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
C3	62	F	Lung	Mucinous adenocarcinoma (sparse)	T2N0M0	1	I	Malignant
C4	51	M	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
C5	37	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
C6	66	F	Lung	Adenocarcinoma	T2N1M0	2	II	Malignant
C7	67	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
C8	50	F	Lung	Adenocarcinoma	T3N0M0	3	IIIa	Malignant
C9	54	F	Lung	Adenocarcinoma with necrosis (sparse)	T3N0M0	3	IIIa	Malignant
C10	49	F	Lung	Adenocarcinoma	T2N1M0	3	II	Malignant
C11	51	F	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
C12	61	M	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
C13	65	M	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
C14	61	M	Lung	Adenocarcinoma	T1N0M0	3	I	Malignant

C15	67	F	Lung	Adenosquamous carcinoma	T2N1M0	-	II	Malignant
D1	62	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
D2	60	M	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
D3	62	F	Lung	Mucinous adenocarcinoma	T2N0M0	1	I	Malignant
D4	51	M	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
D5	37	F	Lung	Adenocarcinoma	T2N0M0	2	I	Malignant
D6	66	F	Lung	Adenocarcinoma	T2N1M0	2	II	Malignant
D7	67	F	Lung	Adenocarcinoma with necrosis	T2N0M0	2	I	Malignant
D8	50	F	Lung	Adenocarcinoma	T3N0M0	3	IIIa	Malignant
D9	54	F	Lung	Adenocarcinoma	T3N0M0	3	IIIa	Malignant
D10	49	F	Lung	Adenocarcinoma	T2N1M0	3	II	Malignant
D11	51	F	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
D12	61	M	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
D13	65	M	Lung	Adenocarcinoma	T2N0M0	3	I	Malignant
D14	61	M	Lung	Adenocarcinoma	T1N0M0	3	I	Malignant
D15	67	F	Lung	Adenosquamous carcinoma	T2N1M0	-	II	Malignant
E1	43	M	Lung	Adenosquamous carcinoma	T2N0M0	-	I	Malignant
E2	70	M	Lung	Adenosquamous carcinoma	T2N0M0	-	I	Malignant
E3	54	M	Lung	Squamous cell carcinoma	T2N3M0	1	IIIa	Malignant
E4	56	M	Lung	Squamous cell carcinoma	T3N1M0	1	IIIa	Malignant
E5	56	M	Lung	Squamous cell carcinoma	T3N0M0	1	IIIa	Malignant
E6	59	M	Lung	Squamous cell carcinoma	T2N1M0	2	II	Malignant
E7	67	M	Lung	Squamous cell carcinoma	T1N0M0	2	I	Malignant
E8	50	M	Lung	Squamous cell carcinoma (tumoral necrosis)	T2N0M0	-	I	Malignant
E9	53	M	Lung	Squamous cell carcinoma with necrosis	T2N0M0	3	I	Malignant
E10	61	M	Lung	Squamous cell carcinoma	T2N0M0	3	II	Malignant
E11	71	F	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant
E12	56	M	Lung	Squamous cell carcinoma	T2N1M0	1	I	Malignant
E13	70	F	Lung	Squamous cell carcinoma with necrosis	T2N0M0	2	II	Malignant

E14	48	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
E15	44	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
F1	43	M	Lung	Adenosquamous carcinoma (carcinoma sparse)	T2N0M0	-	I	Malignant
F2	70	M	Lung	Adenosquamous carcinoma with necrosis (sparse)	T2N3M0	1	I	Malignant
F3	54	M	Lung	Squamous cell carcinoma	T3N1M	1	IIIa	Malignant
F4	56		Lung	Squamous cell carcinoma	T3N0M	1	IIIa	Malignant
F5	56	M	Lung	Squamous cell carcinoma	T2N1M0	2	IIIa	Malignant
F6	59	M	Lung	Squamous cell carcinoma	T1N0M0	2	II	Malignant
F7	67	M	Lung	Squamous cell carcinoma	T2N0M0	-	I	Malignant
F8	50	M	Lung	Squamous cell carcinoma with necrosis (sparse)	T2N0M0	3	I	Malignant
F9	53	M	Lung	Squamous cell carcinoma with necrosis	T2N1M0	3	I	Malignant
F10	61	M	Lung	Squamous cell carcinoma	T2N0M0	3	II	Malignant
F11	71	F	Lung	Squamous cell carcinoma	T2N0M0	1	I	Malignant
F12	56	M	Lung	Squamous cell carcinoma	T2N1M0	2	I	Malignant
F13	70	F	Lung	Squamous cell carcinoma with necrosis	T2N0M0	2	II	Malignant
F14	48	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
F15	44	M	Lung	Squamous cell carcinoma	T3N1M0	-	I	Malignant
G1	50	M	Lung	Squamous cell carcinoma	T2N1M0	2	IIIa	Malignant
G2	57	M	Lung	Squamous cell carcinoma	T2N1M0	2	II	Malignant
G3	65	M	Lung	Squamous cell carcinoma	T2N0M0	1	II	Malignant
G4	60	M	Lung	Squamous cell carcinoma (interstitial pneumonia)	T2N0M0	-	I	Malignant
G5	65	M	Lung	Squamous cell carcinoma	T3N0M0	1	IIIa	Malignant

G6	60	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
G7	54	F	Lung	Squamous cell carcinoma	T2N1M0	3	II	Malignant
G8	61	F	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
G9	61	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
G10	71	M	Lung	Squamous cell carcinoma	T2N1M0	3	II	Malignant
G11	49	F	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant
G12	69	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
G13	49	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
G14	55	F	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant
G15	65	M	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant
H1	50	M	Lung	Squamous cell carcinoma with necrosis	T2N1M0	2	IIIa	Malignant
H2	57	M	Lung	Squamous cell carcinoma (fibrous tissue and blood vessel)	T2N1M0	2	II	Malignant
H3	65	M	Lung	Squamous cell carcinoma	T2N0M0	1	II	Malignant
H4	60	M	Lung	Squamous cell carcinoma (interstitial pneumonia)	T2N0M0	-	I	Malignant
H5	65	M	Lung	Squamous cell carcinoma	T3N0M0	1	IIIa	Malignant
H6	60	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
H7	54	F	Lung	Squamous cell carcinoma	T2N1M0	3	II	Malignant
H8	61	F	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
H9	61	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
H10	71	M	Lung	Squamous cell carcinoma	T2N1M0	3	II	Malignant
H11	49	F	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant
H12	69	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
H13	49	M	Lung	Squamous cell carcinoma	T2N0M0	2	I	Malignant
H14	55	F	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant

H15	65	M	Lung	Squamous cell carcinoma	T2N0M0	3	I	Malignant
I1	68	M	Lung	Squamous cell carcinoma	T3N0M0	3	IIIa	Malignant
I2	46	M	Lung	Bronchioalveolar carcinoma	T2N0M0	-	I	Malignant
I3	55	F	Lung	Bronchioalveolar carcinoma	T2N0M0	-	I	Malignant
I4	54	M	Lung	Small cell undifferentiated carcinoma	T2N1M0	-	II	Malignant
I5	73	M	Lung	Small cell undifferentiated carcinoma with necrosis	T3N1M0	-	IIIa	Malignant
I6	66	F	Lung	Small cell undifferentiated carcinoma	T2N1M0	-	II	Malignant
I7	51	F	Lung	Small cell undifferentiated carcinoma with necrosis	T1N0M0	-	I	Malignant
I8	62	M	Lung	Large cell carcinoma	T3N0M0	-	IIIa	Malignant
I9	57	M	Lung	Large cell carcinoma	T3N0M0	-	IIIa	Malignant
I10	64	M	Lung	Neuroendocrine carcinoma	T2N2M0	-	IIIa	Malignant
I11	22	M	Lung	Normal lung tissue	-	-	-	Normal
I12	25	M	Lung	Normal lung tissue	-	-	-	Normal
I13	46	M	Lung	Normal lung tissue	-	-	-	Normal
I14	41	F	Lung	Normal lung tissue	-	-	-	Normal
I15	40	F	Lung	Normal lung tissue	-	-	-	Normal
J1	68	M	Lung	Squamous cell carcinoma	T3N0M0	3	IIIa	Malignant
J2	46	M	Lung	Bronchioalveolar carcinoma with necrosis	T2N0M0	-	I	Malignant
J3	55	F	Lung	Bronchioalveolar carcinoma	T2N0M0	-	I	Malignant
J4	54	M	Lung	Small cell undifferentiated carcinoma	T2N1M0	-	II	Malignant
J5	73	M	Lung	Small cell undifferentiated carcinoma with necrosis	T3N1M0	-	IIIa	Malignant

J6	66	F	Lung	Small cell un-differentiated carcinoma	T2N1M0	-	II	Malignant
J7	51	F	Lung	Small cell undifferentiated carcinoma with necrosis	T1N0M0	-	I	Malignant
J8	62	M	Lung	Large cell carcinoma	T3N0M0	-	IIIa	Malignant
J9	57	M	Lung	Large cell carcinoma	T3N0M0	-	IIIa	Malignant
J10	64	M	Lung	Neuroendocrine carcinoma	T2N2M0	-	IIIa	Malignant
J11	22	M	Lung	Normal lung tissue	-	-	-	Normal
J12	25	M	Lung	Normal lung tissue	-	-	-	Normal
J13	46	M	Lung	Normal lung tissue	-	-	-	Normal
J14	41	F	Lung	Normal lung tissue	-	-	-	Normal
J15	40	F	Lung	Normal lung tissue	-	-	-	Normal

2.2.2. Immunohistochemistry

Histostain-Plus IHC Kit, 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. Immunohistochemistry (IHC) was performed as per the manufacturer's protocol: i.e.: deparaffinization, rehydration, peroxidase quenching, blocking, primary antibody incubation, secondary antibody-peroxidase conjugate incubation, addition of DAB chromogen and counterstaining with hematoxylin. Finally, the slide is dehydrated and mounted with coverslip using D.P.X. mountant (Cat No DC4DF64352; Merck, New Jersey, USA). The primary antibodies anti-TNFAIP8 antibody (Cat. No. ab64988), anti-TNFAIP8L1 antibody (Cat. No. ab85409), anti-TNFAIP8L2 antibody (Cat. No. ab110389) and anti-TNFAIP8L3 antibody (Cat. No. ab111524) were obtained from abcam®, Cambridge, USA and used

in the dilution of 1:25, 1:50, 1:50 and 1:100 respectively, for immunohistochemical analysis.

2.2.3. Scoring

The immunostained microarray slides were analyzed under Olympus light microscope. Tissues that are stained brown are considered as positive for the presence of antigen of interest and given a score according to the staining intensity and number of positive cells. Score for percentage of positive cells is scaled from 0 to 4+ and staining intensity is scaled from 1 to 3 (McDonald *et al.*, 1999; Shiao *et al.*, 2000; Charafe-Jauffret *et al.*, 2004; Monisha *et al.*, 2018) (Table 2.2).

Table 2.2. Scoring method for IHC.

Score (P)	**0**	**1+**	**2+**	**3+**	**4+**
Positive Cells	<10%	10-25%	25-50%	50-75%	>75%
Score (I)	1	2	3	Total expression score **Q= P*I**	
Intensity of Stain	weak stain	moderate stain	strong stain		

2.2.4. Statistical analysis

Student's *t*-test was performed to determine the statistical significance. *p* value <0.05 was considered as statistically significant.

2.3. Results and Discussion

Aforementioned, despite the presence of a significant sequence homology among the four members of this family, they are found to be involved in different biological functions. Expression studies of these proteins show notable variability among themselves in different cancers. Therefore, we analyzed the expression of this family

of proteins (TIPE, TIPE1, TIPE2 and TIPE3) on the basis of different lung cancer types, pathological conditions, age, sex, grade and stages of lung cancer with respect to normal. The TMA slide constitutes tissues of both the types of lung cancer i.e. SCLC and NSCLC. They are of different pathologies such as small cell undifferentiated carcinoma, adenocarcinoma, bronchioalveloar carcinoma, squamous cell carcinoma, adenosquamous carcinoma, neuroendocrine carcinoma and large cell carcinoma. Further, they are of different clinical stages (stage I, II and IIIa), grades (grade 1, 2 and 3) and TNM status (T1N0M0, T2N0M0, T2N1M0, T3N0M0, T3N1M0). The tissue samples consists of patients of different age groups and include both the sexes. The total score (Q) of IHC is considered as the expression score of a particular protein for a tissue.

2.3.1. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 in human lung cancer tissues

The first aim of this study was to analyze the expression of the TIPE family of proteins in lung cancer tissues compared to normal lung tissues. Our analysis revealed that TIPE, TIPE2 and TIPE3 were significantly upregulated in lung cancer tissues compared to normal lung tissues. On the other hand, TIPE1 showed significant downregulation in malignant lung tissues than the normal tissues. The expression score for TIPE, TIPE2 and TIPE3 in the normal tissues were found to be almost same, whereas in case of TIPE1, the expression score in normal tissues was found to be around 2.5-3 fold more compared to those of TIPE, TIPE2 and TIPE3. Additionally, in case of TIPE, TIPE2 and TIPE3, around 2 fold increase in their expression was observed in the malignant lung tissues compared to normal lung tissues. Further, the difference in the expression score of TIPE1 in normal and malignant tissues was found to be less than those of the

other three proteins. However, TIPE1 was found to be significantly downregulated in lung cancer tissues when compared to normal (Figure 2.1). TIPE, the most widely studied member of this family is a transcription factor NF-κB-inducible, anti-apoptotic and oncogenic molecule which is associated with prognosis of several human malignancies (Sun *et al.,* 2016; Dong *et al.,* 2017). It has been found to be overexpressed in various human cancers such as breast cancer, cervical cancer, colon cancer, endometrial cancer, ESCC etc. via modulation of different signaling cascades and hence is predicted to serve as a novel prognostic indicator for patients with those malignancies (Xiao *et al.,* 2017; Shi *et al.,* 2013; Miao *et al.,* 2012; Liu *et al.,* 2014a; Sun *et al.,* 2016). Moreover, TIPE was found to be overexpressed in 76.3% human lung cancer samples and correlated with lymph node metastasis, p-TNM stage, Ki-67 expression and poor survival (Dong *et al.,* 2010). Further, TIPE expression was also reported to be linked with advanced pT (pathological T) stage and p-TNM stage, lymph node metastasis and poor patient survival in case of NSCLC (Xing *et al.,* 2018). In line with the previous findings, we also found that the expression of TIPE is significantly high in lung cancer tissues than the normal lung tissues. This indicates TIPE as a positive regulator of the cancers of lung. Further, TIPE1 is identified as a regulator of cell death. Its expression was significantly less in gastric cancer tissues which inversely correlated with differentiation status and distant metastasis (Liu *et al.,* 2018). Further, it 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 (Zhang *et al.,* 2015b). Additionally, in a recent study, the expression of TIPE1 was found to be decreased in the lung tumor tissues notably, and exerted positive correlation with patient survival (Wu *et al.,* 2017). As per our analysis as well, in case of lung cancer tissues, TIPE1 showed significant downregulation in comparison with normal

lung tissues clearly implying the potent anti-tumor role of TIPE1 in lung cancer. In addition, TIPE2, a negative regulator of innate as well as cellular immunity was reported to be significantly downregulated in human breast cancer cells as well as tissues and it's over expression inhibited the proliferation of tumor cells and tumor xenograft growth (Wang *et al.*, 2017). Its expression was found to be remarkably less in glioma cells (Liu *et al.*, 2016). Further, it suppressed the metastasis of gastric cancer cells through downregulation of β-catenin signaling pathway (Wu *et al.*, 2016). Contrary to the above mentioned findings, we found that this protein was significantly upregulated in malignant lung tissues than the normal tissues. Similar observation was reported by Hao and colleagues where enhanced expression of TIPE2 was found in both diffuse large B-cell lymphoma and peripheral T-cell lymphoma (Hao *et al.*, 2016). In line with our findings, another study carried out by Li and group also showed TIPE2 to be up-regulated in NSCLC tumor tissues when compared with adjacent normal tissues (Li *et al.*, 2016). Furthermore, TIPE3 functions as a transfer protein for lipid second messengers; PIP2 and PIP3 and increases their level in the plasma membrane (Fayngerts *et al.*, 2014). It is expressed in several human organs and is highly upregulated in various human cancers such as esophageal cancer, cervical cancer, colon cancer etc. (Lian *et al.*, 2017). Further, expression of TIPE3 in the plasma membrane was found to exert a positive correlation with the T stage of NSCLC (Wang *et al.*, 2018a). In our finding as well, TIPE3 showed significant upregulation in lung cancer tissues compared to the normal tissues and thus it can be considered as a positive regulator of lung cancer. Thus, altogether, it can be hypothesized that upregulation of TIPE, TIPE2 and TIPE3 as well as downregulation of TIPE1 might be involved in the malignant transformation of lung tissues.

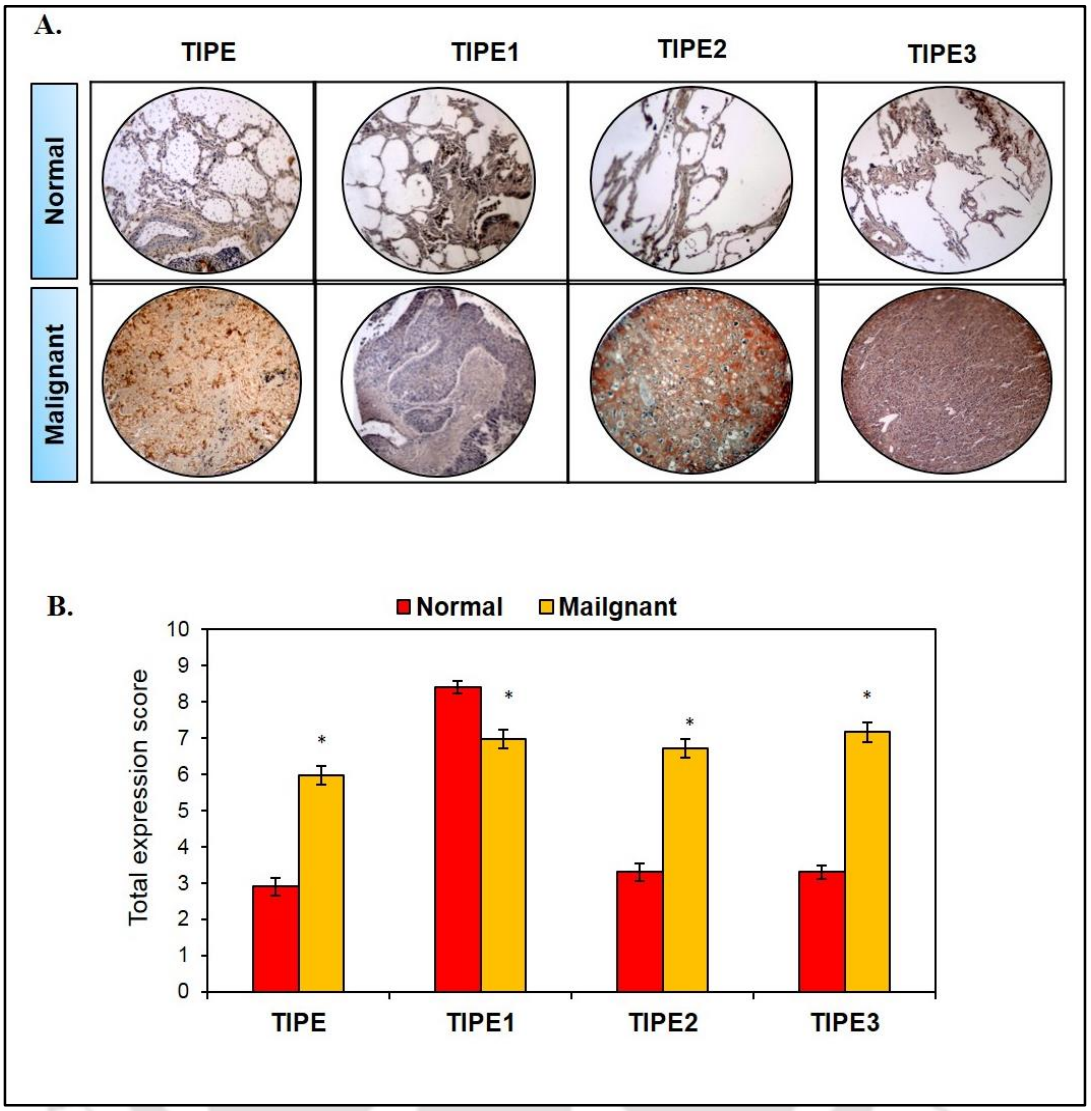

Figure 2.1. Expression of TIPE family of proteins in lung cancer. A. Representative images of the expression of TIPE, TIPE1, TIPE2 and TIPE3 in lung cancer tissues. B. Expression of TIPE, TIPE1, TIPE2 and TIPE3 in lung cancer tissues in terms of expression score. Data are represented as Mean ± SE, * denotes *p*-value < 0.05 compared to normal tissues.

2.3.2. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 in NSCLC and SCLC

Aforementioned, NSCLC accounts for 80-85% of all lung cancer cases and includes 3 major subtypes: (1) adenocarcinoma; (2) squamous cell carcinoma; and (3) large-cell carcinoma. SCLC represents 13% of all newly diagnosed cases of lung cancer worldwide, or more than 180, 000 cases per year (Meerbeeck *et al.*, 2011). The

expression of this family of proteins in both SCLC and NSCLC with respect to normal lung tissues was analyzed. Expression analysis of TIPE family of proteins in normal, SCLC and NSCLC tissue samples showed that TIPE, TIPE2 and TIPE3 were upregulated in both SCLC and NSCLC tissues compared to normal tissues, with more pronounced upregulation in NSCLC type. TIPE, TIPE2 and TIPE3 exerted more than 2 fold increased expression score in NSCLC type compared to normal tissues. In case of TIPE2, a significant 1.4 fold increase in the expression was observed in SCLC type than the normal tissues whereas in TIPE and TIPE3, the increase in their expression was not found to be significant in SCLC tissues than the normal lung tissues. On the other hand, in case of TIPE1, a significant downregulation was observed in both NSCLC and SCLC lung cancer types, where SCLC showed more downregulation (more than 2 fold) compared to NSCLC (Figure 2.2). Exposure to tobacco is the prime risk factor for both the lung cancer types. In particular, NSCLC describes a strong etiologic relation with smoking (Zheng *et al.*, 2007). Further, SCLC is very strongly linked with tobacco consumption. It is quite infrequent in never-smokers and risk factors for SCLC in never-smokers are not completely known. An epidemiologic study carried out in the USA revealed that SCLC cases diagnosed among never- smokers is only 2.5% (Torres-Durán *et al.*, 2016). Therefore, the expression of TIPE family of proteins might possess a correlation with tobacco and tobacco related carcinogens as well which need to be elucidated.

2.3.3. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 in different lung cancer pathologies

Based on pathology, lung cancer can be subdivided into many groups such as adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell

carcinoma, small cell carcinoma, bronchioalveolar carcinoma etc. In the current study, we compared the differential expression of TIPE proteins with respect to disease pathology. TIPE was found to be significantly upregulated in squamous cell carcinoma and adenocarcinoma tissues compared to the normal tissues. Although, it was found to be upregulated in other disease conditions such as adenosquamous cell carcinoma, small cell carcinoma, large cell carcinoma and bronchioalveloar carcinoma, the difference in the expression compared to normal is not significant, probably due to small sample size. Coming to TIPE1, it was found to be significantly downregulated in small cell carcinoma, squamous cell carcinoma and adenocarcinoma. Downregulation in other cases such as adenosquamous carcinoma, bronchioalveloar carcinoma and large cell carcinoma was not found to be significant. Further, TIPE2 exerted significant upregulation in adenocarcinoma, squamous cell carcinoma and bronchioalveloar carcinoma whereas TIPE3 was significantly upregulated in adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma and bronchioalveloar carcinoma (Figure 2.2). Importantly, the expression pattern of TIPE, TIPE2 and TIPE3 in different pathological conditions of lung cancer was observed to be almost same. Precisely, TIPE, TIPE2 and TIPE3 exerted around 2 fold increase in their expression in adenosquamous cell carcinoma tissues than the normal lung tissues. In squamous cell carcinoma, TIPE and TIPE3 showed more than 2 fold increase in their expression, whereas TIPE2 showed relatively less yet significant increase (1.7 fold) in its expression compared to normal lung tissues. In case of adenocarcinoma and bronchioalveloar carcinoma as well, TIPE, TIPE2 and TIPE3 showed marked 2 fold increase in their expression than the normal tissues. In addition, these three proteins were found to exert around 2.5-3 fold increase in their expression in large cell carcinoma tissues in comparison with the normal human lung tissues. In contrast,

TIPE1 might act as a negative regulator in different pathological conditions of lung cancer. In case of squamous cell carcinoma and adenocarcinoma, a significantly decreased expression score of TIPE1 was obtained than the normal lung tissues. Collectively, all the four members of TIPE family can be considered to play important role in lung carcinogenesis though they were found to exert site specific expression. Yet, further studies are required with large number of patient samples to confirm these findings.

2.3.4. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 in lung cancer tissues of different age grouped patients

The expression of different TIPE family of proteins in patients of different age groups ranging from 31 to 80 years was analyzed and it was observed that all the four proteins showed the same pattern of expression with respect to the age. These proteins were found to show higher expression in lower age groups compared to the higher age grouped cancer tissues. An eventual decrease in the expression of TIPE, TIPE1, TIPE2 and TIPE3 was observed in the older patients than the younger ones (Figure 2.3). Smoking habits of people have been found to vary with the ages. For instance, while evaluating the smoking initiation rates by age in New Zealand, it was found that % smokers in aged between 25-34 is 22.9, 35-44 is 21, 45-54 is 17.9, 55-64 is 16.6 and 65+ is 6.6 (Edwards *et al.*, 2013). Again, in the United Nations, as per the reports of 2016, cigarette smoking was found to be higher among persons aged 18–24 years, 25–44 years and 45–64 years than among those aged 65 years and older (https://www.cdc.gov/tobacco/data_statistics/factsheets/adult_data/cig_smoking/index.htm). Thus, high expression of TIPE, TIPE2 and TIPE3 in the tissue samples of lower

age group patients compared to higher age grouped ones, among whom consumption rate of tobacco has been reported to be relatively less provides an indication that they

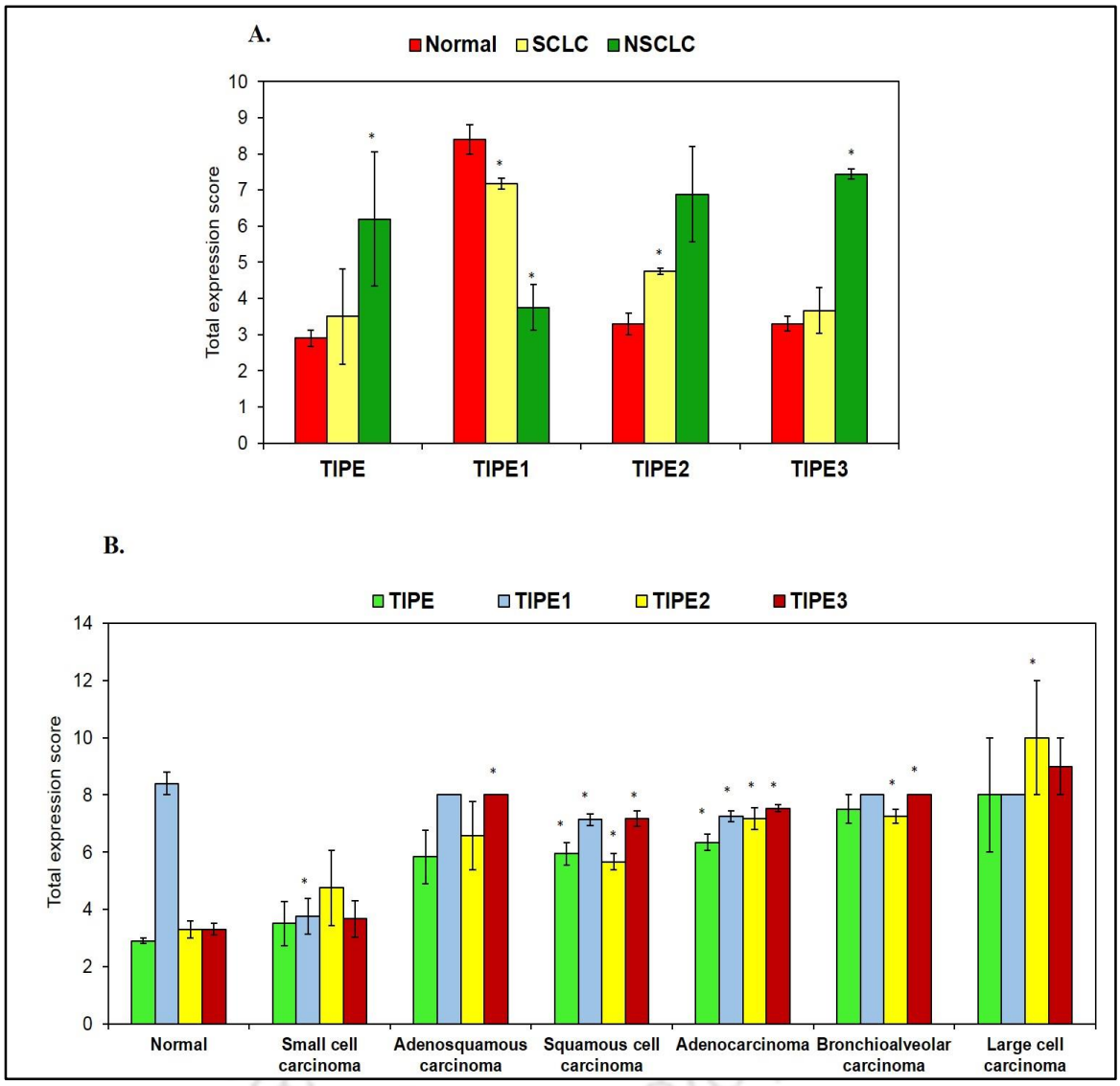

Figure 2.2. Differential expression of TIPE family of proteins in different lung cancer types and pathologies. A. Differential expression of TIPE family of proteins in different lung cancer types i.e. NSCLC and SCLC, B. Expression of TIPE family of proteins in different lung cancer pathologies such as small cell carcinoma, adenocarcinoma, bronchioalveloar carcinoma, squamous cell carcinoma, adenosquamous carcinoma and large cell carcinoma. Data are represented as Mean ± SE, * denotes *p*-value < 0.05 compared to normal tissues.

might play an important role in the positive regulation of tobacco induced lung carcinogenesis.

2.3.5. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 on the basis of sex

The risk of lung cancer is relatively high in males than females (Bain *et al.*, 2004). Smoking might be one of the possible reasons for this increased susceptibility in males, as it is estimated that men smoke nearly five times as much as women worldwide (Hitchman and Fong, 2011). Besides, varied regulatory mode of molecular cascades among males and females might also contribute to this differed cancer risk (Cosgrove *et al.*, 2014). However, there exists a definite association between carcinogen exposure and deregulation of important signaling pathways. Therefore, we analyzed the expression of TIPE family of proteins in different sexes (Figure 2.3). TIPE, TIPE2 and TIPE3 were found to be upregulated in malignant females and males compared to normal females and males respectively. Importantly, although the expression of TIPE and TIPE2 were found to be almost same in the tissue samples of normal male and TIPE2 were found to be almost same in the tissue samples of normal male and female, but significant increase in the expression of both were observed in the tissues of malignant males compared to malignant females. In case of TIPE3 as well, an increase in its expression was observed in malignant males compared to malignant females. As reported, men smoke nearly five times as women worldwide, therefore, it can be hypothesized that TIPE, TIPE2 and TIPE3 might be involved in tobacco induced lung carcinogenesis, again exemplifying the hypothesis of the preceding section. However, in case of TIPE1, no such correlation was noted.

2.3.6. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 in different stages of lung cancer

Cancer staging primarily denotes the anatomic range of the disease spread. Aforementioned, in TNM system; the internationally approved criteria for cancer

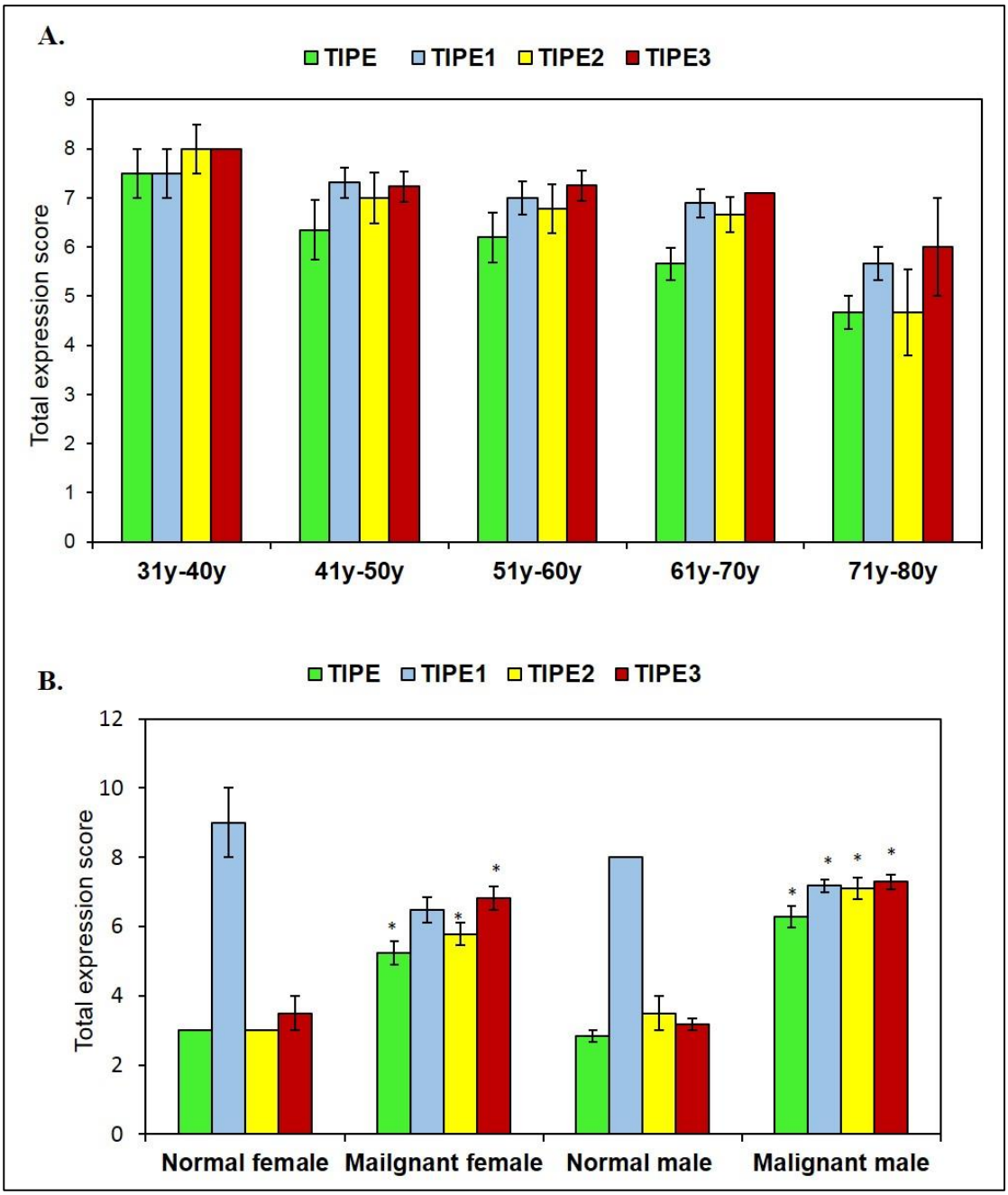

Figure 2.3. Expression of TIPE family of proteins in lung cancer tissues of patients of different age groups and sex. A. Expression of TIPE family of proteins in lung cancer tissues of patients of different age groups i.e. 31y-40y, 41y-50y, 51y-60y, 61y-70y and 71y-80y, B. Differential expression of TIPE family of proteins in different sexes i.e. both males and females. Data are represented as Mean ± SE, * denotes *p*-value < 0.05 compared to normal tissues.

staging, tumor size and local growth, extent of lymph node metastases and occurrence of distant metastases are the parameters which are taken into consideration (Telloni, 2017). Based on that, a patient's cancer can be classified into four different stages between I and IV. The TMA slide contained lung cancer tissues of three different stages which include stage I, II and IIIa. The stage wise difference in the expression of TIPE proteins was calculated and plotted against the expression in normal tissues (Figure 2.4). TIPE, TIPE2 and TIPE3 were found to be upregulated in different stages of lung cancer such as stage I, II and IIIa whereas TIPE1 was found to be downregulated in different stages of lung cancer than the normal tissues. Several studies showed the association between TIPE expression and TNM stage of tumor. For instance, upregulation of TIPE in colon cancer specimens was found to be strongly linked with TNM stage, lymph node metastasis and proliferation index (Miao *et al.*, 2012). Further, Hadisaputri and group also suggested TIPE to have a strong impact on different clinicopathological features such as TNM stage, lymph node involvement, tumor depth, lymphatic and venous invasion, and distant metastasis in case of ESCC patients (Hadisaputri *et al.*, 2012). The expression of TIPE was further correlated with TNM stage and lymphatic metastasis of gastric cancer suggesting TIPE as a potential biomarker for gastric cancer progression (Li *et al.*, 2015; Hu *et al.*, 2016). Further, a study conducted by Dong and group showed TIPE to be positively correlated with p-TNM stage, lymph node metastasis, expression of Ki-67, and poor survival in lung cancer (Dong *et al*., 2010). Contrary to TIPE, overexpression of TIPE2 was found to be negatively correlated with advanced clinical stage of lung cancer (Li *et al*., 2016). In case of our analysis, although we found upregulation in the expression of TIPE in all the three stages of lung tumor, we did not observe any significant difference in the expression of it among these different stages. Similar observation was obtained in case

of TIPE2 and TIPE3 as well. In case of TIPE1 which was found to be downregulated in all the three stages, no significant difference in its expression among the three stages was observed. Thus, based on our finding, these proteins can be predicted to have significant role in the initiation of lung cancer. However, its role in progression needs to be confirmed with more advanced conditions or metastatic tissues.

2.3.7. Expression analysis of TIPE, TIPE1, TIPE2 and TIPE3 in different grades of lung tumor

Like cancer staging, grading of tumors is also of vital importance. Tumor grading denotes their status of differentiation and is done based on the histology and architecture of tumor tissues. Thus, the well differentiated, moderately differentiated and poorly differentiated tumors are classified as grade 1, grade 2 and grade 3 cancers respectively. Further, with the increasing grade of tumor, aggressiveness of the disease also gets proportionally increased (Telloni, 2017). The TMA slide used in the present study contained tissues from grade 1, 2 and 3 tumors. Therefore, we analyzed the expression of TIPE family of proteins with respect to different tumor grades or degree of differentiation (Figure 2.4). Similar to different lung cancer stages, TIPE, TIPE2 and TIPE3 were found to be upregulated in different grades of lung cancer such as grade 1, 2 and 3 compared to normal tissues, whereas TIPE1 was found to be downregulated in different grades of lung cancer than normal. The association between tumor grade and TIPE expression was studied in EC, ovarian cancer and prostate cancer. In case of EC, overexpression of TIPE strongly correlated with various clinico-pathologic characteristics including higher histologic grade and lymph node metastasis (Liu *et al.*, 2014). In ovarian cancer as well, overexpression of TIPE was associated with high histologic grade and large residual size of tumor (Liu *et al.*, 2013). Moreover, both

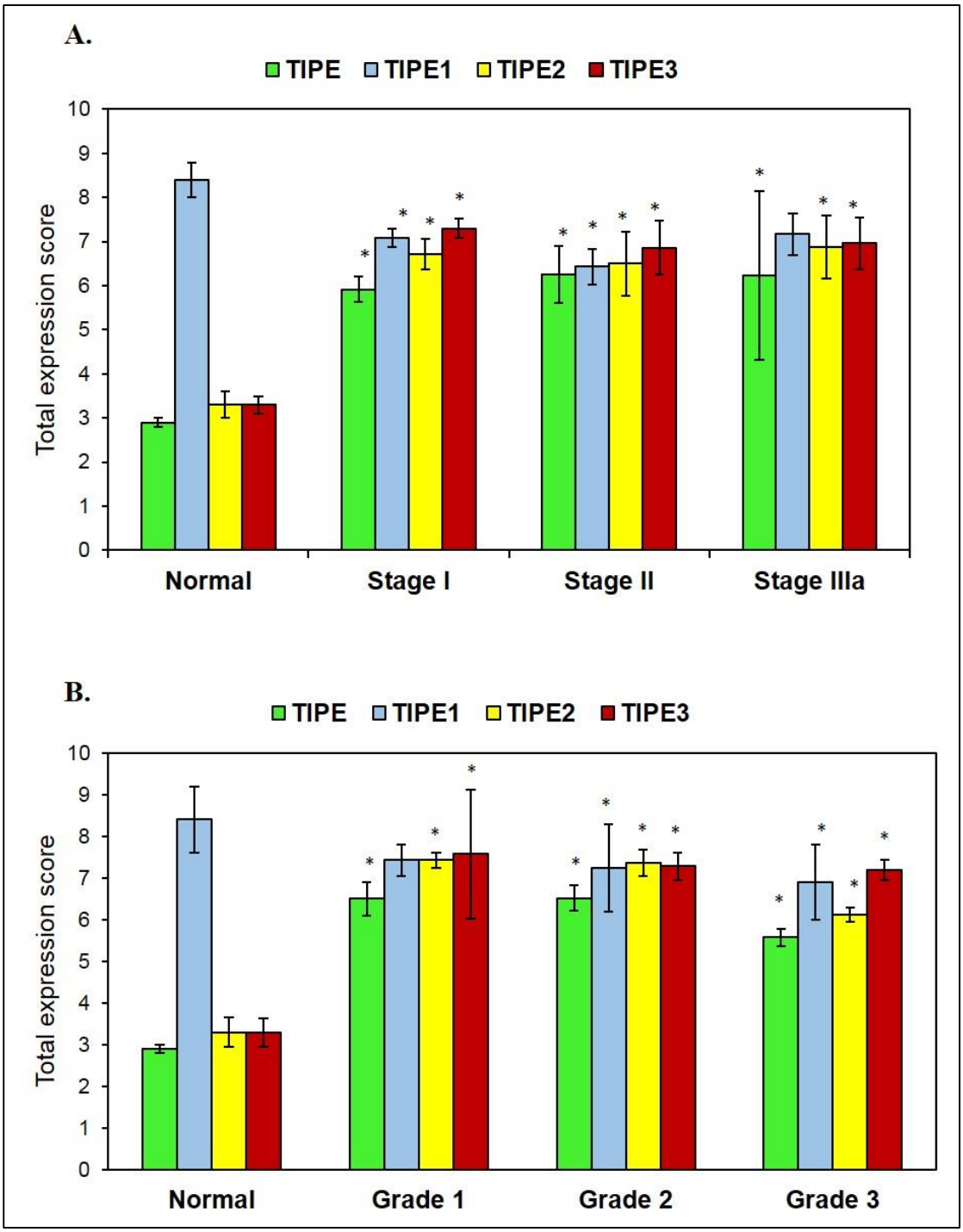

Figure 2.4. Differential expression of TIPE family of proteins in different stages and grades of lung cancer. A. Differential expression of TIPE family of proteins in different stages (Stage I, II, IIIa) of lung cancer, B. Differential expression of TIPE family of proteins in different grades (Grade 1, 2, 3) of lung tumor. Data are represented as Mean ± SE, * denotes *p*-value < 0.05 compared to normal tissues.

cytoplasmic and nuclear overexpression of TIPE was linked with high grade prostatic adenocarcinomas whereas nuclear overexpression was reported as a prediction indicator of disease recurrence and tumor grade (Zhang *et al.*, 2013). However, in our finding, no significant difference in the expression of TIPE family of proteins among grade 1, 2 and 3 was observed. Hence, these proteins can again be predicted to play crucial role in tumor initiation. Nevertheless, further confirmation is warranted with lager number of samples.

2.4. Conclusion

This chapter aimed at evaluating the expression of TIPE family of proteins individually as well as their comparative expression analysis in lung cancer tissues of different types, pathologies, stages, grades, different age groups and sexes. Our results suggest that TIPE, TIPE2 and TIPE3 were strongly associated with the positive regulation of lung carcinogenesis whereas TIPE1 might negatively regulate lung cancer. Further, the expression of TIPE family of proteins were found to be different in different pathological conditions. However, further confirmations are warranted to find the exact molecular mechanisms involved in case of these proteins in the pathogenesis of lung cancer, in particularly tobacco induced lung carcinogenesis which plausibly will provide new insights to the effective management of this deadly disease.

Chapter 3

Effect of tobacco and different tobacco components on the expression of TIPEs in human lung epithelial cells

3.1. Introduction

In the previous chapter, we have shown that TIPE, TIPE2 and TIPE3 were strongly associated with the positive regulation of lung carcinogenesis. Further, our results also showed that TIPE, TIPE2 and TIPE3 were significantly upregulated in both SCLC and NSCLC tissues compared to the normal lung tissues. Therefore, in this chapter we examined what leads to the modulation of these proteins in lung cancer. It is now well-established that tobacco use is the prime cause of lung cancer in both males (90% cases) and females (79% cases). It is well known that tobacco smoke is a toxic and carcinogenic mixture of above 5,000 chemicals (Talhout *et al.*, 2011). More than 60 carcinogens are found to be present in cigarette smoke whereas near about 16 carcinogens were identified in unburned tobacco. Among them, the most important ones include tobacco-specific nitrosamines, PAH etc. These compounds exert their carcinogenic effect through formation of DNA adducts (Petti, 2009). NNK and N-nitrosonornicotine (NNN), two most important tobacco specific nitrosamines induce both systemic and local tumors and hence are used as strong carcinogenic agents in different laboratory animal models. The organospecificity of NNK for the lung is especially distinguished (Hecht and Hoffmann, 1988). PAHs are another class of compounds comprising of two or more fused benzenoid rings and possess strong carcinogenic and mutagenic properties. Notably, there are above five hundred different PAHs present in tobacco smoke. Among them, benzo[a]pyrene (BaP) is classified as the most potent carcinogen. It is well reported to induce lung tumors through its local administration or even inhalation (Vu *et al.*, 2015). Apart from them, nicotine is another component present in tobacco which is principally associated with addiction. Nicotine exerts its biological effects through the binding and activation of nicotinic acetylcholine

receptors (nAChRs), which are strongly implicated in the pathogenesis of cancer. Further, reports suggest that nicotine and its metabolites exert proliferative effects through activation of mitogen-activated protein kinase (MAPK) and ERK 1/2 (Warren and Singh, 2013). In addition, NNK and NNN also function as activators of proliferation and tumor promotion via activation of nAChRs and β-AdrRs (Warren and Singh, 2013). Further, 90% of deaths due to lung cancer can be primarily attributed to smoking (Ozlü and Bülbül, 2005). This provides an indication that TIPE, TIPE2 and TIPE3 might possess certain correlation with this prime risk factor of lung cancer. This hypothesis is further strengthened by another finding reported in the previous chapter that all these proteins showed notable upregulation in the tissue samples of malignant males compared to malignant females despite the fact that they exerted similar expression profile in both the genders in normal conditions. As discussed earlier, men tend to consume tobacco products at higher rates compared to women; in fact nearly five times as much as women worldwide. Thus these findings provide a strong basis for the existence of an association between TIPE, TIPE2 and TIPE3 with tobacco and its related carcinogens. However, the role of tobacco components in the regulation of the expression of TIPE family is not studied till date. Therefore, in this chapter we focuses on examining the role of tobacco extract and various tobacco components on the expression of TIPE, TIPE2 and TIPE3 in human lung epithelial cells. As from the previous study, we observed upregulation in the expression of TIPE, TIPE2 and TIPE3 in lung cancer tissues, therefore we focused on evaluating the association of these three members of TIPE family only with tobacco and its components.

3.2. Materials and methods

3.2.1. Tobacco extract and tobacco components

NNK (Cat No. 78013), NNN (Cat No. 75285), Nicotine (Cat No. N3876) and BaP (Cat No. B1760) were purchased from Sigma- Aldrich, Missouri, USA. 'Tuibur', tobacco smoke infused water, was collected from the local market of Aizwal, Mizoram; filtered, lyophilized and then dissolved in sterile distilled water for use.

3.2.2. Cell culture

L132 human lung epithelial cell line was procured 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 1X Pen-Strep (Invitrogen, CA, USA). The cells were cultured and maintained at 37 °C in a CO_2-regulated incubator (5% CO_2 and 95% humidity).

3.2.3. MTT assay

To determine the non –toxic concentrations of tuibur and different tobacco components, MTT assay was performed. L132 human lung epithelial cells were seeded at a density of $2X10^3$ cells/well in 96 well plates in sextuplicates. After overnight incubation, the cells were treated with different concentrations of tuibur (0, 0.25, 0.5 and 0.75 µg/ml) and tobacco components such as NNK (0, 0.05, 0.1, 0.25 and 0.5 µM), NNN (0, 0.05, 0.1, 0.25 and 0.5 µM), nicotine (0, 0.25, 0.5, 0.75 and 1 µM) and BaP (0, 0.05, 0.1, 0.25 and 0.05 µg/ml) for 0 and 24 h. After each time point, 10 µL of 5 mg/mL MTT (Cat No. M2128, Sigma-Aldrich, Missouri, USA) was added to the cells and then incubated

for 2 h at 37 °C. Following this, MTT containing media was removed and then 100 µL of dimethyl sulfoxide (DMSO) (Cat. No. 1.16743.0521, Merck, Darmstadt, Germany) was added to all the wells and incubated in dark at room temperature to dissolve the MTT-formazan product. Finally, absorbance of the colored solution was measured at 570 nm with the help of a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland). The percentage of proliferation was calculated by normalizing the absorbance value of 24 h with 0 h absorbance while considering the absorbance of untreated control to be 100%.

3.2.4. Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze the expression of TIPEs in tuibur and different tobacco components' treated human lung epithelial cells. Total RNA was extracted using Trizol reagent (Cat No. T9424-200 ML, Sigma-Aldrich, Missouri, USA) and then cDNA synthesis was done using 1µg of RNA with the help of High-Capacity cDNA Reverse Transcription Kit (Cat No. 4368814, Applied Biosystems™, USA) as per the manufacturer's protocol. Primers for amplifying TIPEs were obtained from Intergrated DNA Technologies (IDT; Coralville, Iowa), and 2x Hot Start Taq Master Mix (Cat No. M0496L) from New England Biolabs® (NEB, USA) (Table 3.1). The TIPEs were then amplified by 35 cycles of PCR using specific primers and 1µL of cDNA as template. The amplicons obtained were then resolved in 1% agarose gel electrophoresis and the band intensity was determined by Image lab software. 100 bp DNA Ladder (Cat No. N3231S, NEB, USA) was run as a standard. Housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal gene control.

Table 3.1. Forward primers (FP) and reverse primers (RP) used for the amplification of *TIPE*, *TIPE2* and *TIPE3* genes.

Gene	Primer Sequence		Melting temperature Tm (°C)	Amplicon size
TIPE	FP	5′ ATGCACTCCGAAGCAGAAGA 3′	64	601 bp
	RP	5′ GTGCTCATATGTTCTCTTCATCC 3′	61.1	
TIPE2	FP	5′ ATGGAGTCCTTCAGCTCAAA 3′	61.5	555 bp
	RP	5′ TCAGAGCTTCCCTTCGTCTA 3′	62.3	
TIPE3	FP	5′ GTGGGAGGAGCACTAAACCA 3′	62.8	472 bp
	RP	5′ GCCAGGCTGATCTTGAACTC 3′	61.8	
GAPDH	FP	5′ AGGTCGGAGTCAACGGATTTG 3′	66.1	532 BP
	RP	5′ GTGATGGCATGGACTGTGGT 3′	67.3	

3.2.5. Statistical analysis

Statistical analysis was performed using Student's *t*-test and *p*-value < 0.05 was denoted as statistically significant.

3.3. Results and discussion

3.3.1. Effect of tuibur on the expression of TIPEs in human lung epithelial cells

Tuibur is a unique form of tobacco smoke-saturated aqueous concentrate which is traditionally consumed in the North-Eastern states of India, especially in Mizoram. It is highly addictive and deep-rooted in the culture of the region (Madathil *et al.*, 2018). Tuibur is prepared locally by passing smoke which is generated via burning of tobacco, through water until the preparation becomes cognac in color and gives a pungent smell. Tuibur users generally take around 5- 10 ml of it orally and keep in their mouth for some time and then spit it out. Many users take it quite a few times a day even (Phukan *et al.*, 2005). In a survey it was reported that around 7% people in Aizawl (872 of 12

185) and Churchandpur (139 of 2137) used tobacco water, which is stored and sold in bottles (Sinha *et al.,* 2004). While examining the toxicity of tuibur using Allium test, even the dilute solutions of tuibur were found to be extremely toxic (Mahanta *et al.*, 1998). In that scenario, humans consuming undiluted tuibur regularly is a cause for major concern (Sinha and Gupta, 2006). Another report suggested that heavy metals such as cadmium (0.011–0.012 mg/g), lead (0.015–0.018 mg/g), nickel (0.01–0.03 mg/g), arsenic (1.02–1.08 mg/g) and triethylene glycol (2321.42–2399.72 mg/g) were present in tobacco water. Further, a high concentration of NNN (19.65–20.12 mg/g) was also present in tuibur; in fact NNN was reported to be the most abundant as well as the strongest carcinogen found in tuibur (Sinha and Gupta, 2006). Hence, determining whether tuibur has any effect on the expression of *TIPE*s would help in better understanding of the mechanism involved in lung carcinogenesis through tobacco. In order to determine the non-toxic concentrations, L132 cells were treated with various concentrations of tuibur as mentioned above and it was found that 0.25, 0.5 and 0.75µg/ml of tuibur did not induce any cytotoxicity to the lung epithelial cells. Our results showed that the mRNA expression of *TIPE* and *TIPE2* was upregulated in a dose dependent manner in tuibur treated L132 cells. *TIPE3* was also found to be upregulated dose dependently upto 0.5 µg/ml of tuibur treatment in L132 cells (Figure 3.1). As there is a complete lack of information on tuibur mediated cancer signaling cascades and its role in lung carcinogenesis, therefore further studies are warranted to unveil the downstream targets of TIPE proteins upon treatment with tuibur. Nevertheless, our results provide an understanding that TIPE, TIPE2 and TIPE3 are strongly involved in tobacco mediated lung carcinogenesis which held through our hypothesis based on IHC data discussed in the previous chapter. In addition, as mentioned earlier, tobacco constitutes more than 5000 chemicals and cigarette smoke

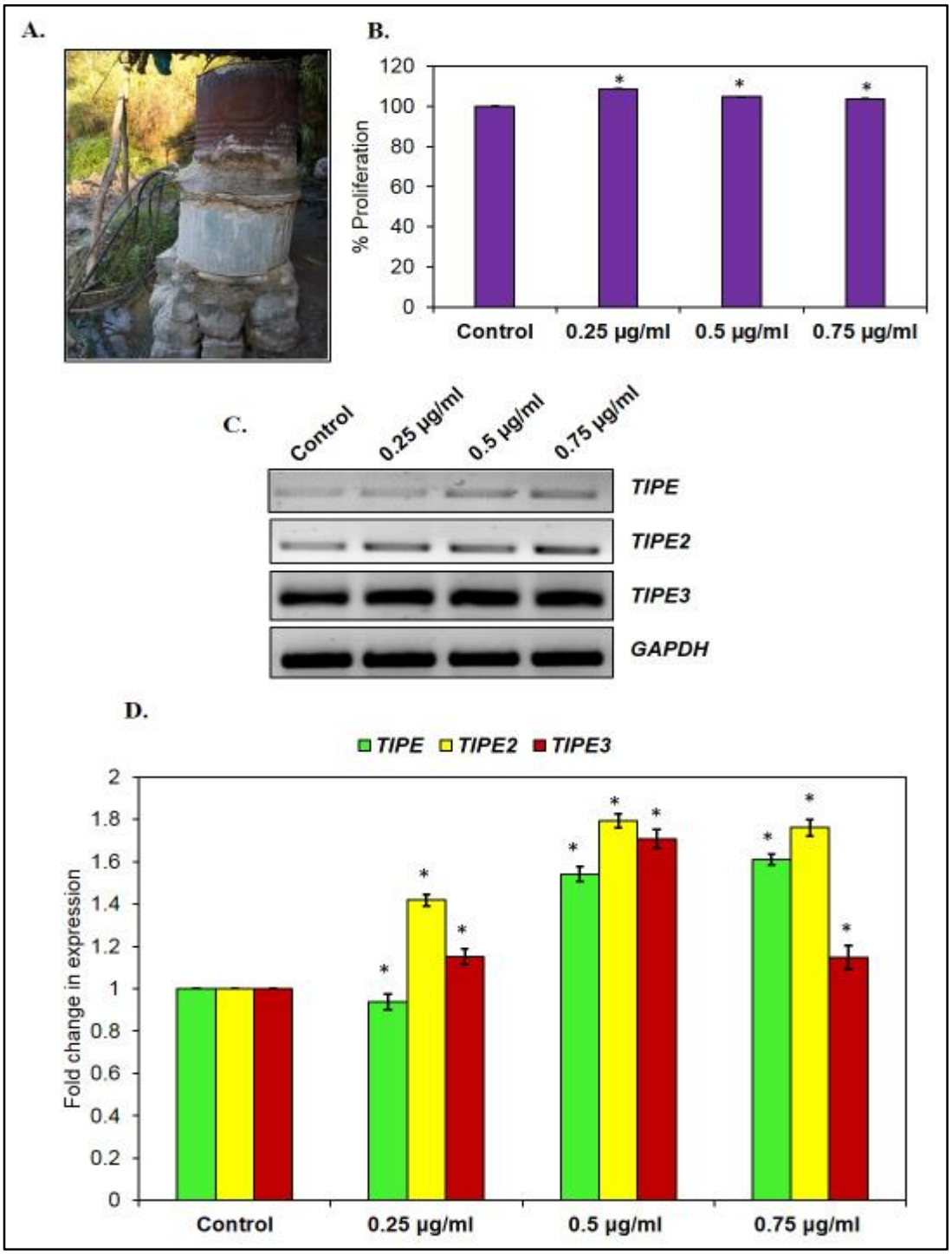

Figure 3.1. Effect of tuibur on the expression of *TIPE*, *TIPE2* and *TIPE3* in lung epithelial cells A. Preparation of tuibur; it requires tobacco ash, dried tobacco stalk, filter to prevent tobacco from falling into the lower compartment, alkaline feedstock; and small pipe to inhale the tobacco smoke (Madathil *et al.*, 2018), B. MTT assay showing non-cytotoxic concentrations of tuibur in L132 lung epithelial cells, C. RT-PCR analysis of the expression of *TIPE*, *TIPE2* and *TIPE3* after treating L132 cells with tuibur for 24 h , D. Graphical representation of the expression of *TIPE*, *TIPE2* and *TIPE3* in tuibur treated L132 cells as measured by densitometry scanning. GAPDH was used as internal control. Data are represented as Mean±SE, * denotes $p<0.05$ compared to control.

contains around 60 carcinogens. Therefore, studying the effect of these constituents on the expression of *TIPE*s in human lung cells will strengthen our understanding towards tobacco mediated lung carcinogenesis. For this purpose, three potent carcinogens present in tobacco such as NNK, NNN and BaP and the principal addictive chemical present in tobacco namely nicotine were chosen and their effect on the expression of *TIPE*s was observed.

3.3.2. Effect of NNK on the expression of TIPEs in human lung epithelial cells

NNK, a tobacco specific nitrosamine which is formed by nitrosation of nicotine is listed as group 1 human carcinogens by the IARC (Gupta *et al.*, 2019; Shen *et al.*, 2012). It is considered to be the strongest carcinogen among all the tobacco-specific nitrosamines (Gankhuyag *et al.*, 2017). Naturally occurring NNK in tobacco smoke is basically a procarcinogen, which entails metabolic activation to exhibit its carcinogenic properties. Different CYPs are involved in activating NNK to DNA-reactive metabolites which in turn induce methylation, pyridyloxobutylation and pyridylhydroxybutylation of nucleobases in DNA and leads to the formation of DNA adducts (Xue *et al.*, 2014). Further, reports suggest that NNK promotes tumor metastasis through regulation of cell motility and also causes enhanced migration and invasion of human lung cancer cells through activation of a protein kinase cascade which include c-Src, PKCι and Focal adhesion kinase (FAK) (Shen *et al.*, 2012). Further, NNK is also reported to cause enhanced aldehyde dehydrogenase (ALDH) -positive cells through reactive oxygen species (ROS)-Wnt signaling cascade in human lung cancer cells (Hirata *et al.*, 2017). Besides, it also increased the expression as well as activity of DNA methyltransferase 1 (DNMT1) via Akt pathway (Lin *et al.*, 2010). Notably, NNK has been found to effectively induce various types of lung cancer in several animal models such as A/J

mice, rat, hamster, and ferret (Ge *et al.*, 2015; Zheng and Takano, 2011). For instance, it induced lung carcinogenesis in female A/J mice via MEK1/2-ERK1/2 activation (Yamakawa *et al.*, 2016). Further, NNK was also reported to enhance lung cancer risk in patients bearing p53 mutations through perturbation of chromosome integrity and appropriate mitotic progression (Park *et al.*, 2017). Apart from the above mentioned, various other mechanisms are involved in NNK mediated lung carcinogenesis such as activation of TxAS/TxA2/TP/PI3K/AKT/CREB and β-AR/arachidonic acid signaling pathways, induction of heme oxygenase-1 and immune suppression etc. (Ge *et al.*, 2015). Therefore, treatment of lung cells with NNK will help us to have insights into the molecular mechanism of NNK-induced lung carcinogenesis and also to find out the novel targets to prevent NNK-associated lung cancer. Firstly, we determined the non-toxic concentrations of NNK on human lung epithelial cells with the help of MTT assay and found that the concentrations 0.05, 0.1, 0.25 and 0.5 µM did not induce any cytotoxicity to the L132 cells. Therefore, L132 cells were treated with those concentrations for 24 hour and then RT-PCR was performed to analyze the expression of *TIPE*, *TIPE2* and *TIPE3*. Our findings showed that *TIPE* and *TIPE2* exerted a complete dose dependent upregulation in their expression upon treatment with different concentrations of NNK. In the case of *TIPE3* expression as well, a significant upregulation after treating L132 cells with different concentrations of NNK was observed (Figure 3.2). NNK is well evinced to play a vital role in the development of smoking associated lung carcinogenesis through activation of PI3K/Akt, protein kinase C (PKC), NF-κB, and diverse other signaling pathways which are responsible for the proliferation, survival, and angiogenesis of lung cancer cells (Ge *et al.*, 2015). Further, stable overexpression of plasma membrane localized TIPE3 with a C-terminal flag resulted in the induction of growth and migration of NSCLC cells via activation of Akt

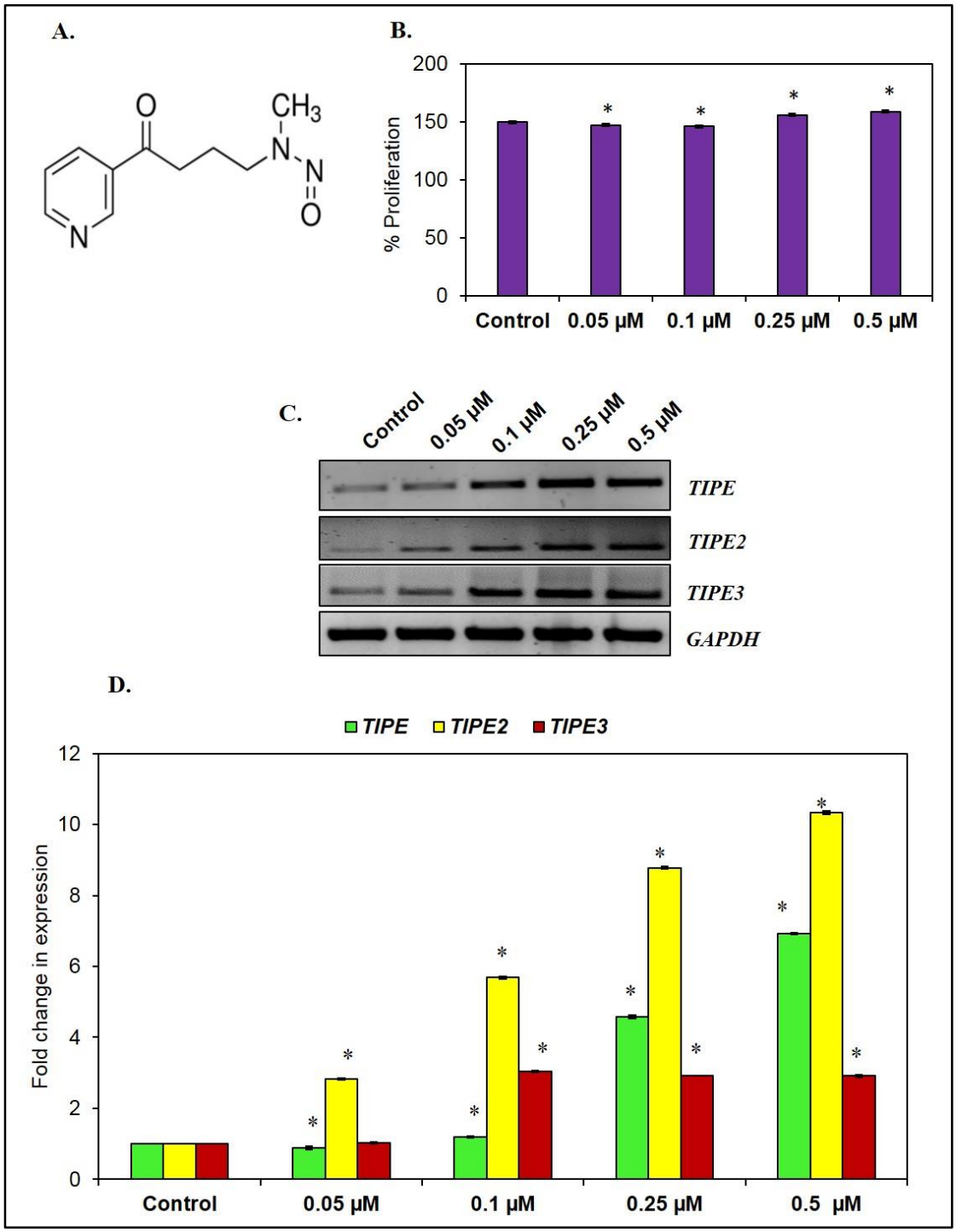

Figure 3.2. Effect of NNK on the expression of *TIPE*, *TIPE2* and *TIPE3* in lung epithelial cells A. Structure of NNK, B. MTT assay showing non-cytotoxic concentrations of NNK in L132 lung epithelial cells, C. RT-PCR analysis of the expression of *TIPE*, *TIPE2* and *TIPE3* after treating L132 cells with NNK for 24 h, D. Graphical representation of the expression of *TIPE*, *TIPE2* and *TIPE3* in NNK treated L132 cells as measured by densitometry scanning. GAPDH was used as internal control. Data are represented as Mean±SE, * denotes p<0.05 compared to control.

and ERK pathway (Bordoloi *et al.*, 2018, Padmavathi *et al.*, 2018). Therefore, Akt might serve as a downstream target of TIPE3 in NNK mediated lung cancer. Altogether, NNK induced upregulation of *TIPE, TIPE2* and *TIPE3* might play a critical role in the development as well as progression of human lung cancer. Nonetheless, further in-depth analysis is certainly vital for establishing the detailed mechanisms associated.

3.3.3. Effect of NNN on the expression of TIPEs in human lung epithelial cells

NNN is a powerful pulmonary carcinogen present in tobacco product and is primarily responsible for lung cancer (Vijayaraj *et al.*, 2014). It is formed from nicotine metabolites of the nitrosamines, reacts with DNA to form adducts accountable for genotoxic effects. The structure of NNN resembles with that of nicotine (Schuller, 2007). Notably, it is the first organic carcinogen isolated from unburned tobacco. Besides, it is also present in smoking and chewing tobaccos and snuff in concentrations ranging from 0.3 to 90.0 mug (Hoffmann *et al.*, 1976). NNN has been reported to produce tumors in the lungs of mice, in the oesophagus and nasal cavity of rats and also in the trachea and nasal cavity of Syrian golden hamsters (Konstantinou *et al.*, 2018). The potent carcinogenic effect of NNN in mice was shown for the first time by Boyland. Subsequently, it was reported as an esophageal carcinogen in rats. NNN is extensively metabolized in laboratory animals, with only a very little amount excreted out with urine (Hecht, 2014). Metabolic activation of NNN is principally initiated via CYPs through 2'-hydroxylation or 5'-hydroxylation (Fan *et al.*, 2019). Upon activation, NNN induces harmful mutations in oncogenes and tumor suppressor genes via formation of DNA adducts leading to tumor initiation (Xue *et al.*, 2014). Vijayaraj and group showed that *in vitro* exposure of this tobacco specific nitrosamine caused reduction in rat lung phospholipids with the help of enhanced phospholipase A2 function (Vijayaraj

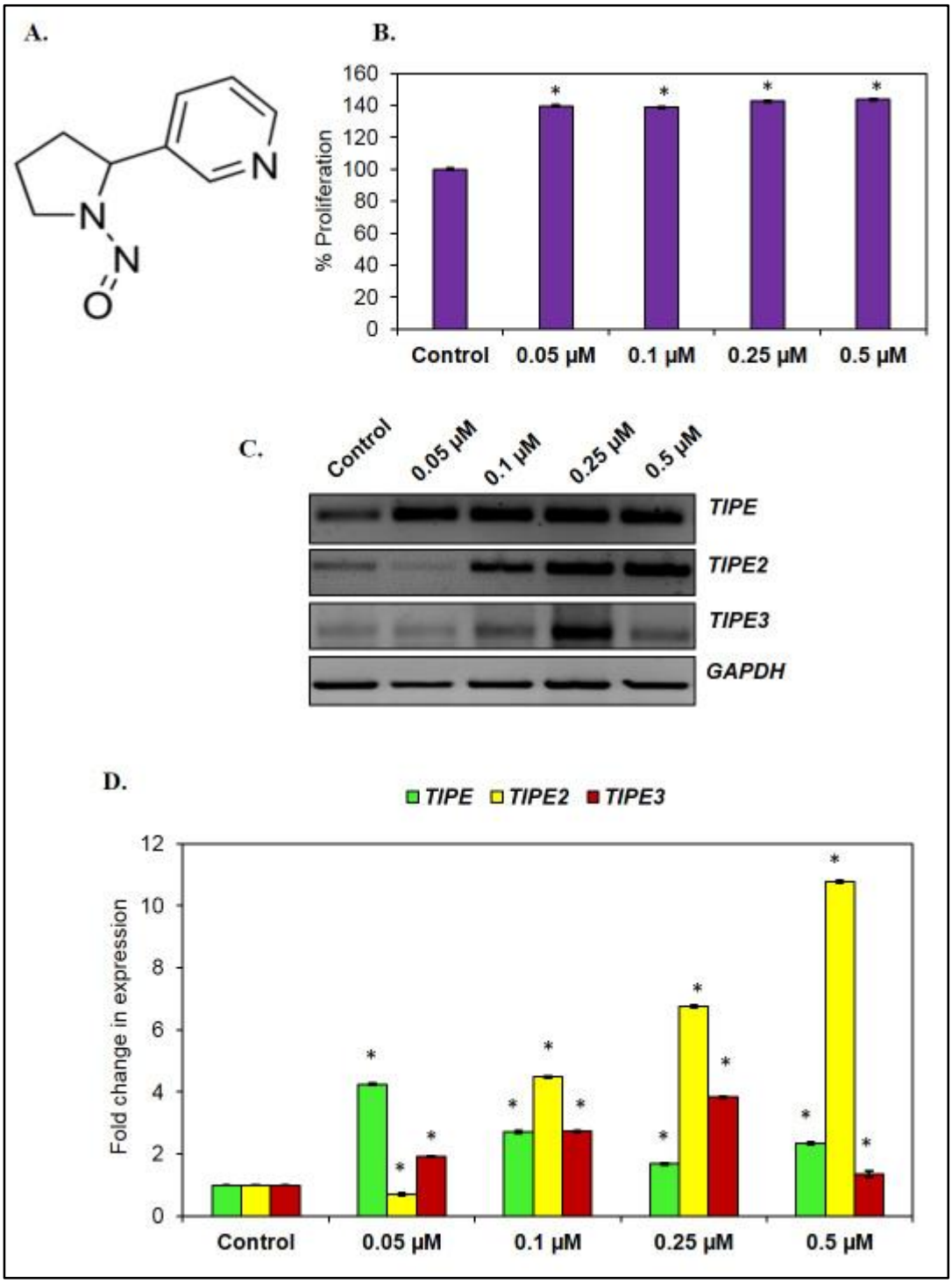

Figure 3.3. Effect of NNN on the expression of *TIPE*, *TIPE2* and *TIPE3* in lung epithelial cells A. Structure of NNN, B. MTT assay showing non-cytotoxic concentrations of NNN in L132 lung epithelial cells, C. RT-PCR analysis of the expression of *TIPE*, *TIPE2* and *TIPE3* after treating L132 cells with NNN for 24 h, D. Graphical representation of the expression of *TIPE*, *TIPE2* and *TIPE3* in NNN treated L132 cells as measured by densitometry scanning. GAPDH was used as internal control. Data are represented as Mean±SE, * denotes p<0.05 compared to control.

et al., 2014). Further, intraperitoneal administration of NNN resulted in genotoxic effects in indicator bacteria recovered from different organs of nitrosamine-pretreated mice in a dose dependent manner (Knasmüller *et al.*, 1994). Further, NNN binds to the nAChR which results in tumor growth through deregulated cell proliferation, survival, invasion and migration (Xue *et al.*, 2014). Therefore, it is essential to determine if the carcinogenic effect of NNN is mediated through any of the TIPEs. To determine the non-toxic concentrations of NNN, L132 cells were treated with various concentrations of NNN as mentioned above for 24 h. 0.05, 0.10, 0.25 and 0.5 µM of NNN were found not to induce cytotoxicity to L132 human lung epithelial cells. To determine the mRNA levels of *TIPE*s, L132 cells were treated with the non-toxic concentrations of NNN followed by expressions analysis with the help of RT-PCR. The results showed that *TIPE*, *TIPE2* and *TIPE3* were significantly upregulated in NNN treated lung epithelial cells. In case of *TIPE2*, a dose dependent upregulation was observed, whereas *TIPE3* was also found to be upregulated dose dependently upto 0.25µM in NNN treated L132 cells. However, in case of TIPE, no such dose dependency was observed in L132 cells after treating with NNN (Figure 3.3). Aforementioned, NNN stimulates nAChRs, which can induce the constitutive activation of various transcription factors like GATA3, NF-κB, and STAT-1 which in turn enhances the expression of various proliferative and anti-apoptotic genes leading to increased cell proliferation (Arredondo *et al.*, 2006). Thus, these results again suggest that *TIPE*, *TIPE2* and *TIPE3* are probably involved in NNN mediated lung cancer. However, further studies are required to establish the exact molecular mechanism involved.

3.3.5. Effect of nicotine on the expression of *TIPE*s in human lung epithelial cells

Among the different chemical constituents present in the cigarette, nicotine is considered to be the principal addictive component (Sugavanesh and Pushpanjali, 2018). Although it does not play role in tumor initiation, but is strongly involved in promoting proliferation, migration, and invasion of cells *in vitro* and tumor growth and metastasis *in vivo* plausibly via activation of nAChRs. Further, nicotine has been reported to be involved in promoting self-renewal of stem-like side-population cells from lung cancers (Schaal *et al.,* 2018). The nAChRs were first identified as regulators of the nervous system. However, increasing lines of evidence suggest that they are involved in diverse processes in almost all non-neuronal mammalian cells (Wang and Hu, 2018). They play immensely important function in lung carcinogenesis through modulation of either stimulatory or inhibitory signaling cascades. Among the different members of nicotinic receptors family, alpha7-subtype of nAChR (α7nAChR) is a critical mediator involved not only in inflammatory responses but also in cancers and plays important role in proliferative, pro-angiogenic and pro-metastatic effects of nicotine in lung cancer (Hajiasgharzadeh *et al.,* 2019; Wang and Hu, 2018). In case of NSCLC, blockade of α7 nAChRs led to the inhibition of nicotine-induced tumor growth and vimentin expression via MEK/ERK signaling pathway (Zhang *et al.,* 2017a). Further, nicotine increased the levels of α5-nAChR mRNA and protein in NSCLC cells and activated the janus-activated kinase (JAK) 2/ signal transducer and activator of transcription (STAT) 3 signaling pathway (Zhang *et al.,* 2017b). Nicotine was also reported to induce chemoresistance in lung cancer cells via stimulation of Mcl-1 phosphorylation and its interaction with Bak (Liu *et al.,* 2019). In addition, nicotine also promoted the progressive properties of lung cancer cells through inhibition of the expression of cystic fibrosis transmembrane conductance regulator (CFTR) and plasma membrane localization (Li *et al.,* 2018). Further, nicotine also enhanced store‑operated

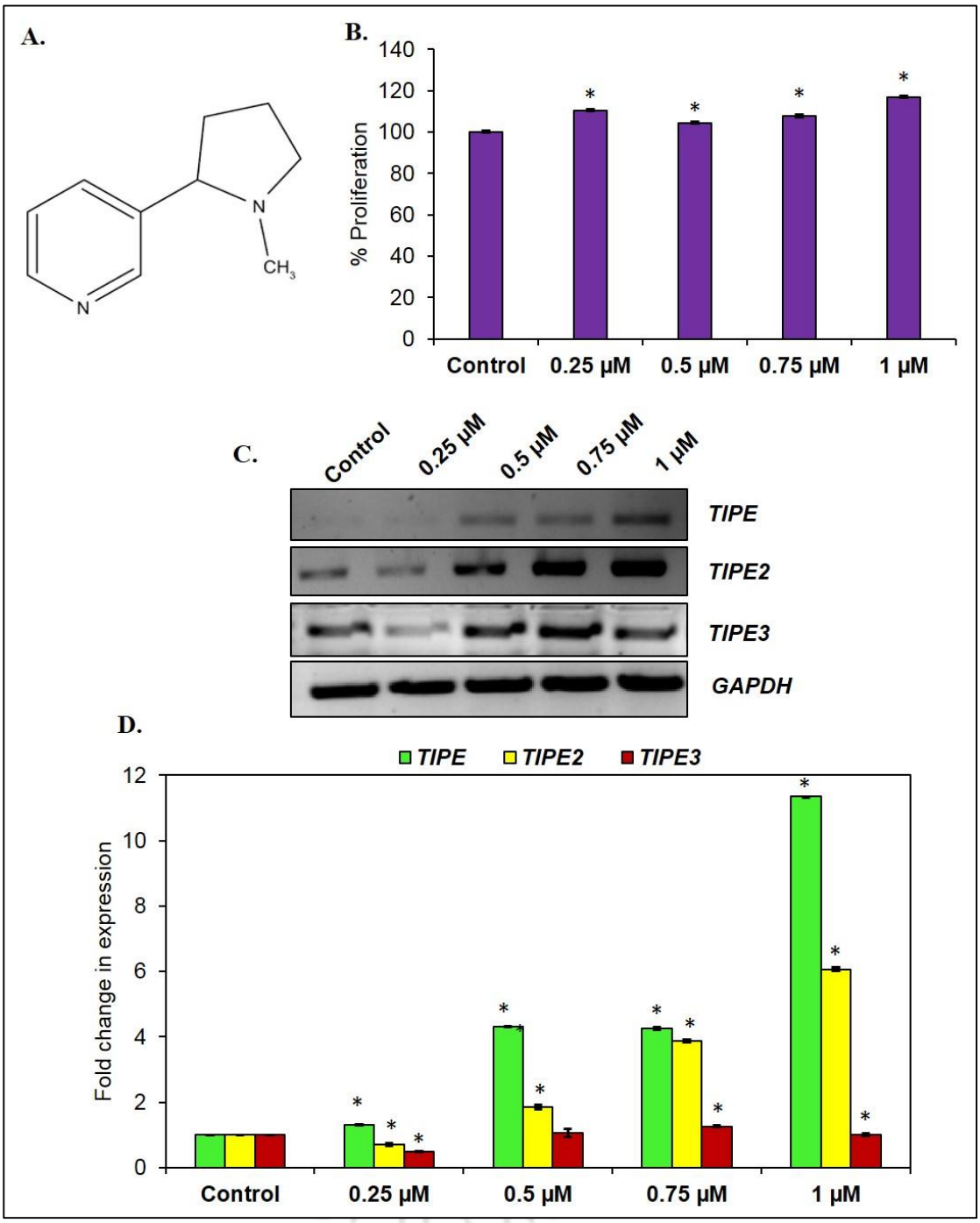

Figure 3.4. Effect of Nicotine on the expression of *TIPE*, *TIPE2* and *TIPE3* in lung epithelial cells A. Structure of Nicotine, B. MTT assay showing non-cytotoxic concentrations of Nicotine in L132 lung epithelial cells, C. RT-PCR analysis of the expression of *TIPE*, *TIPE2* and *TIPE3* after treating L132 cells with Nicotine for 24 h, D. Graphical representation of the expression of *TIPE*, *TIPE2* and *TIPE3* in Nicotine treated L132 cells as measured by densitometry scanning. GAPDH was used as internal control. Data are represented as Mean±SE, * denotes p<0.05 compared to control.

calcium entry through upregulation of hypoxia-inducible factor 1-alpha (HIF‑1α) and store‑operated calcium channel (SOCC) components in NSCLC cells (Wang *et al.,* 2018b). Additionally, nicotine inhibited the expression of the cancer stem cell marker, CD24 in Lewis lung carcinoma cells via upregulation of RAS (Liu *et al.,* 2018a). As nicotine is responsible for the regulation of diverse genes involved in lung pathogenesis, we determined its effect on the expression of *TIPE*s. Firstly we determined the non-toxic concentrations of nicotine on human lung cells and found that the concentrations 0.25, 0.5, 0.75 and 1 µM did not induce any cytotoxicity to the lung epithelial cells. Therefore, we used the above mentioned concentrations of nicotine for treating the L132 cells for 24 h which was followed by expression analysis of *TIPE*, *TIPE2* and *TIPE3* with the help of RT-PCR. Our results showed that nicotine upregulated the expression of *TIPE* and *TIPE2* significantly in L132 lung cells in a dose dependent manner. In case of *TIPE3,* upregulation was observed at 0.5 and 0.75 µM though not much difference in fold change compared to control was observed (Figure 3.4). Thus, it provides an indication that *TIPE*s are involved in nicotine induced carcinogenesis of lung. Findings revealed that nicotine plays an important role in the stimulation of Akt-dependent proliferation and NF-κB-dependent survival in lung cancer cells (Tsurutani *et al.,* 2005). Furthermore, TIPEs are also known to be the activators of NF-κB (Padmavathi *et al.,* 2018) and thus they might plausibly be associated with nicotine mediated lung carcinogenesis. Additionally, nicotine induces cancer cell proliferation and tumor progression through modulation of diverse signaling cascades through nAChRs which include MAPK/ERK, PI3K/Akt pathway, and JAK/STAT signaling etc. (Schaal and Chellappan, 2014). Therefore, it can be concluded that nicotine mediated upregulation of *TIPE*, *TIPE2* and *TIPE3* might play a critical role in the development

and progression of lung cancer. However, further in depth studies are requisite to unveil the underlined molecular mechanism of action.

3.3.5. Effect of BaP on the expression of TIPEs in human lung epithelial cells

BaP is a chief constituent present in tobacco smoke which plays an important role in the development of lung cancer. BaP itself is a procarcinogen, however it can function as a complete carcinogen in animals upon its metabolic activation and cause mutations in different genes through binding with DNA. Further, BaP also helps in the promotion of tumor with the help of a signal transduction mediated by aryl hydrocarbon receptor (AhR), which plays an important role in BaP induced carcinogenesis (Kasala *et al.*, 2015). Metabolically active BaP is well evinced to exert different cytotoxic, teratogenic, genotoxic, mutagenic and carcinogenic effects in diverse cell types and tissue samples (Shi *et al.,* 2010). BaP gets converted to benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE) in liver and lung with the help of CYP enzyme superfamily which include CYP1A1/1A2 and CYP1B1. The BPDE produced then reacts with DNA and results in the formation of benzo(a)pyrene-7,8-diol-9,10-epoxide-N(2)-deoxyguanosine (BPDE-dG) adduct, which serves as a potent risk factor for lung cancer (Alexandrov *et al,* 2010). Further, BaP mediated activation of AhR induces MAPK signaling pathway in diverse cell types. It also activates AhR/Src/ERK cascade in lung cells which aids in the induction of CYP1A1 and formation of DNA adducts (Vázquez-Gómez *et al.*, 2018). In addition, BaP stimulates the migration and invasion of lung cancer cells through up-regulation of the expression of IL8, chemokines such as CCL2 and CCL3, Twist and TG-interacting factor (TGIF) (Zhang *et al.,* 2016b; Wang *et al.,* 2015c; Chen *et al.,* 2017; Yang *et al.*,2018). It also induced cell cycle progression through ERK-mediated activation of Chk1 pathway in lung cancer cells (Wang *et al.*,

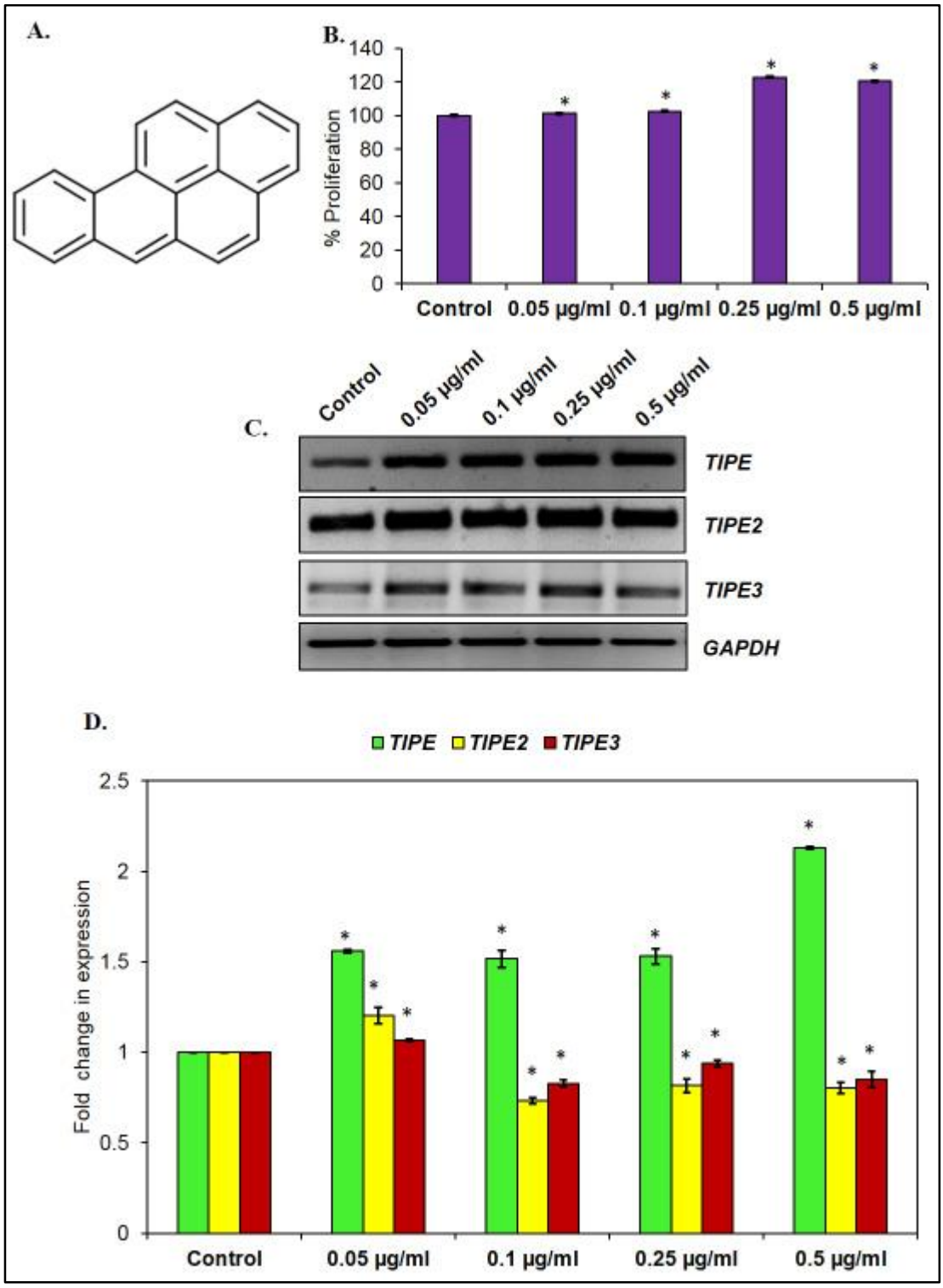

Figure 3.5. Effect of BaP on the expression of *TIPE*, *TIPE2* and *TIPE3* in lung epithelial cells A. Structure of BaP, B. MTT assay showing non-cytotoxic concentrations of BaP in L132 lung epithelial cells, C. RT-PCR analysis of the expression of *TIPE*, *TIPE2* and *TIPE3* after treating L132 cells with BaP for 24 h, D. Graphical representation of the expression of *TIPE*, *TIPE2* and *TIPE3* in BaP treated L132 cells as measured by densitometry scanning. GAPDH was used as internal control. Data are represented as Mean±SE, * denotes $p<0.05$ compared to control.

2015a). Besides, this constituent of tobacco was also reported to cause upregulation of mesenchymal markers such as N-cadherin and vimentin and reduce the expression of E-cadherin, an epithelial marker (Chen *et al.*, 2017). In addition, BaP is reported to cause alteration in p53 signaling cascade in human lung cancer cells as well cancer stem Cells (Bak *et al.*, 2018). Additionally, NF-κB is also reported to have strong involvement in the BaP-promoted p53 expression (Pei *et al.*,1999). Therefore, examining the effect of BaP on the expression of TIPEs may give insight towards understanding the role of TIPEs in BaP induced lung carcinogenesis. Therefore, initially we determined the non-toxic concentrations of BaP which were found to be 0.05, 0.1, 0.25 and 0.5 µg/ml. Hence, those concentrations were used to treat L132 human lung epithelial cells for 24h and then expression of *TIPE*, *TIPE2* and *TIPE3* were analyzed with the help of RT-PCR. The mRNA level of *TIPE* was found to be upregulated in a dose dependent manner. In the case of *TIPE2* and *TIPE3*, slight upregulation was observed at 0.05 µg/ml; however beyond that concentration, downregulation of both the genes were observed (Figure 3.5). As mentioned earlier, BaP activates MAPK signaling pathway through AhR. Deregulated MAPK pathway in turn gives rise to alterations in diverse cellular processes like differentiation, proliferation, apoptosis etc. (Vázquez-Gómez *et al.*, 2018). Therefore, MAPK can be presumed as a downstream target of *TIPE* in BaP mediated lung carcinogenesis. Further, BaP is reported to exert varied effect on the basis of the growth kinetics of the target cell population, tissue type and also genetic variations (Hamouchene *et al.*, 2011). Therefore, it can be considered to be responsible in part for *TIPE2* and *TIPE3* to get upregulated at lower concentration i.e. 0.05 µg/ml followed by their downregulation beyond that concentration when checked upto 0.5 µg/ml of BaP.

3.4. Conclusion

This is the first report which showed the correlation between tobacco and its constituents and the regulation of *TIPE*, *TIPE2* and *TIPE3* in human lung cancer. These results are in accordance with our previous findings from IHC data. Treatment of L132 human lung epithelial cells with the water extract of tobacco called tuibur and other important constituents of tobacco such as NNK, NNN, nicotine and BaP resulted in upregulation of *TIPE2* and *TIPE3* notably. Additionally, tuibur, NNK, NNN and nicotine treated L132 cells showed upregulation of TIPE as well. This supports our IHC results, where upregulation of TIPE, TIPE2 and TIPE3 was observed in lung cancer tissues compared to normal lung tissues. Therefore, it can be presumed that deregulation of TIPE proteins may serve as one of the key molecular events in the development and progression of lung cancer as tobacco, which is responsible for 90% of all lung cancer cases is strongly involved in the modulation of the expression of these proteins. Nevertheless, our results provide only a preliminary indication of the involvement of TIPE, TIPE2 and TIPE3 in tobacco induced lung carcinogenesis. Therefore, mechanistic studies are certainly warranted to decipher the upstream regulators as well as downstream targets of TIPEs in tobacco induced lung carcinogenesis.

Chapter 4

Role of TIPE family of proteins in the development and progression of lung cancer

4.1. Introduction

In the preceding chapters, we have shown that TIPE family of proteins were differentially expressed in lung cancer tissues. TIPE, TIPE2 and TIPE3 were found to be significantly upregulated and TIPE1 showed marked downregulation in lung cancer tissues compared to the normal lung tissues. Further, treatment with tuibur and other tobacco related components were found to upregulate the expression of *TIPE*, *TIPE2* and *TIPE3* markedly. Noteworthy, treatment with NNK, the potent lung carcinogen which has been used customarily for inducing lung carcinogenesis in different animal models resulted in significant upregulation of *TIPE*, *TIPE2* and *TIPE3* in human lung epithelial cells. Altogether, these studies provide us a clear idea that TIPE, TIPE2 and TIPE3 are strongly involved in the positive regulation of lung carcinogenesis, in particular tobacco induced lung cancer. On the other hand, TIPE1 is found to be involved in the negative regulation of lung tumorigenesis. However, to elucidate the exact role of this family of proteins and their downstream targets, it is imperative to silence or disrupt them. Notably, siRNA or shRNA mediated silencing of these proteins was found to influence cell growth, proliferation, invasion and metastasis of lung cancer cells as evinced by a few studies carried out thus far which are discussed in the first chapter. However, no study reported the comparative analysis upon individual knockout of all the four proteins. Therefore, in this chapter, we have done knockout of TIPE, TIPE1, TIPE2 and TIPE3 individually in NCIH460 human lung cancer cells using CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas9-method of gene editing and determined the effect of individual gene knockout on different hallmarks of cancer as well as a comparative analysis of them in lung cancer cells. Further, we determined their downstream targets which are involved in the

pathogenesis of lung cancer. In addition, we found their involvement in tobacco mediated lung carcinogenesis and the underlined mechanism of action.

4.2 Materials and Methods

4.2.1 Cell culture

NCIH460 human NSCLC cells were procured from NCCS, Pune, India. These cells were maintained in DMEM medium (Gibco™; Life Technologies, NY, USA) supplemented with 10% FBS (Gibco®, NY, USA) and 1X Penstrep (Invitrogen, CA, USA). The cells were cultured and maintained at 37°C in 5% CO_2 and 95% humidity.

4.2.2. CRISPR/Cas9 mediated gene knockout

For disrupting TIPE, TIPE1, TIPE2 and TIPE3 genes individually, we used CRISPR/Cas9 mediated gene editing method (Figure 4.1). To carry out that, we obtained commercially available CRISPR/Cas9 All-in-One Lentivector sets (Human) expressing both human Cas9 and respective sgRNAs viz. scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (Cat. No. K010), TNFAIP8 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414505), TNFAIP8L1 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414605), TNFAIP8L2 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414705) and TNFAIP8L3 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414805) from Applied Biological Materials, Richmond, BC, Canada. The sequences of sgRNA target are given in Table 4.1. Briefly, NCIH460 cells were seeded at a density of 25000 cells/well in a 24 well plate and allowed to attain confluency by 70-80%. Following this, the cells were transfected with 1 µg of respective plasmids with the help of Lentifectin™ transfection reagent (Cat. No. G074,

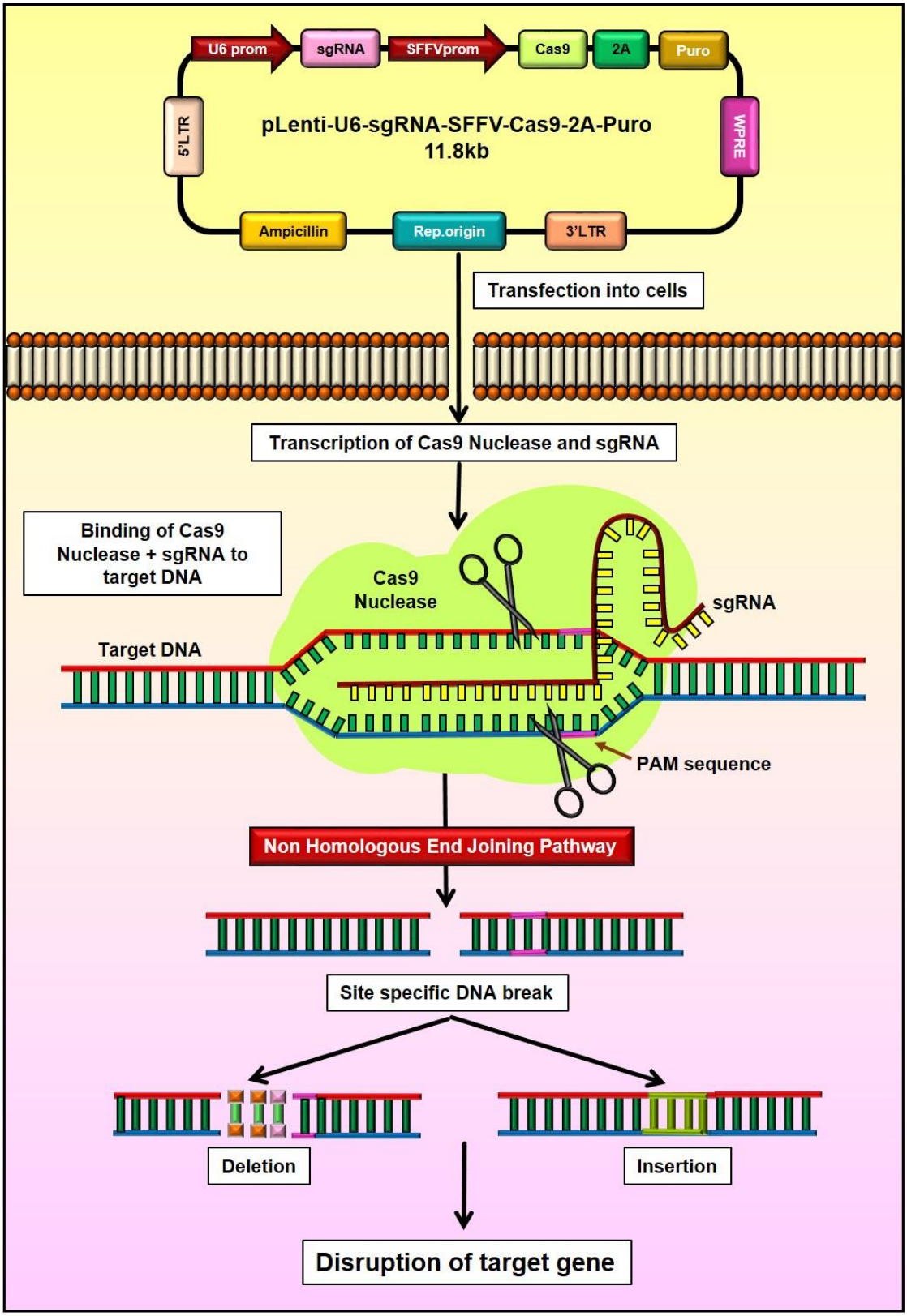

Figure 4.1. Mechanism of CRISPR/Cas 9 mediated gene disruption

Applied Biological Materials, Richmond, BC, Canada) in incomplete opti-MEM media. The transfected cells were then incubated at 37°C in 5% CO_2 and 95% humidity for around 5-8 h. Subsequently, 10% FBS (Gibco®, NY, USA) was added to the cells and then again incubated for 24 h. After that, the media containing plasmid was removed and fresh DMEM medium (with 10% FBS and 1X Penstrep) was added to the wells and kept for recovery. After 24 h recovery, positive selection of cells were carried out by adding 2.5 µg/ml of puromycin (Cat. No. P8833, Sigma-Aldrich, Missouri, USA). As the CRISPR/Cas9 plasmids carry puromycin resistance gene, only the transfected cells will show resistance against puromycin. The puromycin resistant cells were allowed to form colonies and then single cell selection was carried out. Confirmation of knock out of the selected single cell clones was done by Western blot analysis. Clones exhibiting complete inhibition of the expression of respective proteins were considered as the ones with successful knockout and they were grown further for future studies.

Table 4.1. sgRNA target sequences.

Gene	Target	sgRNA target sequence
TNFAIP8 (TIPE)	Target 1	ACTTGTGTCGTCTATTAAGG
	Target 2	TCATCAGCTTGCTATGACCG
	Target 3	CAAAGGTATAATCCACCTGA
TNFAIP8L1 (TIPE1)	Target 1	TGAGCTGTACCGCGCCACCA
	Target 2	GTCCACCTGGTGGAAGCTGA
	Target 3	CACCTGACCGCCAAGTCCCA
TNFAIP8L2 (TIPE2)	Target 1	CCAAGGAGTACACGCACAGC
	Target 2	CAGGTCCTTGATCACGCGCT
	Target 3	CCCGCTTTCGCCAGAAGCTG

TNFAIP8L3 (TIPE3)	Target 1	AACGGATATGCAGGGACCCA
	Target 2	ACGTGGCATCCCTTGTGCCT
	Target 3	CGGAATCCGAATCCATGCTG
Scramble	-	GCACTCACATCGCTACATCA

4.2.3. MTT assay

The effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the proliferation of human lung cancer cells was determined with the help of an MTT assay. Briefly, the scrambled sgRNA transfected cells (represented as CRISPR/Cas9 scramble), TIPE knockout cells (represented as CRISPR/Cas9 TIPE), TIPE1 knockout cells (represented as CRISPR/Cas9 TIPE1), TIPE2 knockout cells (represented as CRISPR/Cas9 TIPE2) and TIPE3 knockout cells (represented as CRISPR/Cas9 TIPE3) were seeded at a density of $2X10^3$ cells/well in 96 well plates and incubated for 24h at 37°C in a CO_2 incubator. The MTT assay was performed at 0 and 72 h. After each time point, 10µl of 5mg/ml of MTT (Cat. No. M2128, Sigma-Aldrich, Missouri, USA) was added to each well and incubated for 2h. After that the culture medium was removed and 100µl of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added to all the wells and incubated at room temperature (RT) for 1h to dissolve the MTT-formazan product. Finally, absorbance of the colored solution was measured with a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland) at 570 nm. The inhibition in proliferation caused due to the knockout of each TIPEs was then calculated by normalizing the absorbance value of 72 h with 0 h while considering the absorbance of CRISPR/Cas9 scramble as 100%. Further, the effect on the proliferation of tobacco components' treated TIPE, TIPE2 and TIPE3 knockout cells was also evaluated using this assay, in which after 24 hour incubation of the seeded cells in 96 well plates,

different tobacco components such as NNK (0.05 µM), NNN (0.05 µM), nicotine (1 µM) and BaP (0.25 µg/ml) were added to the CRISPR/Cas9 scramble as well as CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells. The MTT assay was performed at 0 and 24 h after the addition of the tobacco components and the same procedure was followed as mentioned. Finally, the inhibition in proliferation of tobacco components' treated knockout cells was calculated by normalizing the absorbance value of 24 h with 0 h while considering the absorbance of CRISPR/Cas9 scramble with the respective treatment of tobacco components as 100%.

4.2.4. Colony formation assay

The clonogenic potential of TIPE, TIPE1, TIPE2 and TIPE3 knockout NCIH460 cells was determined with the help of a colony formation assay. Briefly, CRISPR/Cas9 scramble, CRISPR/Cas9 TIPE, , CRISPR/Cas9 TIPE1, , CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells were seeded in 6-well plates at low density (~1000 cells per well). Then they were allowed to grow for 15 days with replenishing of media as required. At the end of 15 days, the plates were then washed with 1X phosphate buffer saline (PBS) and colonies were fixed with 70% ethanol. After fixation, washing was done with IX PBS again and then stained with 0.01% (w/v) crystal violet (Cat No: 548-6209; SRL Pvt. Ltd., India). To remove the excess stain, again a gentle washing with 1X PBS was done. The images of each well were captured, the individual clone types were identified, and survival fraction was determined as per the following formula:

PE, Plating efficiency = (Number of colonies counted/ Number of cells plated) × 100

SF, Survival fraction = (PE of treated sample/ PE of control) × 100

Additionally, the effect on the clonogenic potential of tobacco components' treated TIPE, TIPE2 and TIPE3 knockout cells was also evaluated with the help of this assay, in which after 24 h incubation of the seeded cells in 6 well plates, different tobacco components such as NNK (0.05 µM), NNN(0.05 µM), nicotine (1 µM) and BaP (0.25 µg/ml) were added to the CRISPR/Cas9 scramble as well as CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells. After incubating for 24 h, the media of all the wells were changed, cells were allowed to grow for 10 days and the same procedure was followed henceforth.

4.2.5. Migration assay

This assay was performed to evaluate the migration of NCIH460 cells after individual knockout of TIPE, TIPE1, TIPE2 and TIPE3 compared to scrambled control. For this, CRISPR/Cas9 scramble, CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE1, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells were seeded at a density of $6x10^5$ cells/well in 12 well plates. After the formation of monolayer, the medium was replaced with serum free DMEM medium and the cells were incubated for 6-8h. Then the medium was removed and a pipette tip (200 µl) was used to scratch a wound in the midline of the culture well. Following this, the cells were washed twice with PBS to remove any debris. The migration of the cells were evaluated by observing the difference in the area of the scratch wounds with the help of an inverted microscope (Nikon T1-SM, Japan). Images were taken at different time intervals and then analyzed using Image J software. Migration assay was also performed to analyze the effect of tobacco components on the migration potential of TIPE, TIPE2 and TIPE3 knockout cells. For that, after serum starvation followed by scratching of wound, different tobacco components such as NNK (0.05 µM), NNN (0.05 µM), nicotine (1 µM) and BaP (0.25 µg/ml) were added

to the CRISPR/Cas9 scramble as well as CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells and then the migration of the cells was evaluated as discussed above.

4.2.6. Invasion assay

Invasion is a critical step in tumor metastasis. Therefore, the invasive potential of NCIH460 cells after knock out of TIPE, TIPE1, TIPE2 and TIPE3 was analyzed using a Boyden chamber assay. Briefly, CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE1, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells were serum starved for 18h before seeding in transwell migration chambers. 24-well, 8mm pore transwell inserts (Cat No. 354480, Corning, USA) pre-coated with Matrigel were used for this assay. After serum starvation, the cells were trypsinized and then $5x10^4$ cells were seeded in 500µl of serum free medium in the upper chamber of the transwell insert. Then 750µl of DMEM medium containing 10% FBS was added as a chemo-attractant to the lower chamber. Cells were then incubated for 24 h at 37°C in a CO_2 regulated incubator. The non-migrating cells present on the upper surface of the membrane were then scraped off with cotton swabs. The migrated cells at the bottom of the transwell insert were fixed in 70% ethanol and then stained with 0.01 % (w/v) crystal violet. Stained cells were visualized under an inverted microscope (Nicon Eclipse TS100, Japan) and images were captured using a Nikon 500 camera.

4.2.7. Western blot

Western blot analysis was carried out for the confirmation of TIPE, TIPE1, TIPE2 and TIPE3 knockout in NCIH460 cells. Further, it was also done to determine the expression of their different targets. For this purpose, briefly, CRISPR/Cas9 scramble,

CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE1, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells were lysed with the help of whole cell lysis buffer containing protease inhibitors (20mM HEPES, 2mM EDTA, 250mM NaCl, 0.1% (v/v) Triton-X100, 2µg/ml Leupeptin hemisulfate, 2µg/ml Aprotinin, 1mM PMSF, 1mM DTT). The protein concentration of the lysates were measured using Bradford reagent (Cat. No. 500-0205; Bio-Rad, California, USA) where the standard used was Bovine serum albumin (BSA). 50µg of proteins were resolved after mixing with 5X Laemmli Buffer (250mM Tris HCl, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% Bromophenol blue) in a 12% or 8% SDS-acrylamide gel. Then they were transferred to nitrocellulose membrane (Bio-Rad, California, USA). After confirmation of transfer using Ponceau-S stain (Cat. No. ML045; HIMEDIA), the membranes were blocked with 5% non-fat milk in tris-buffered saline with 1% tween 20 (TBST). In case of phospho (p) antibodies, blocking was done using 5% BSA in TBST. After proper washing with 1X TBST, the blots were then probed with appropriate primary antibodies for overnight (Table 4.2). Subsequently, the blots were again washed with 1X TBST and then incubated with suitable horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 4.2). The bands representing different proteins were visualized using Clarity Western ECL Substrate (Cat. No. 1705061; Bio-Rad, California, USA) in a ChemiDoc™ XRS System (Bio-Rad, California, USA). The house keeping gene α-tubulin was used as the loading control. Further, to determine the expression of different targets in tobacco components' treated TIPE, TIPE2 and TIPE3 knockout cells, Western blot was carried out in which lysis of the cells were done after 24 h of treatment with NNK (0.05 µM), NNN (0.05 µM), nicotine (1 µM) and BaP (0.25 µg/ml) and the same procedure was followed thereafter.

Table 4.2. Details of the primary and secondary antibodies used for Western blot.

Name	Details	Dilutions used
Anti-TNFAIP8 antibody	ab64988; abcam®, Cambridge, USA	1:2000
Anti-TNFAIP8L1 antibody	ab85409; abcam®, Cambridge, USA	1:1000
Anti-TNFAIP8L2 antibody	ab110389; abcam®, Cambridge, USA	1:4000
Anti-TNFAIP8L3 antibody	ab111524; abcam®, Cambridge, USA	1:4000
Anti-α-Tubulin antibody	2144S; Cell Signaling Technology, Massachusetts, USA	1:2000
Anti-Phospho- Akt (Ser473) antibody	4060S; Cell Signaling Technology, Massachusetts, USA	1: 4000
Anti- Akt1 antibody	2938S; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- mTOR protein (Ser2448) antibody	5536T; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-mTOR antibody	2983T; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- S6 Ribosomal protein (Ser235/236) antibody	4858T; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-S6 Ribosomal protein-antibody	2317S; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- STAT-3 (Ser727) antibody	9134T; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-STAT-3 antibody	9139T; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- NF-κB p65 (Ser536) antibody	3033P; Cell Signaling Technology, Massachusetts, USA	1: 5000
Anti- NF-κB p65 antibody	8242P; Cell Signaling Technology, Massachusetts, USA	1: 4000
Anti-p53 antibody	2524T; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-p21 antibody	10-7526; ABGENEX Pvt. Ltd., Odisha , India	1:1000
Anti-PTEN antibody	11-7539; ABGENEX Pvt. Ltd., Odisha , India	1: 1000
Anti-Redd1 antibody	2516S; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-LC-3B antibody	2775S; Cell Signaling Technology, Massachusetts, USA	1: 1000

Anti-Bcl-2 antibody	15071; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-Cox-2 antibody	12282P; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-survivin antibody	2808BC; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-XIAP antibody	20-1106; ABGENEX Pvt. Ltd., India	1: 1000
Anti-cIAP-1/HIAP-2 antibody	20-1054; ABGENEX Pvt. Ltd., India	1: 1000
Anti-c-Myc antibody	10-10013; ABGENEX Pvt. Ltd., India	1:2000
Anti-Caspase 9 antibody	9508T; Cell Signaling Technology, Massachusetts, USA	1:2000
Anti-CXCR4 antibody	ab124824; abcam®, Cambridge, USA	1: 2000
Anti-MMP-9 antibody	13667P; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-VEGF-A antibody	ab46154; abcam®, Cambridge, USA	1: 2000
Anti-rabbit secondary antibody	ab97080; abcam®, Cambridge, USA	1: 6000
Anti-mouse secondary antibody	ab97040; abcam®, Cambridge, USA	1: 6000

4.2.8. Statistical analysis

Statistical analysis was performed using Student's *t*-test. All the data are represented as Mean±SE. p-value < 0.05 was denoted as statistically significant.

4.3. Results and discussion

In this chapter, we determined the role of TIPE, TIPE1, TIPE2 and TIPE3 on the regulation of diverse cancer hallmarks. At first, we established stable knockout of TIPE, TIPE1, TIPE2 and TIPE3 in NCIH460 cells separately. Subsequently, we studied the effect of the knockout of TIPEs on the proliferation, survival, invasion and migration of NCIH460 lung cancer cells. Further, we identified the downstream molecular targets

of TIPEs. We have also studied the effect on the proliferation, survival and migration of tobacco components' treated TIPE, TIPE2 and TIPE3 knockout cells and also the underlined mechanism of action of TIPEs in tobacco induced lung carcinogenesis.

4.3.1. Confirmation of knock out of TIPEs

The knockout of TIPEs in NCIH460 human lung cancer cells were done with the help of CRISPR/Cas9 method of gene editing. Among three targets provided for each one of the TIPEs, transfection with target 2 sgRNA/Cas9 plasmids generated successful knockout clones for TIPE, TIPE1 and TIPE2 whereas target 1 sgRNA/Cas9 plasmids transfected clones engendered successful knockout of TIPE3. For the confirmation of knockout, Western blot was performed (Figure 4.2). Those cells were allowed to grow further and used for different studies.

4.3.2. Effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the proliferation of lung cancer cells

Increased proliferation and survival, reduced apoptosis are some of the major characteristics exhibited by cancer cells which are attained through inflection of diverse signaling cascades (Kurgan *et al.*, 2017). Therefore, we determined the effect of knockout of TIPEs on the proliferation of human lung cancer cells with the help of MTT assay. Our results showed that knockout of TIPE, TIPE2 and TIPE3 resulted in significantly decreased proliferation whereas knockout of TIPE1 led to the increased proliferation of NCIH460 cells compared to scrambled control (Figure 4.2). However, the reduction in proliferation obtained for TIPE, TIPE2 and TIPE3 knockout cells were found to be different. The highest inhibition in the proliferation of NCIH460 cells was

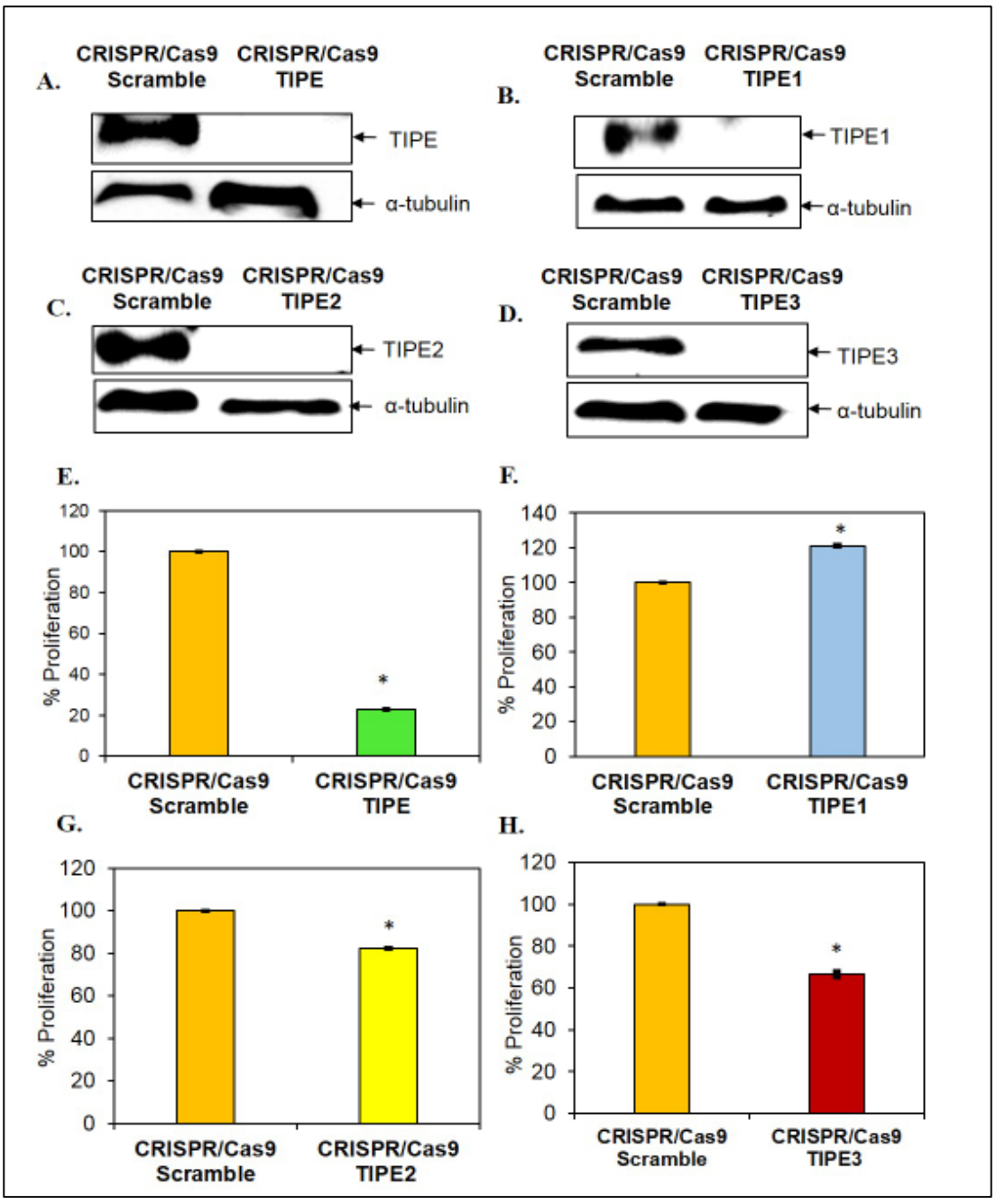

Figure 4.2: Knockout TIPEs in lung cancer cells and their effect on lung cancer cells' proliferation. A-D. Representative Western blot images of TIPE, TIPE1, TIPE2 and TIPE3 knockout compared with scrambled control, E-H. Percentage change in cell proliferation of TIPE, TIPE1, TIPE2 and TIPE3 knockout cells compared with scrambled control cells evaluated using MTT assay. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

obtained upon knockout of TIPE (77% inhibition), followed by TIPE3 knockout (33.4% inhibition) and TIPE2 knockout (17.7 %) respectively. Our results are supported by the previous findings as well. For instance, Miao and group showed that knockdown of TIPE led to reduction in proliferation of colon cancer cells along with downregulation of Cyclin D1 and p-Rb (Miao *et al.*, 2012). Further, in esophageal and gastric cancer cells as well, depletion of TIPE resulted in decreased proliferation and motility of ESCC cells (Sun *et al.*, 2016; Hadisaputri *et al.*, 2012; Hu *et al.*, 2016). In addition, TIPE was found to promote the proliferation of lung cancer cells as well. Han and group also showed that overexpression of TIPE caused enhanced cell proliferation and upregulation of Cyclin and connective tissue growth factor proteins (Han *et al.*, 2018; Dong *et al.*, 2017). However, we obtained contrasting results in case of TIPE2 than the existing findings. It was reported to be downregulated in human breast cancer and its overexpression inhibited the proliferation of tumor cells and tumor xenograft growth (Wang *et al.*, 2017). In addition, in line with our findings, knockdown of TIPE3 was shown to inhibit the proliferation of breast cancer cells (Lian *et al.*, 2017). Further, knockdown of endogenous TIPE3; generally expressed in the plasma membrane, also caused inhibition in the proliferation of NSCLC cells (Wang *et al.*, 2018a). In contrary to TIPE, TIPE2 and TIPE3, our findings showed that knockout of TIPE1 resulted in markedly increased proliferation of NCIH460 cells. This is supported by a previous study carried out by Wu and group where they showed that TIPE1 overexpression led to the reduced cell growth and proliferation, but induced apoptosis in TIPE1-downregulated lung cancer cells via modulation of Cyclins, Caspases and MMPs (Wu *et al.*, 2017). Altogether, the results obtained are in accordance with our previous findings and can be said that TIPE, TIPE2 and TIPE3 are plausibly involved in the

positive regulation of lung cancer whereas TIPE1 negatively regulates lung carcinogenesis.

4.3.3. Effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the clonogenic potential of lung cancer cells

Aforementioned, apart from enhanced proliferation, increase in survival is also a prime characteristic of cancer cells. Therefore, to determine the effect of the knockout of TIPEs on the survival of NCIH460 cells, colony formation assay was performed. This assay determines the clonogenic potential of cells, which can be defined as the ability of a cell to proliferate indefinitely and retain its reproducibility to give rise to a large colony or a clone which in turn gives the measure of cell survival fraction (Munshi *et al.,* 2005). The results of our study showed that knock out of TIPE, TIPE2 and TIPE3 resulted in reduced clonogenic potential of NCIH460 cells compared to scrambled control implying that TIPE, TIPE2 and TIPE3 are involved in increasing the survival of lung cancer cells. Highest reduction in the colony formation ability was observed in TIPE knockout cells followed by TIPE2 and TIPE3 knockout NCIH460 cells respectively. Contrary to those, 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 (Figure 4.3). In line with our results, knockdown of TIPE was found to reduce the colony formation ability of colon cancer cells (Miao *et al.,* 2012). Further, contradicting our finding, TIPE1 was reported to inhibit the growth and colony forming ability of HCC cells through modulation of Rac1 (Zhang *et al.,* 2015b). However, a study carried out in lung cancer supports our finding where they have shown that overexpression of TIPE1 resulted in reduced colony

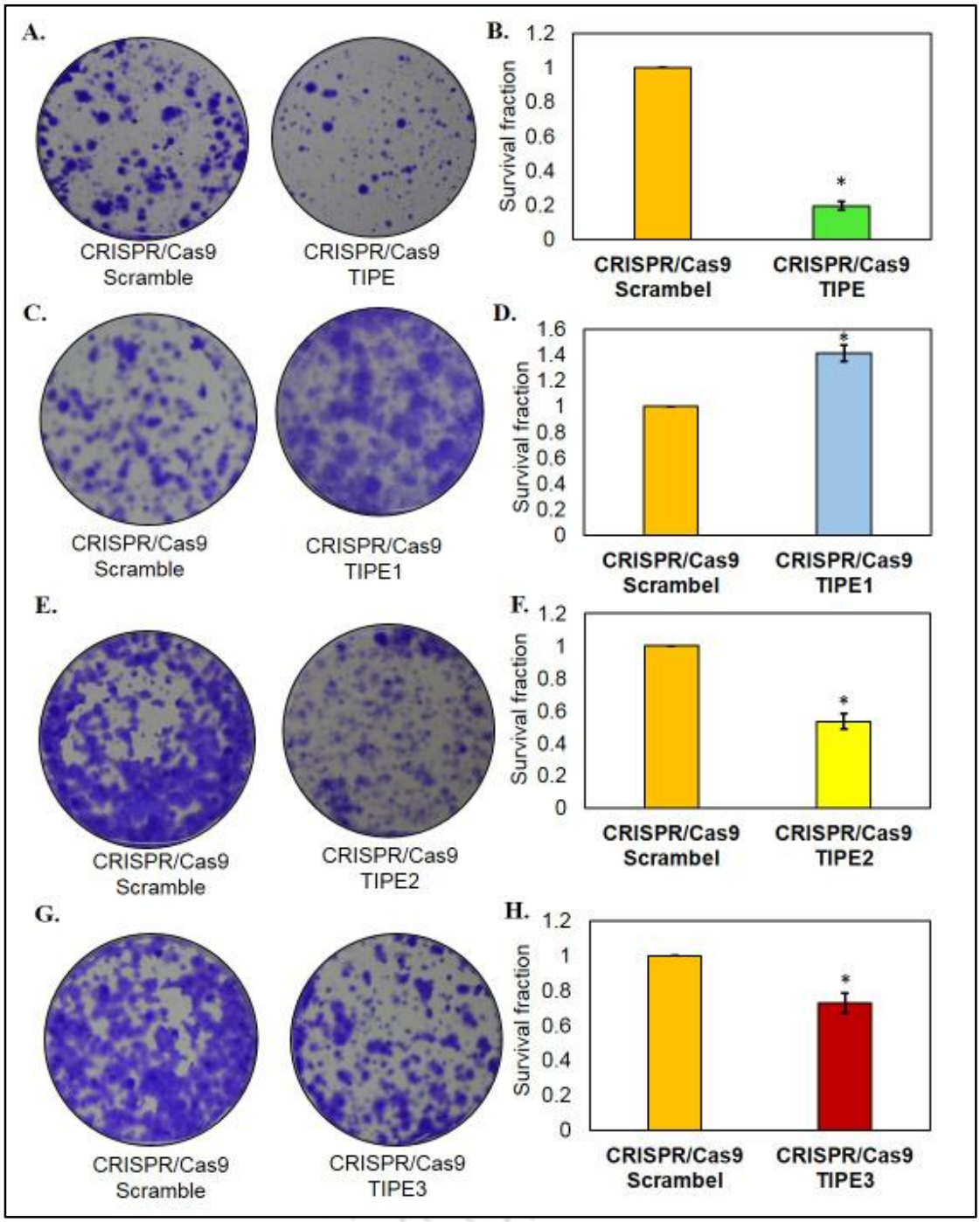

Figure 4.3. Effect of CRISPR/Cas9 mediated knockout of TIPEs on the survival of lung cancer cells. A, C, E & G represents the images of the colonies formed in TIPE, TIPE1, TIPE2 and TIPE3 knockout cells along with scrambled control; B, D, F & H shows the graphical representation of clonogenic potential of TIPE, TIPE1, TIPE2 & TIPE3 knockout cells in terms of survival fraction compared to scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

formation of lung cancer cells (Wu *et al.*, 2017). In addition, overexpression of TIPE2 was reported to exert an inhibitory effect on the colony formation ability of human NSCLC cells in a study conducted by Li and group, whereas our results revealed the opposite effect (Li *et al.*, 2016). Taken together, our results suggest that TIPE, TIPE2 and TIPE3 are responsible for the positive regulation of the lung cancer cell survival whereas TIPE1 negatively regulates the proliferation and survival of lung cancer cells.

4.3.4. Effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the migration of lung cancer cells

As mentioned earlier, in lung cancer, the most lethal cancer type worldwide, majority of the patients are presented with a highly advanced stage of the disease. The ability acquired by lung cancer cells to migrate and invade nearby cells is strongly linked with their high metastatic potential (Tungsukruthai *et al.*, 2017; Millar *et al.*, 2017). In the course of metastasis, primary tumor cells migrate and invade nearby tissues and thus form secondary tumor sites. Mounting evidence suggest that different signaling molecules present in the tumor microenvironment play significant role in modulating the migratory properties of the cancer cells (Luanpitpong *et al.*,2010). Therefore, in order to know whether TIPEs have any role in the migration of lung cancer cells, migration assay was carried out. The results showed that loss of TIPE, TIPE2 and TIPE3 inhibited the migration potential of lung cancer cells effectively. In case of scrambled control, almost complete healing of wound was observed at 24 h, whereas in TIPE, TIPE2 and TIPE3 knockout NCIH460 cells, remarkably less healing in the wound was observed. In TIPE knockout cells, the wound area remaining was around 92% whereas in TIPE2 and TIPE3 knockout cells, more than 60% wound area was

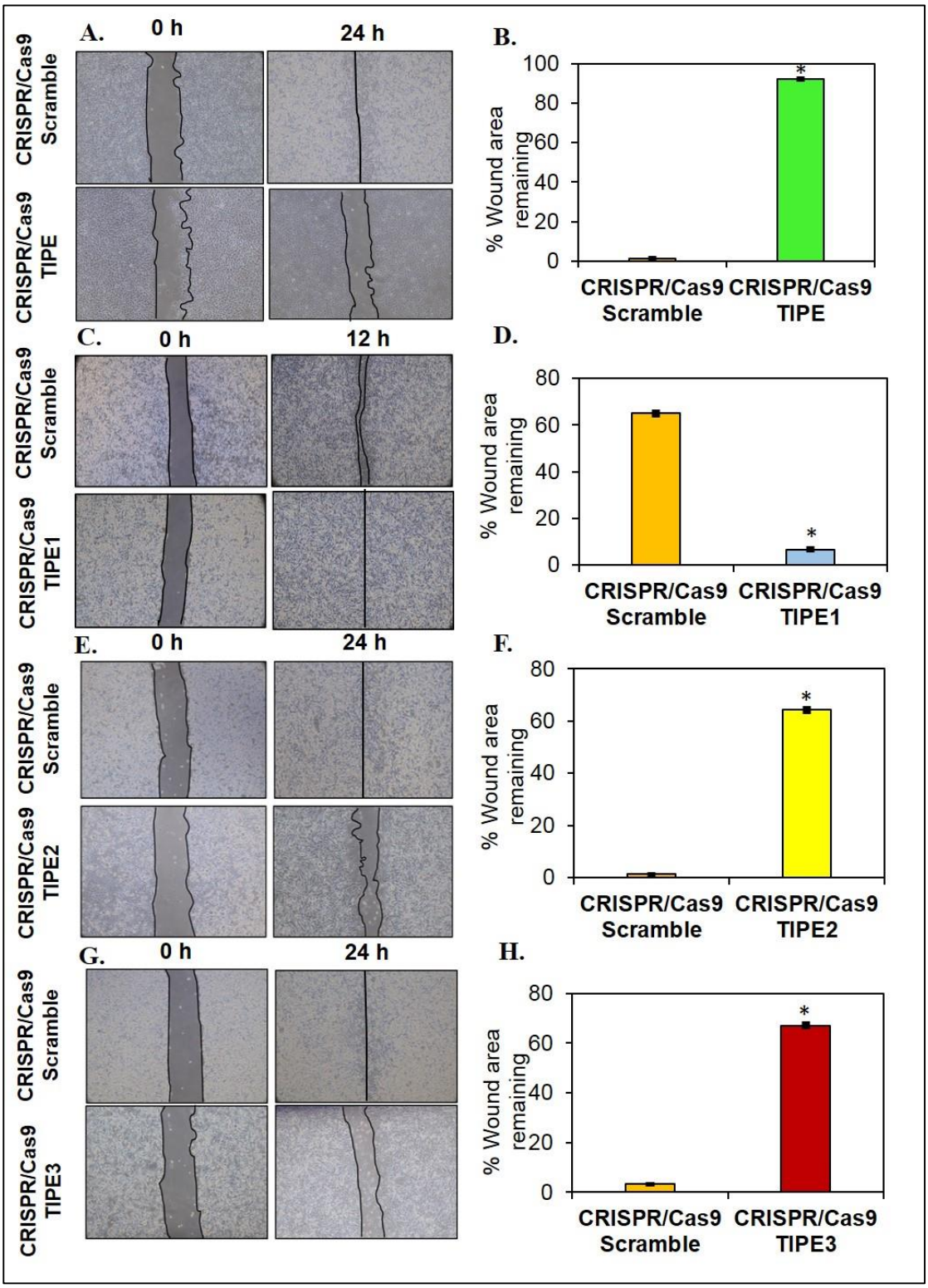

Figure 4.4. Effect of CRISPR/Cas9 mediated knockout of TIPEs on the migration of lung cancer cells. A, C, E & G shows the representative images of the effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the migration of lung cancer cells; B, D, F & H shows the graphical representation of percent wound area remaining in TIPE, TIPE1, TIPE2 & TIPE3 knockout cells compared to scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

found to remain at 24 h. In case of TIPE1 knockout NCIH460 cells, almost complete healing of the wound was observed at 12 h itself which is 12 h prior to the healing time of wound created in scrambled control (Figure 4.4). The effects of TIPE family of proteins on the migration potential of different cancers were reported earlier as well. For instance, similar to our results, decreased expression of TIPE was reported to inhibit the growth and migration of gastric cancer cells effectively (Li *et al.*, 2015). Again, contrary to our results, overexpression of TIPE2 was reported to exert an inhibitory effect on the migration of lung and prostate cancer cells (Li *et al.*, 2016; Lu *et al.*, 2016). Additionally, stable overexpression of TIPE3 with a C-terminal flag, plausibly localized in the plasma membrane, induced the growth and migration of NSCLC cells through modulation of ERK and Akt pathway (Wang *et al.*, 2018a). Altogether, TIPE proteins were found to be responsible for the regulation of migration of lung cancer cells. Knockout of TIPE, TIPE2 and TIPE3 led to the negative regulation of lung cancer cells' migration whereas loss of TIPE1 was found to promote the migration of lung cancer cells effectively.

4.3.5. Effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the invasion of lung cancer cells

Multiple genetic and epigenetic alterations in cancer cells result in the activation of different signaling cascades which in turn promote growth, survival, migration and invasion of cancer cells (Rao *et al.*, 2017). Invasion of neighboring tissue which occurs locally or distally through metastasis is considered as one among the most critical hallmarks of cancer. It is primarily mediated via interactions between tumor and extracellular matrix and cancer related fibroblasts (Haney *et al.*, 2018; Hanahan and Weinberg, 2011). Therefore, we have performed Boyden chamber assay to determine

whether knockout of TIPEs have any effect on the invasive potential of human lung cancer cells. We observed that the number of cells that invaded to the lower part of the transwell insert were remarkably less in TIPE knockout NCIH460 cells compared to scrambled control cells. Further, in TIPE1 knockout cells, a notably higher number of cells were found to get invaded to the lower part of the transwell insert compared to control cells. However, in case of TIPE2 and TIPE3 knockout cells, not significant difference in the number of invaded cells was observed when compared with control (Figure 4.5). Thus, TIPE and TIPE1 are involved in the regulation of invasive potential of lung cancer cells. Loss of TIPE caused decreased invasion of NCIH460 cells whereas knockout of TIPE1 was found to increase the invasion of lung cancer cells. The effect of TIPEs on the invasion of different cancers was studied by different groups. The expression of TIPE in breast cancer cells was reported to have strong correlation with enhanced invasion as well as frequency of pulmonary colonization of tumor cells (Zhang *et al.,* 2006). Further, silencing of TIPE was also found to cause decreased invasion in gastric cancer as well as ESCC derived cells (Li *et al.,* 2015; Sun *et al.,* 2016). Additionally, TIPE was also found to promote invasion potential of lung cancer cells along with other hallmarks such as motility, proliferation etc. through modulation of the Hippo pathway (Han *et al.,* 2018). These results support our finding and thus suggest the tumorigenic potential of TIPE. Further, in case of TIPE1, its overexpression was found to be associated with decreased invasion of lung cancer cells (Wu *et al.,* 2017) which again correlates with our findings clearly suggesting the anti-tumor role of TIPE1 in lung cancer. Collectively, TIPE and TIPE1 are found to be involved not only in the proliferation, survival and migration, but also in the invasion of lung cancer cells exemplifying their potential in the progression and metastasis of lung cancer and their immense therapeutic implications.

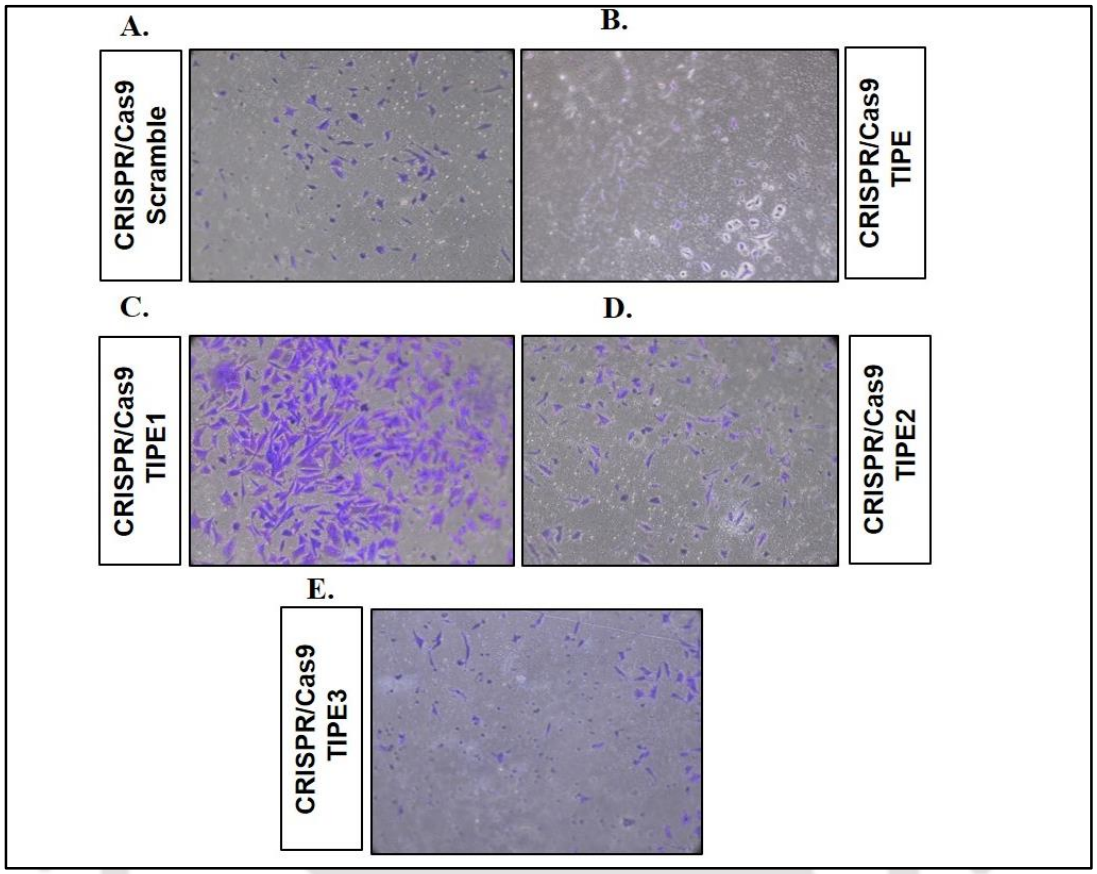

Figure 4.5. Effect of CRISPR/Cas9 mediated knockout of TIPEs on the invasion of lung cancer cells. A-E. Representative images of the effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the invasion of lung cancer cells analysed by Boyden chamber assay.

4.3.6. Effect of TIPE knockout in the modulation of Akt/mTOR/S6/NF-κB/STAT-3 signaling

The findings of our previous studies demonstrate TIPE to have profound role in the promotion of lung cancer cell proliferation, survival, migration and invasion. Notably, there are different signaling molecules/pathways associated with cancer hallmarks (Li and Mansmann, 2014). In other words, modulation of these pathways impacts cancer cell growth, proliferation, survival, migration, invasion etc. Therefore, it is important to know the involvement of these signaling molecules/pathways to decipher the exact molecular mechanism of action of TIPE in human lung cancer cells. Therefore, to

determine the mechanism of action of TIPE, we performed expression analysis of different proteins with the help of Western blot (Figure 4.6). Loss of TIPE downregulated the expression of apoptosis regulatory proteins such as survivin, cIAP-1, XIAP, Bcl-2 and upregulated the expression of caspase 9. Survivin, cIAP-1 and XIAP are the members of inhibitor of apoptosis protein (IAP) family and are involved in apoptosis inhibition (Erkanli *et al.*, 2007). Bcl-2, an anti-apoptotic protein is responsible for the suppression of both Caspase-dependent and Caspase-independent apoptotic pathways. Additionally, Caspase-9 is a member of Caspase family of cysteine proteases and are involved in cytokine processing and apoptosis (Perfettini *et al.*, 2002). Further, loss of TIPE resulted in the downregulation of Cox-2, which plays important role in cellular growth, differentiation and inflammation (Erkanli *et al.*, 2007). c-Myc is an oncogene which functions as a key regulator of cell growth as well as metabolism and Cyclin D1 is involved in the regulation of cell cycle progression (Inoue and Fry, 2015; Miller *et al.*, 2012). Knockout of TIPE also downregulated the expression of c-Myc and Cyclin D1 effectively. In addition, autophagy is a catabolic cellular mechanism in which degradation of dysfunctional components of cell occurs through autophagosomes which in turn allows cell survival even under stress conditions, by maintaining suitable immune homeostatic conditions. LC-3B is considered to be the most effective marker of autophagosome formation (Mortezavi *et al.*, 2017). Notably, in TIPE knockout cells, downregulation in the expression of LC-3B was observed which can be predicted to contribute towards reduced cell proliferation and survival of lung cancer cells. 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 (Hao *et al.*, 2007). Further, p53 is a tumor suppressor protein which regulates diverse cell functions such as promotion of apoptosis,

senescence, reduced cell growth, migration, and invasion. p21 is a target of p53 which plays an important role in inhibiting cell growth and also aids in reducing invasive potential of tumor cells through its interaction with p53 (Kim *et al.*,2017a). Importantly, loss of TIPE expression led to the upregulation of these two tumor suppressors effectively. Emerging evidence suggest that PI3K/Akt/mTOR pathway plays a vital role in oncogenesis and is often found to be activated in lung cancer (Cheng *et al.*, 2014). Aberrations in different messenger molecules of this pathway lead to proliferation, inhibition of apoptosis, angiogenesis and metastasis of tumor cells (Sarris *et al.*, 2012). Therefore, we determined whether TIPE mediated lung cancer has any involvement with this signaling axis. The results showed that knockout of TIPE affected the important components of Akt/mTOR pathway. Loss of TIPE downregulated the expression of Akt1, p-Akt^{S473}, Akt^{T308}, mTOR, p-mTOR^{S2448}, S6 and p-S6$^{S235/236}$. Phosphorylation at two sites, i.e. T308 and S473, the activation domain and C-terminal hydrophobic motif respectively denotes full activation of Akt (West *et al.*, 2003). Further, an upregulation in the expression of PTEN was observed. PTEN, a lipid and protein phosphatase, is a negative regulator of Akt and the loss of its function results in the constitutive activation of Akt (Sarris *et al.*, 2012). Thus, TIPE is found to activate Akt/mTOR signaling pathway which in turn contributes to the pathogenesis of lung cancer. Further, reports suggest that interaction between STAT-3 and Akt signaling pathway plays an important role in tumor development and progression in various cancers (Blando *et al.*, 2011). Constitutive activation of the PI3K/Akt leads to the aberrant activity of NF-κB and STAT-3 (Han *et al.*, 2010; Dan *et al.*, 2008). Our results showed that knock out of TIPE down regulated the expression of NF-κB, p-NF-κB^{S536}, STAT-3 and p-STAT-3^{S727} notably. Altogether, loss of TIPE is found to reduce the proliferation, survival, invasion and migration of lung cancer cells through inactivation

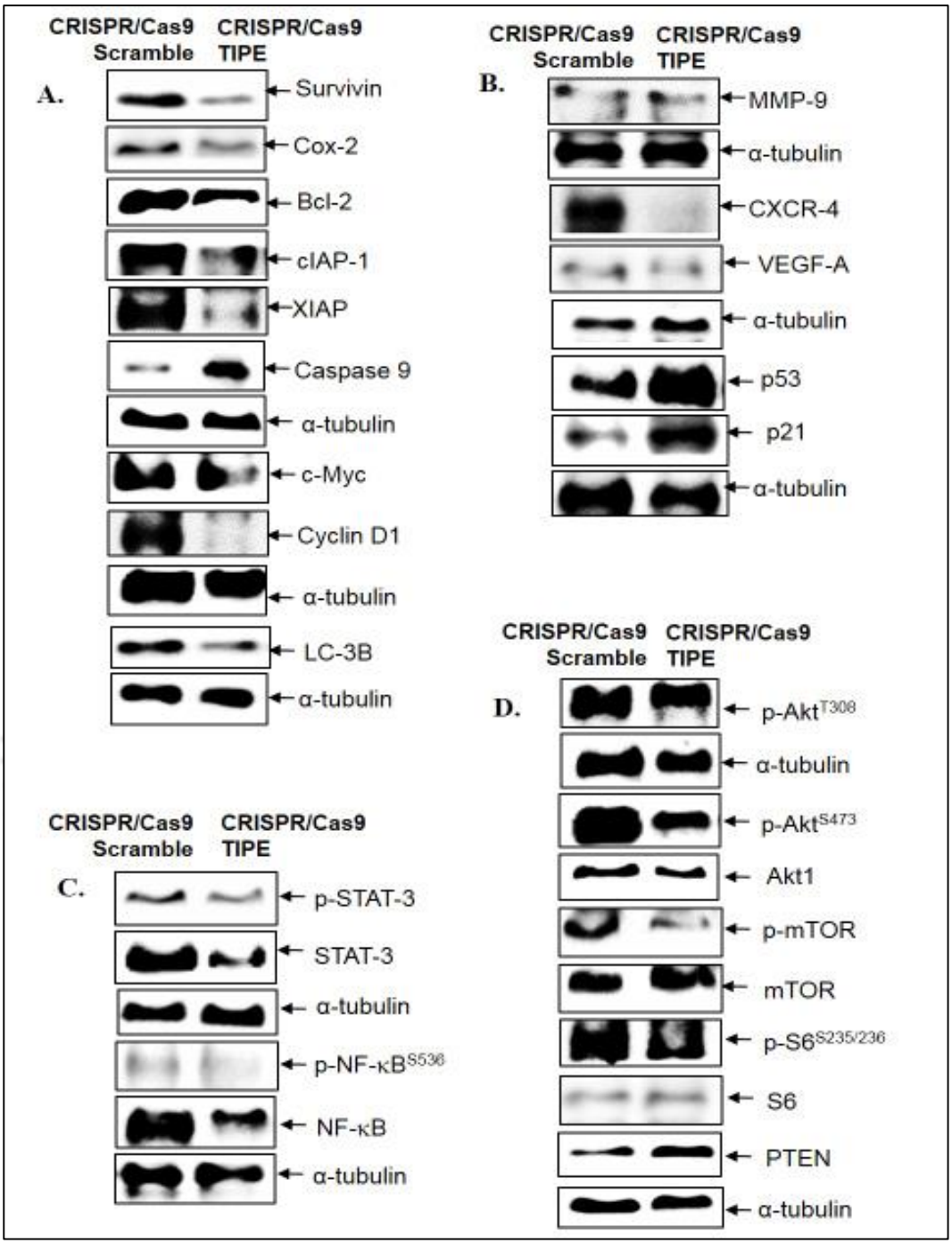

Figure 4.6. Effect of CRISPR/Cas9 mediated knockout of TIPE on different signaling molecules/pathways. A. Effect of TIPE knockout on the expression of proteins involved in cell growth, proliferation, survival and apoptosis regulation; B. Effect of TIPE knockout on the expression of proteins involved in migration, metastasis, angiogenesis and effect on tumor suppressors' p53 and p21, C&D. Effect of TIPE knockout on Akt/mTOR/S6/NF-κB/STAT-3 signaling. α-tubulin was used as loading control.

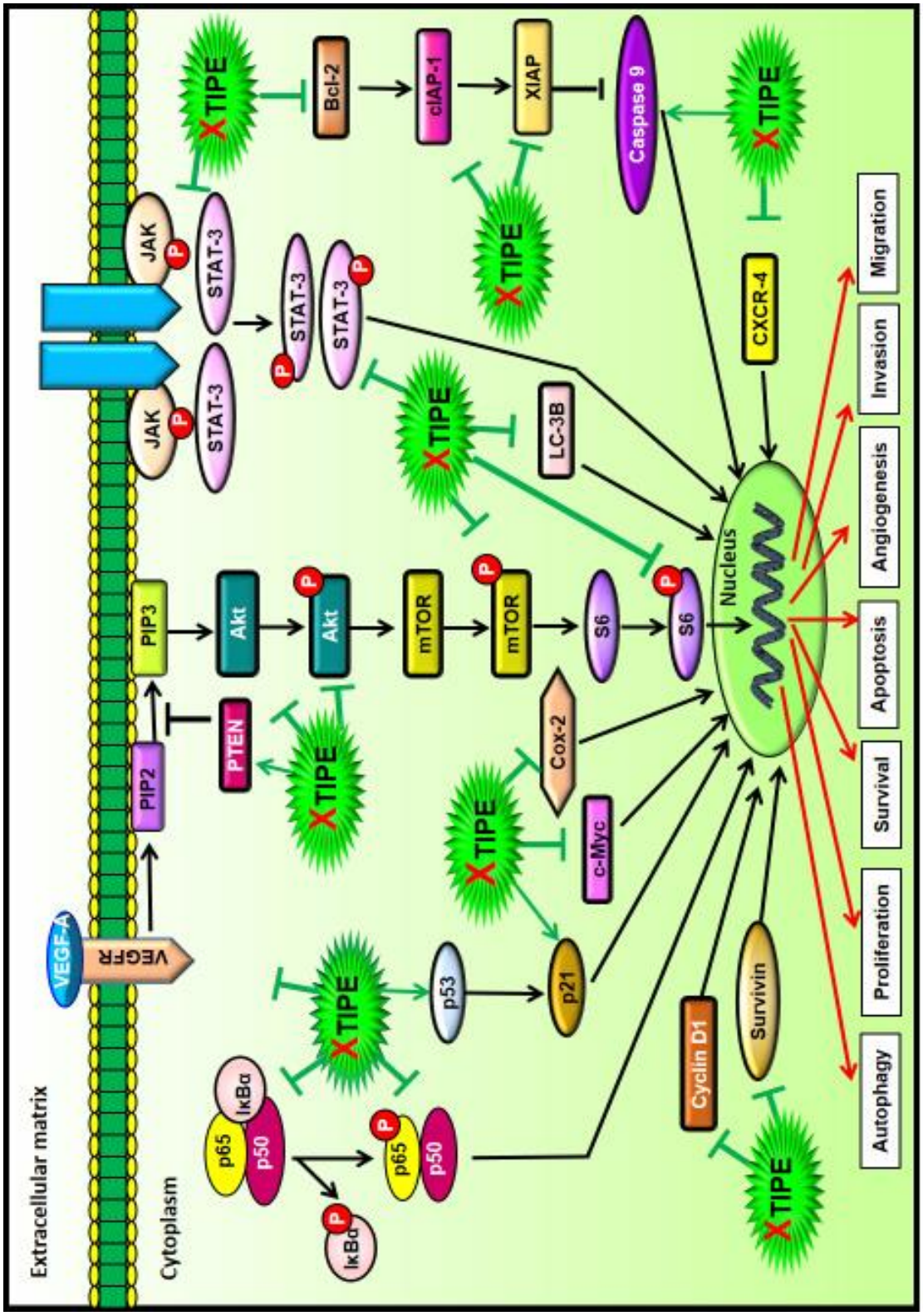

Figure 4.7. Knockout of TIPE modulates Akt/mTOR/S6/NF-κB/STAT-3 signaling, 'XTIPE' denotes CRISPR/Cas9 TIPE.

of Akt/mTOR/S6/NF-κB/STAT-3 signaling (Figure 4.7). Noteworthy, this is the first report which shows the involvement of Akt/mTOR/S6/NF-κB/STAT-3 signaling axis in TIPE mediated lung tumorigenesis.

4.3.7. Effect of TIPE1 knockout in the modulation of Akt/mTOR/S6/STAT-3 signaling

The results of the previous studies revealed TIPE1 to possess anti-tumorigenic effect on human lung cancer cells. The characteristics exhibited by this protein was found to be opposite to those observed in case of the rest three members of TIPE family. In this study, we determined the downstream targets of TIPE1 as well by analyzing the expression of different proteins involved in diverse processes in cancer cells (Figure 4.8). In contrary to TIPE, knockout of TIPE1 upregulated the expression of apoptosis regulatory proteins such as survivin and XIAP. Further, the expression of Caspase 9, which is also involved in apoptosis was found to be downregulated. In addition, TIPE1 knockout cells exerted increased expression of Cox-2 and Cyclin D1, which are involved in cell growth and proliferation, compared to scrambled control cells. Further, knockout of TIPE1 led to the upregulation of the oncogene c-Myc and downregulation of tumor suppressors' such as p53 and p21. Thus, modulation in the expression of all these proteins are responsible for the negative regulation of lung carcinogenesis through TIPE1. In addition, LC-3B, the marker of autophagosomes' formation, has also shown upregulation in NCHI460 cells upon knockout of TIPE1, which in turn might promote lung cancer cell proliferation and survival. Further, knockout of TIPE1 induced the expression of MMP-9 and VEGF-A which are involved in cancer cell migration, metastasis and angiogenesis. Liu and group also reported TIPE1 to be involved in the regulation of MMP-2 and MMP-9, which play important roles in tumor progression and

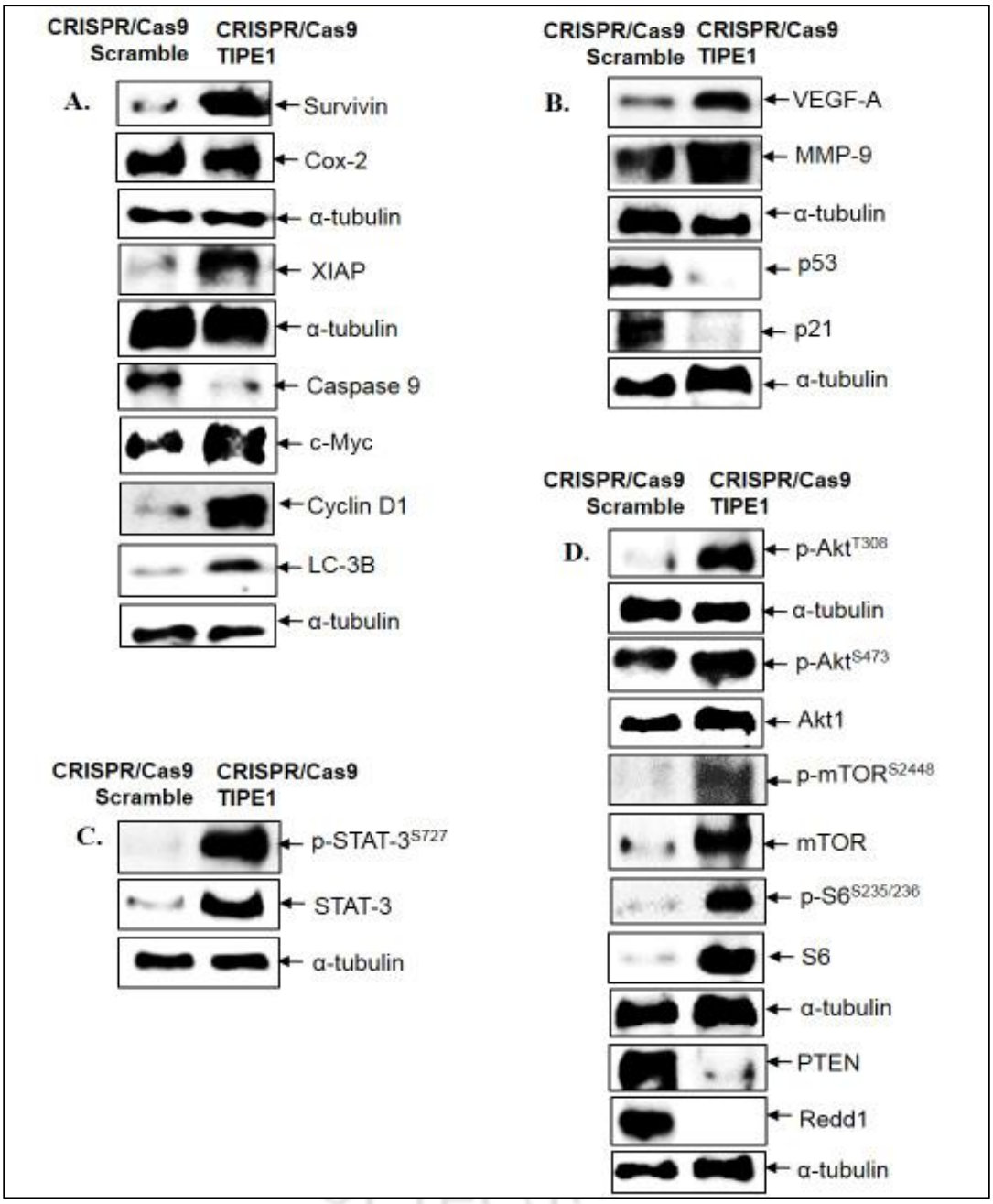

Figure 4.8. Effect of CRISPR/Cas9 mediated knockout of TIPE1 on different signaling molecules/pathways. A. Effect of TIPE1 knockout on the expression of proteins involved in cell growth, proliferation, survival and apoptosis regulation, B. Effect of TIPE1 knockout on the expression of proteins involved in migration, metastasis, angiogenesis and effect on tumor suppressors' p53 and p21, C&D. Effect of TIPE1 knockout on Akt/mTOR/S6/STAT-3 signaling. α-tubulin was used as loading control.

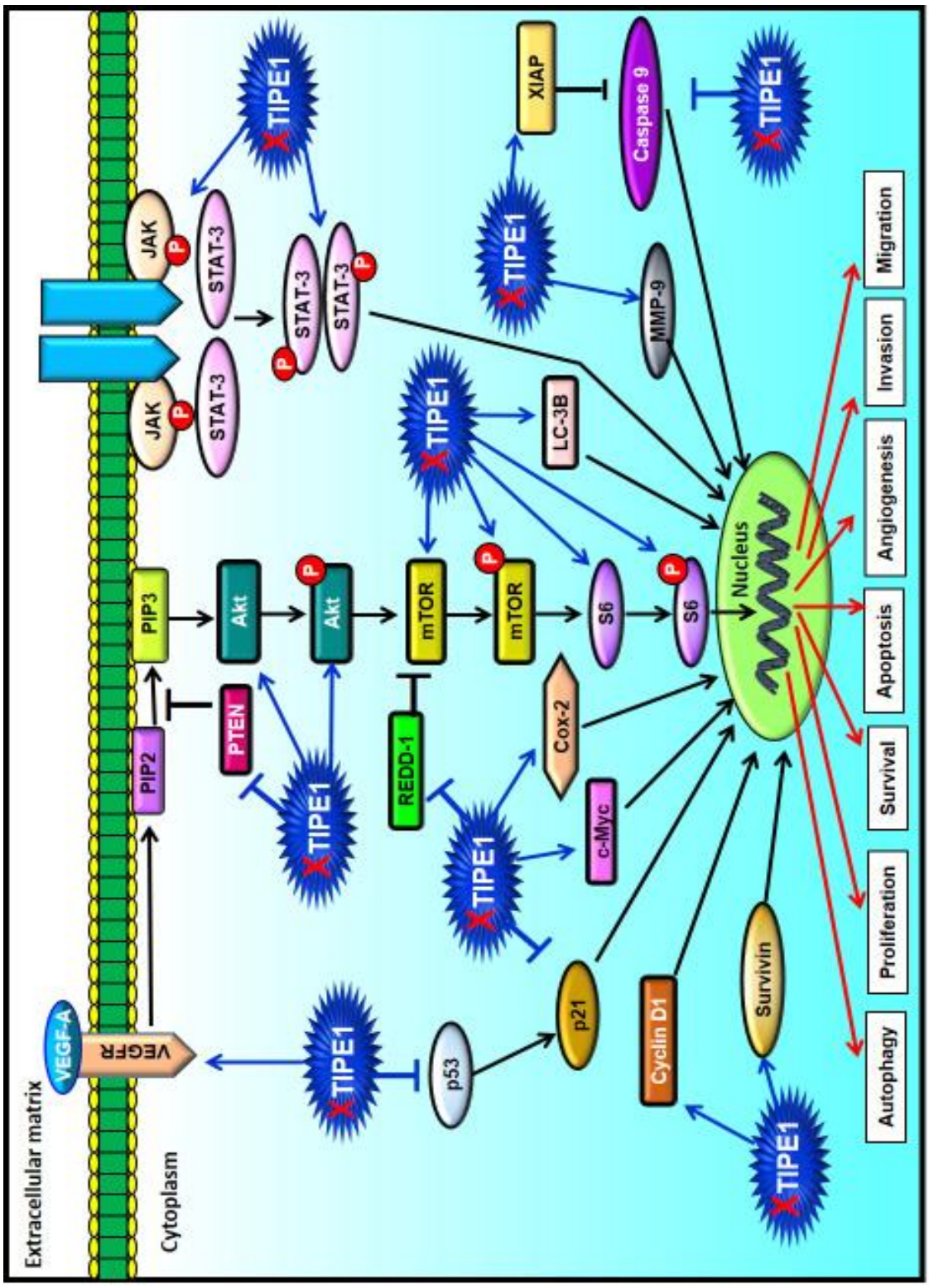

Figure 4.9. Knockout of TIPE1 modulates Akt/mTOR/S6/STAT-3 signaling, 'XTIPE1' denotes CRISPR/Cas9 TIPE1.

EMT in gastric cancer cells (Liu *et al.*, 2018b). In addition, a study conducted by Wu and group 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 (Wu *et al.*, 2017). Loss of TIPE1 was also found to inflect the important components of Akt/mTOR signaling cascade like TIPE, although the effect of both are completely opposite. 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 PTEN and Redd1. Redd1 is a negative regulator of mTOR and it has an inhibitory role on lung cancer cells' invasion. A study carried out by Ha and group showed that oxidative stress-induced TIPE1 caused reduction in mTOR phosphorylation and an increase in autophagy (Ha *et al.*, 2014). However, no study till date reports the involvement of mTOR in TIPE1 regulated lung cancer. Further, knock out of TIPE1 also suppressed the expression of STAT-3 and p-STAT-3^{S727} notably. Collectively, loss of TIPE1 increased the proliferation, survival, invasion and migration of lung cancer cells through activation of Akt/mTOR/S6/STAT-3 signaling axis (Figure 4.9). As evinced from review of literature, TIPE1 and its association with different cancers and other chronic diseases is least studied compared to the rest three members of TIPE family of proteins. Noteworthy, this is the first report which shows the involvement of Akt/mTOR/S6/STAT-3 signaling axis in TIPE1 mediated lung tumorigenesis.

4.3.8. Effect of TIPE2 knockout in the modulation of Akt/mTOR/S6/NF-κB/STAT-3 signaling

TIPE2, the third member of this family is a latterly discovered negative regulator of immunity. Knockout of TIPE2 caused marked decrease in the proliferation, survival, invasion and migration of human lung cancer cells. To decipher the underlined molecular mechanism of action of TIPE2 mediated lung cancer pathogenesis, we studied the expression of different signaling molecules involved in lung cancer (Figure 4.10). Our results showed that knockout of TIPE2 resulted in the down regulation of proteins involved in cell growth, survival, proliferation and regulation of apoptosis such as Cox-2, survivin, cIAP-1, XIAP and Cyclin D1 and upregulated the expression of Caspase 9. Further, loss of TIPE2 also downregulated the expression of CXCR-4 and MMP-9, which are involved in cancer cell invasion, migration and metastasis. Our results also showed that knockout of TIPE2 led to the upregulation of tumor suppressors' p53 and p21. In addition, autophagosomes' formation marker namely LC-3B showed notable downregulation in TIPE2 knockout NCHI460 cells. The similar findings were observed in case of TIPE as well. In addition, similar to TIPE, knockout of TIPE2 also resulted in down regulation of Akt1, p-Akt^{S473}, p-Akt^{T308}, mTOR, p-mTOR^{S2448}, S6 and p-S6$^{S235/236}$, the critical components of Akt/mTOR signaling cascade. In addition, upregulation in PTEN, the inhibitor of p-Akt was also observed. The involvement of Akt in TIPE2 mediated carcinogenesis is also observed in gastric cancer and prostate cancer. However in those studies, TIPE2 was reported to exert opposite effect of that obtained in our study. For instance, in gastric cancer, TIPE2 overexpression suppressed the growth of gastric cancer cells via reduction of Akt and ERK1/2 (Zhu *et al.,* 2016). Consistent with the previous study, Wu and group also showed that TIPE2 suppressed the metastasis of gastric cancer cells through inhibition of Akt (Wu *et al.,* 2016). In case of prostate cancer cells as well, upregulation of TIPE2

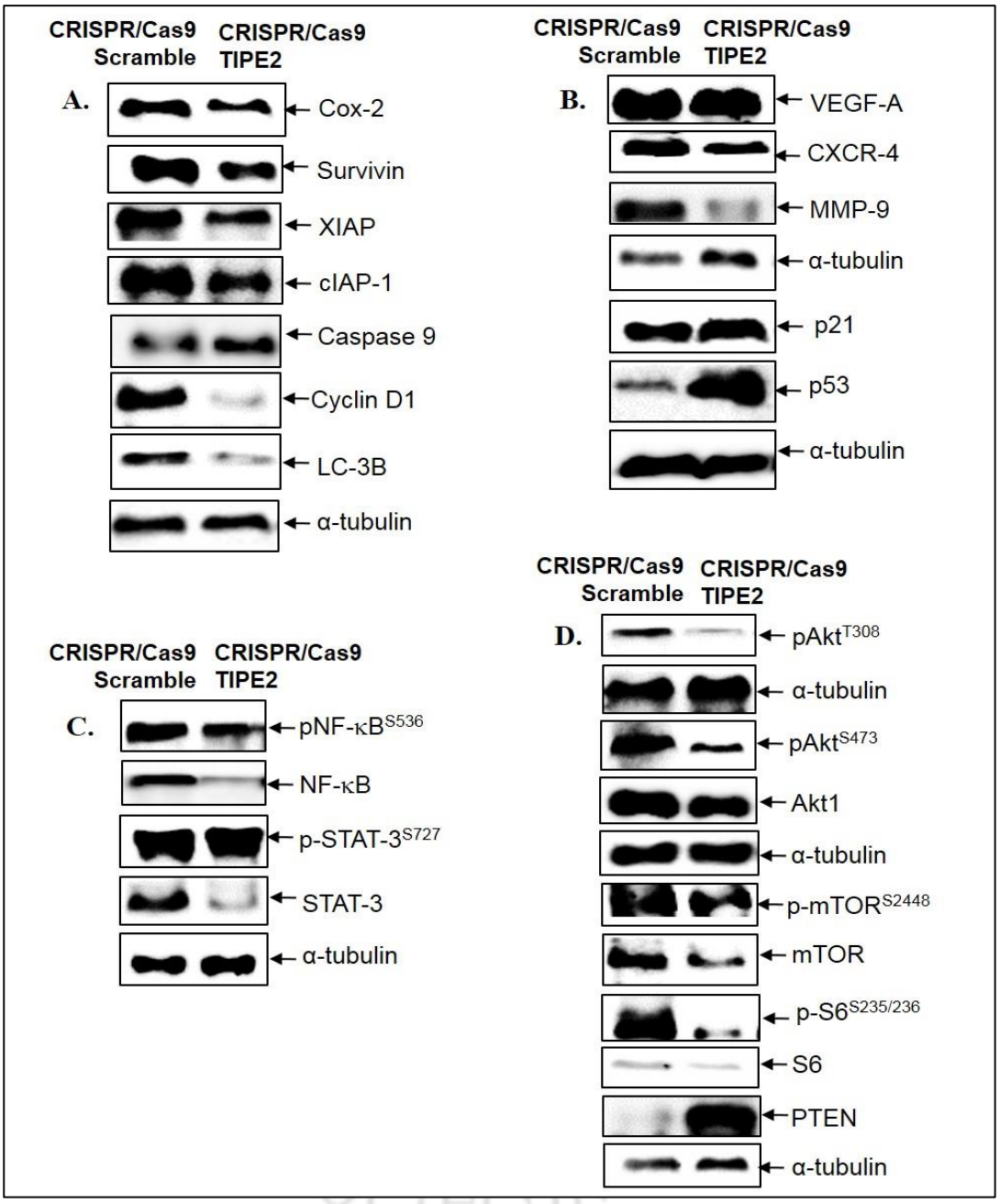

Figure 4.10. Effect of CRISPR/Cas9 mediated knockout of TIPE2 on different signaling molecules/pathways. A. Effect of TIPE2 knockout on the expression of proteins involved in cell growth, proliferation, survival and apoptosis regulation, B. Effect of TIPE2 knockout on the expression of proteins involved in migration, metastasis, angiogenesis and effect on tumor suppressors p53 and p21, C&D. Effect of TIPE2 knockout on Akt/mTOR/S6/NF-κB/STAT-3 signaling. α-tubulin was used as loading control.

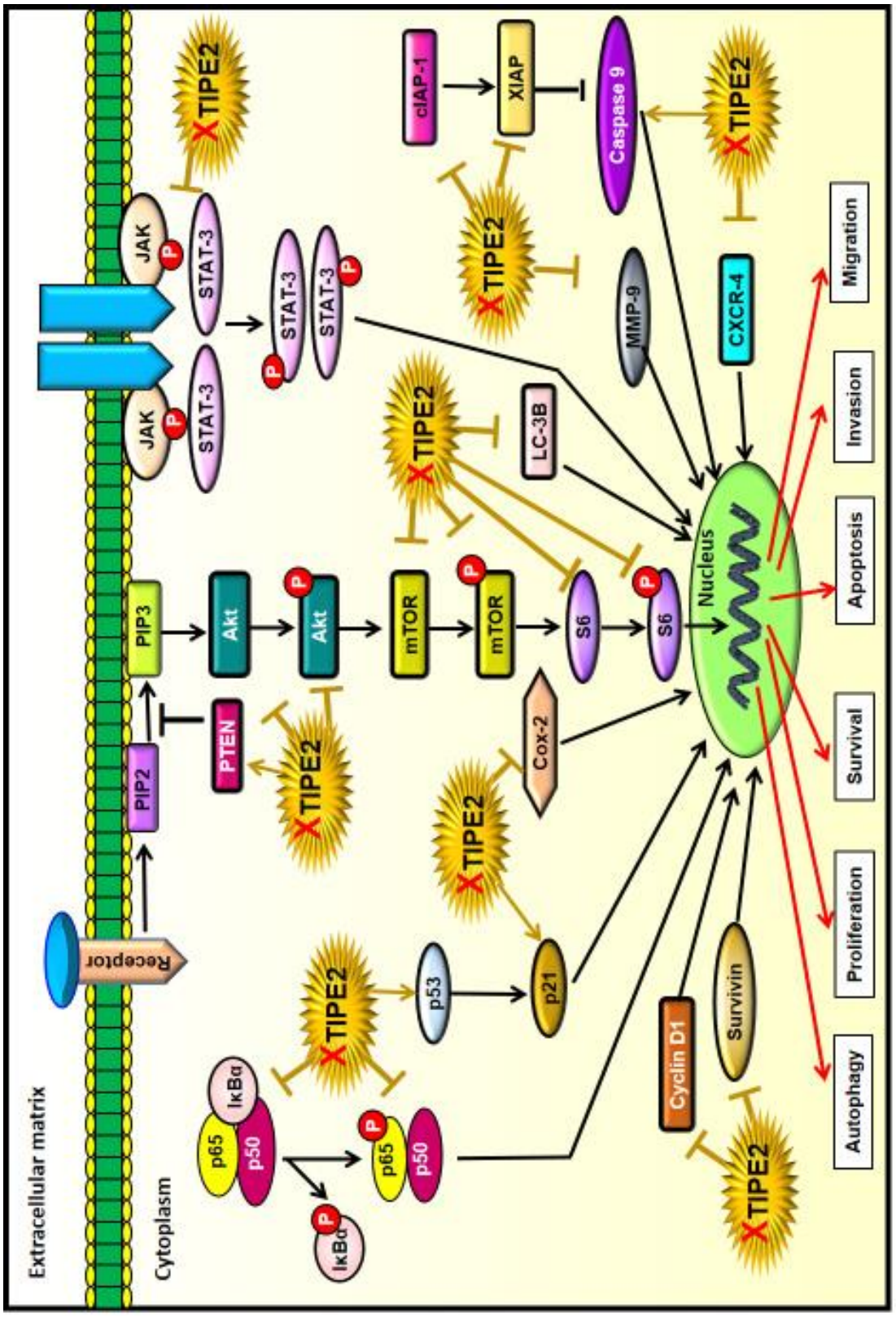

Figure 4.11. Knockout of TIPE2 modulates Akt/mTOR/S6/NF-kB/STAT-3 signaling, 'XTIPE2' denotes CRISPR/Cas9 TIPE2.

caused suppression of migration, invasion, and the process of EMT via blocking of the PI3K/Akt signaling pathway (Lu *et al.,* 2016). Further, we have observed that TIPE2 knockout NCIH460 cells exerted reduced expression of NF-κB, p-NF-κB^{S536} and STAT-3. However, the effect of this knock out on p-STAT-3^{S727} was found to be not significant and hence might need to be determined at other phosphorylation sites. A study conducted on HCC also showed the involvement of NF-κB in TIPE2 mediated carcinogenesis. TIPE2 was found to suppress HCC metastasis induced by TNF-α through inhibition of NF-κB and ERK1/2 (Zhang *et al.,* 2015a). Thus, our findings clearly imply that knock out of TIPE2 led to reduced proliferation, survival, invasion and migration of lung cancer cells via downregulation of Akt/mTOR, S6, NF-κB and STAT-3 signaling and their downstream targets which are involved in diverse cellular processes linked with lung cancer (Figure 4.11). This is the first report which shows the involvement of mTOR, S6, NF-κB and STAT-3 in TIPE2 mediated lung carcinogenesis.

4.3.9. Effect of TIPE3 knockout in the modulation of Akt/mTOR/S6/NF-κB/STAT-3 signaling

TIPE3, the newest member of the TIPE family is reported to be upregulated in several human cancers such as cervical cancer, colon cancer, esophageal cancer, and lung cancer (Bordoloi *et al.,* 2018). TIPE3 is reported as a potent breast cancer metastasis biomarker as it promoted the metastasis of breast cancer cells effectively, augmented the level of uPA and MMP-2, and also activated NF-κB and Akt signaling pathways (Lian *et al.,* 2017). In addition, as mentioned above, stable overexpression of plasma membrane localized TIPE3 with a C-terminal flag, induced the growth and migration of NSCLC cells though activation of the ERK and Akt pathway (Wang *et al.,* 2018a).

In line with these studies, we also found the involvement of Akt pathway in TIPE3 mediated pathogenesis of lung cancer. In case of TIPE3 knockout NCIH460 cells, we observed downregulation of vital constituents of Akt/mTOR signaling cascade such as Akt, p-Akt^{S473}, p-Akt^{T308}, mTOR, p-mTOR^{S2448}, S6 and p-S6$^{S235/236}$. Further, upregulation in the expression of PTEN, a negative regulator of Akt was also observed upon knockout of TIPE3. Aforementioned, constitutive activation of the PI3K/Akt leads to aberrant activity of NF-κB and STAT3. Therefore, we evaluated the effect of Akt inactivation on NF-κB ad STAT-3 activity as well in TIPE3 knockout NCIH460 cells. The results showed that knockout of TIPE3 down regulated the expression of NF-κB, p-NF-κB^{S536}, STAT-3 and p-STAT-3^{S727} as well. This study for the first time shows the involvement of NF-κB and STAT-3 in the tumorigenic effect mediated by TIPE3 in lung cancer cells. Further analyzing the effect on other downstream targets in lung cancer cells after the knockout of TIPE3, it was observed that loss of TIPE3 caused marked downregulation in the expression of diverse proteins involved in cell proliferation, survival, apoptosis regulation, invasion, migration and metastasis such as Cox-2, survivin, cIAP-1, XIAP, Cyclin D1, CXCR-4, MMP-9 and VEGF-A. Additionally, an increase in the expression of Caspase 9, which is also an apoptosis regulatory protein was observed. Unlike TIPE and TIPE2, knockout of TIPE3 did not cause any alteration in the expression of tumor suppressors' p53 and p21 implying non-involvement of these two proteins in TIPE3 mediated lung tumorigenesis (Figure 4.12). Taken together, loss of TIPE3 is found to inhibit the critical hallmarks of cancer such as proliferation, survival, invasion, migration and metastasis through inactivation of Akt/mTOR/S6/NF-κB/STAT-3 signaling axis in NCIH460 lung cancer cells (Figure 4.13). In other words, TIPE3 exerts its tumorigenic effect through modulation of Akt/mTOR/S6/NF-κB/STAT-3 signaling in human lung cancer.

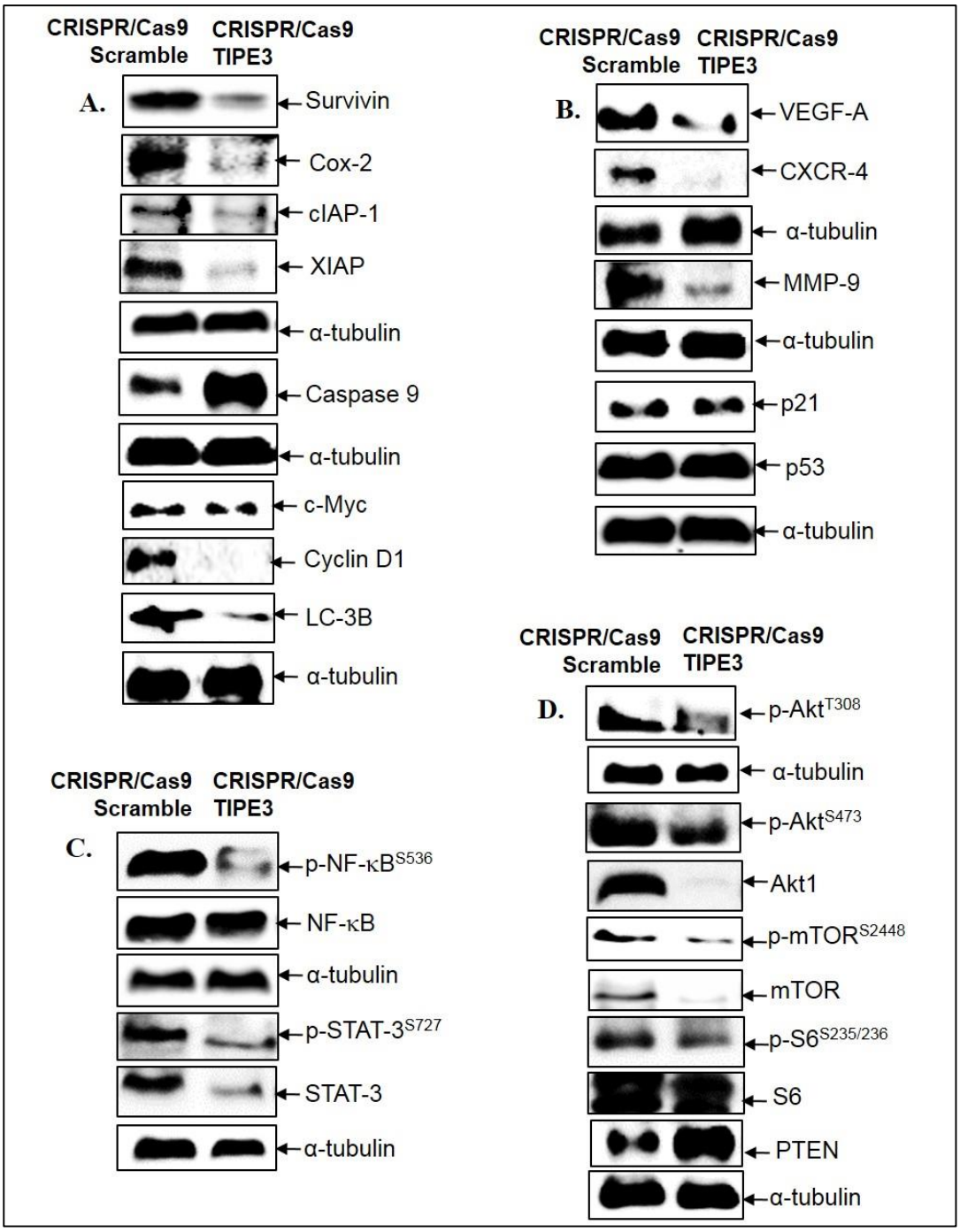

Figure 4.10. Effect of CRISPR/Cas9 mediated knockout of TIPE3 on different signaling molecules/pathways. A. Effect of TIPE3 knockout on the expression of proteins involved in cell growth, proliferation, survival and apoptosis regulation, B. Effect of TIPE3 knockout on the expression of proteins involved in migration, metastasis, angiogenesis and effect on tumor suppressors p53 and p21, C&D. Effect of TIPE3 knockout on Akt/mTOR/S6/NF-κB/STAT-3 signaling. α-tubulin was used as loading control.

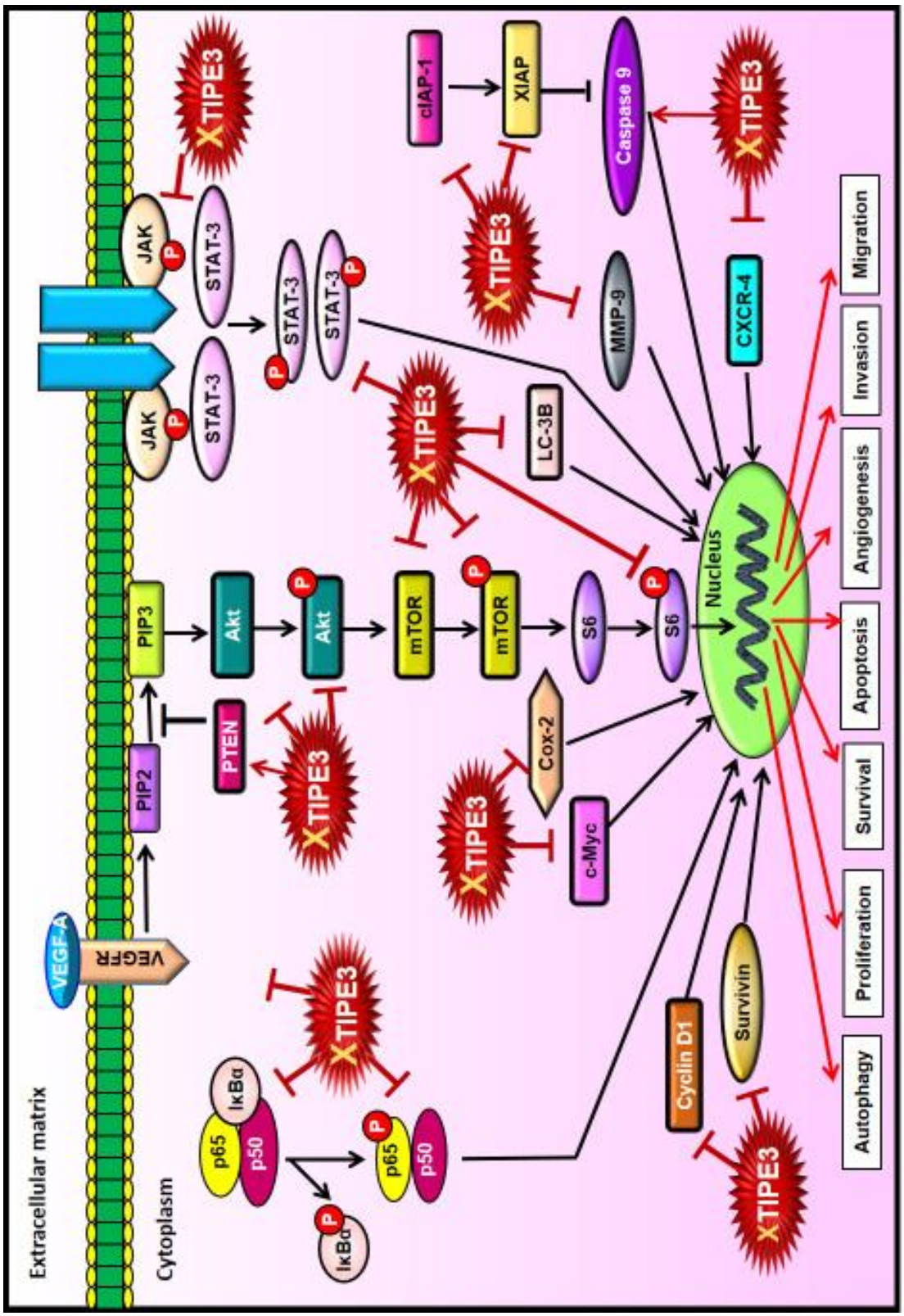

Figure 4.13. Knockout of TIPE3 modulates Akt/mTOR/S6/NF-kB/STAT-3 signaling 'XTIPE3' denotes CRISPR/Cas9 TIPE3.

4.3.10. Role of TIPE, TIPE2 and TIPE3 in tobacco mediated lung carcinogenesis

In the previous two chapters, we have discussed that TIPE, TIPE2 and TIPE3 might be involved in tobacco mediated lung carcinogenesis. In the immunohistochemical analysis, TIPE, TIPE2 and TIPE3 were found to be upregulated in tissue samples of malignant females and males compared to normal females and males respectively. As men smoke nearly five times more than women worldwide, therefore, it was presumed that TIPE, TIPE2 and TIPE3 might be involved in tobacco induced lung carcinogenesis. Further, high expression of TIPE, TIPE2 and TIPE3 in the tissue samples of lower age group patients compared to higher age groups, among which consumption rate of tobacco has been reported to be relatively less provided another strong indication of their involvement in tobacco induced lung carcinogenesis. Our hypothesis was further strengthened in the third chapter where we have shown that treatment of human lung cells with the different tobacco components resulted in significant upregulation of *TIPE*, *TIPE2* and *TIPE3*. Therefore, it is presumed that deregulation of TIPE, TIPE2 and TIPE3 might serve as one of the key molecular events in the development and progression of tobacco induced lung cancer. To confirm the same, we treated TIPE, TIPE2 and TIPE3 knockout NCIH460 cells with four different tobacco components such as NNK, NNN, nicotine and BaP and their effect on the proliferation, survival and migration were studied. In addition, we identified the associated downstream targets as well.

4.3.10.1. Effect of tobacco components on the proliferation of TIPE, TIPE2 and TIPE3 knockout lung cancer cells

Aforementioned, NNK is a potent lung carcinogen. The binding of NNK and NNN to the nAChR induces the cell proliferation, survival, migration, and invasion of tumor

cells (Xue *et al.*, 2014). NNK was found to induce pulmonary adenocarcinoma (PAC) through enhanced cellular growth and proliferation with the involvement of Wnt/*β*-catenin signaling (Zheng *et al.*, 2011). Further, NNK induced proliferation of NSCLC cells was found to be associated with enhanced Cyclin D1 expression (Tsurutani *et al.*, 2005). NNK also stimulated Bcl-2 phosphorylation at serine 70 and c-Myc phosphorylation at threonine 58 and serine 62 concurrently which ultimately resulted in enhanced survival, proliferation, and chemoresistance of human lung cancer cells (Du *et al.*, 2018). Nicotine is also reported to induce proliferation and angiogenesis in varied cellular models (Dasgupta *et al.*, 2006). It promoted the survival and proliferation of lung cancer cells via activation of PKC/Raf/MEK/ERKs cascade (Deng *et al*., 2014). Nicotine induced the proliferation and EMT of NSCLC cells through downregulation of miR-99b and miR-192 (Du *et al.*, 2018). Further, a feedback loop between α5-nAChR and STAT-3 as well as nAChRs containing the α7 or α9 subunits were also reported to contribute to nicotine-induced lung cancer cell proliferation (Zhang *et al.*, 2017b; Mucchietto *et al.*, 2018). In addition, BaP was also found to promote the proliferation of lung cancer cells effectively (Wang *et al.*, 2015c). Therefore, in order to see the effect of NNK, NNN, nicotine and BaP on the proliferation of NCIH460 human lung cancer cells after individual knockout of TIPE, TIPE2 and TIPE3, MTT assay was performed (Figure 4.14). Upon treatment with all the four tobacco components separately, there was a decrease in the proliferation of CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells compared to scrambled control cells treated with the respective components. In the case of CRISPR/Cas9 TIPE3 cells treated with NNK, NNN, nicotine and BaP, highest reduction in proliferation was observed. In case of tobacco components' treated

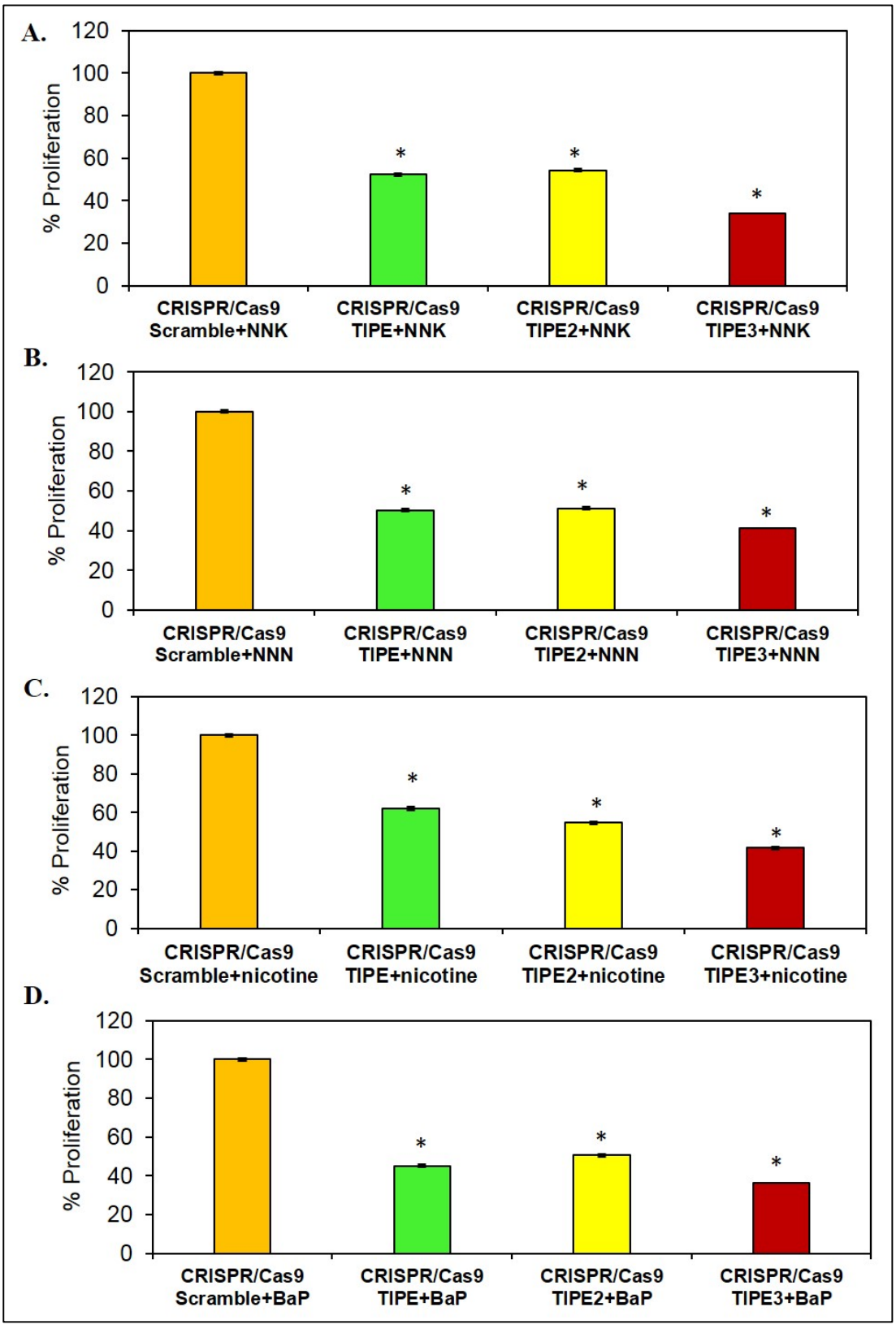

Figure 4.14. Effect of TIPE, TIPE2 and TIPE3 in tobacco induced proliferation of lung cancer cells. A-D. Percent proliferation in NNK, NNN, nicotine and BaP treated TIPE, TIPE2 and TIPE3 knockout cells evaluated using MTT assay. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

CRISPR/Cas9 TIPE and CRISPR/Cas9 TIPE2 cells as well, significant reduction in the proliferation was seen. However, no significant difference in the inhibition of proliferation among them was observed. Thus, these results suggest that TIPE, TIPE2 and TIPE3 are involved in the positive regulation of tobacco induced proliferation of lung cancer cells.

4.3.10.2. Effect of tobacco components on the clonogenic potential of TIPE, TIPE2 and TIPE3 knockout lung cancer cells

Tobacco components namely NNK and nicotine were reported to induce Akt-dependent proliferation and NF-κB-dependent survival of tumor cells (Tsurutani *et al.*, 2005). Further, tobacco-carcinogen transformed human bronchial epithelial cells have been reported to exert enhanced activation of the PI3K/Akt pathway which resulted in increased proliferation as well as survival (West *et al.*, 2004). Puliyappadamba and group reported that nicotine induced clonogenic potential in lung cancer cells via up-regulation of p53 and p21 (Puliyappadamba *et al.*, 2010). For determining the effect of NNK, NNN, nicotine and BaP on the clonogenic potential of NCIH460 human lung cancer cells after individual knockout of TIPE, TIPE2 and TIPE3, colony formation assay was performed (Figure 4.15 & 4.16). Similar to the proliferation assay, in the colony formation assay as well, decreased survival fraction of CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells treated with NNK, NNN, nicotine and BaP were observed compared to scrambled control cells treated with the same. In case of treatment with NNK, CRISPR/Cas9 TIPE2 cells showed the highest reduction in survival fraction as denoted by remarkably decreased number of colonies formed; followed by CRISPR/Cas9 TIPE3 and CRISPR/Cas9 TIPE respectively. In NNN treatment; again CRISPR/Cas9 TIPE2 cells were found to exert the least colony

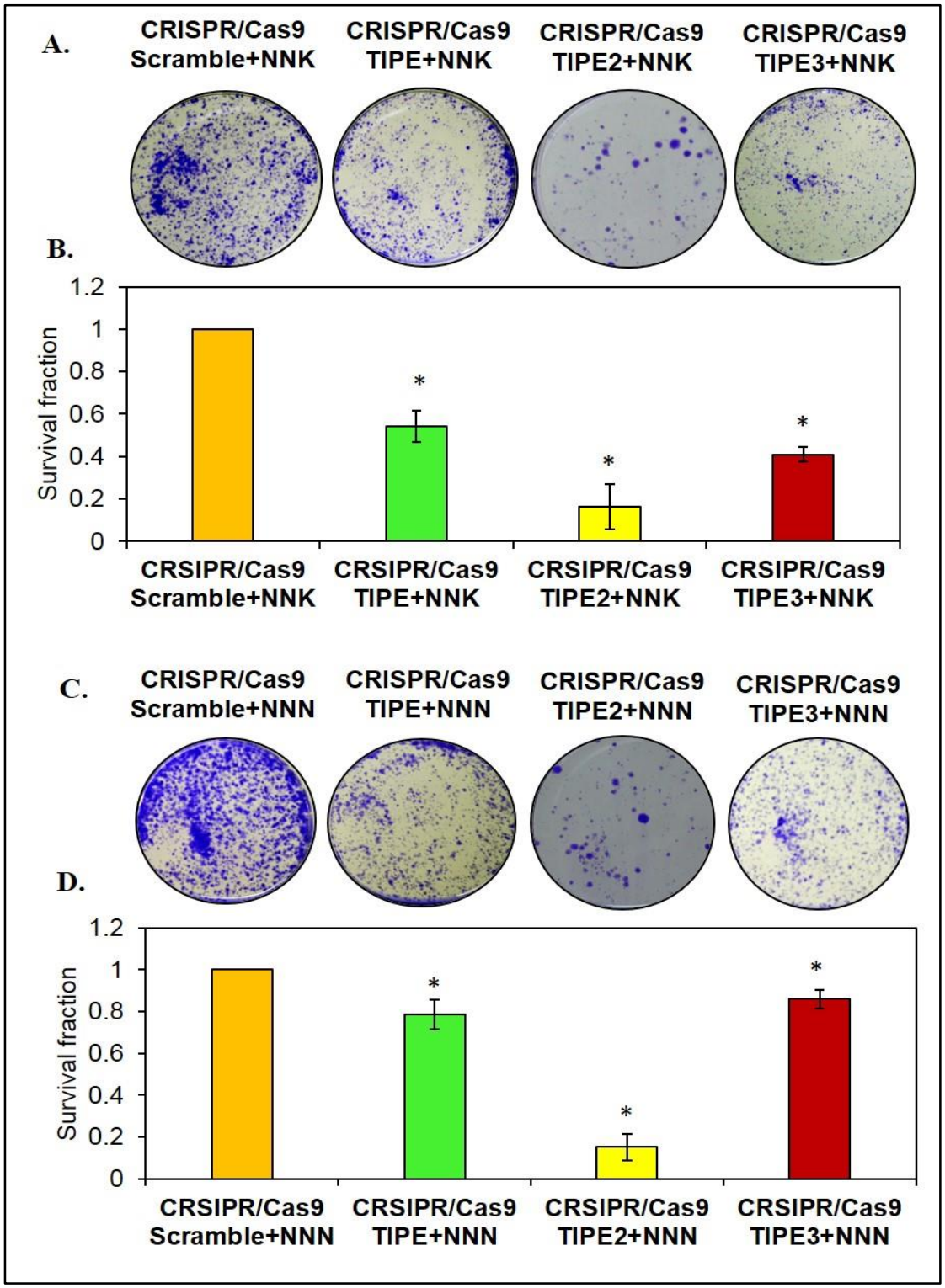

Figure 4.15. Effect of TIPE, TIPE2 and TIPE3 in NNK and NNN induced survival of lung cancer cells. A &C represents the images of the colonies formed in NNK and NNN treated TIPE, TIPE2 and TIPE3 knockout cells along with scrambled control; B & D shows the graphical representation of clonogenic potential of NNK and NNN treated TIPE, TIPE2 & TIPE3 knockout cells in terms of survival fraction compared to scrambled control treated with NNK and NNN. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

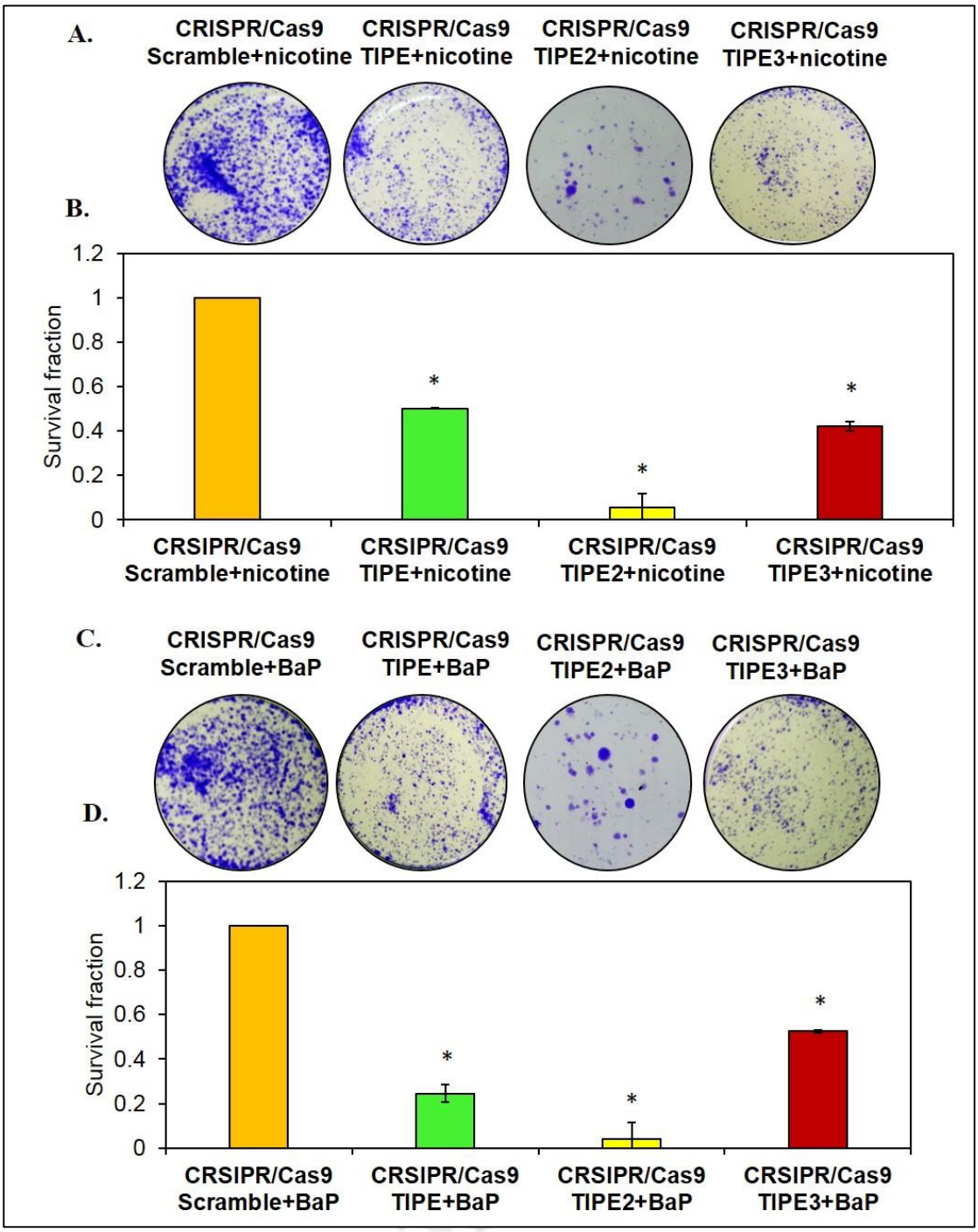

Figure 4.16. Effect of TIPE, TIPE2 and TIPE3 in nicotine and BaP induced survival of lung cancer cells. A &C represents the images of the colonies formed in nicotine and BaP treated TIPE, TIPE2 and TIPE3 knockout cells along with scrambled control; B & D shows the graphical representation of clonogenic potential of nicotine and BaP treated TIPE, TIPE2 & TIPE3 knockout cells in terms of survival fraction compared to scrambled control treated with nicotine and BaP. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

formation and hence is the survival fraction. Further, significantly less survival fraction was observed in case of CRISPR/Cas9 TIPE and CRISPR/Cas9 TIPE3 cells. In case of nicotine and BaP treatment as well, CRISPR/Cas9 TIPE2 cells exhibited highest reduction in the clonogenic potential as the number of the colonies were found to be remarkably less in both the cases. Further, in case of nicotine treatment, survival fraction in CRISPR/Cas9 TIPE and CRISPR/Cas9 TIPE3 cells were found to be almost same. On the other hand, BaP treated CRISPR/Cas9 TIPE cells exerted more reduction in survival fraction compared to CRISPR/Cas9 TIPE3 cells treated with BaP. These results clearly imply that knock out of TIPE, TIPE2 and TIPE3 did not impact proliferation alone, but also reduced the survival fraction of tobacco components' treated TIPE, TIPE2 and TIPE3 knockout cells. Collectively, TIPE, TIPE2 and TIPE3 are involved in the positive regulation of tobacco mediated growth and survival of human lung cancer cells.

4.3.10.3. Effect of tobacco components on the migration potential of TIPE, TIPE2 and TIPE3 knockout lung cancer cells

Tobacco and its components are well known to have significant involvement in cancer cell migration. Chronic exposure to cigarette smoke resulted in the activation of p21 - activated kinase 6 (PAK6) in NSCLC cells, which in turn regulates different processes in cancer including metastasis (Raja *et al.*, 2016). Further, components present in tobacco such as nicotine and NNK are also reported to influence the migration of lung cancer cells. Nicotine was found to induce invasion and migration of NSCLC cells in a α7-receptor and Src-dependent manner (Dasgupta *et al.*, 2009; Zhang *et al.*, 2016a). In addition, NNK induced chemokine CCL20 production, which resulted in the promotion of lung cancer cell proliferation and migration (Wang *et al.*, 2015b). To

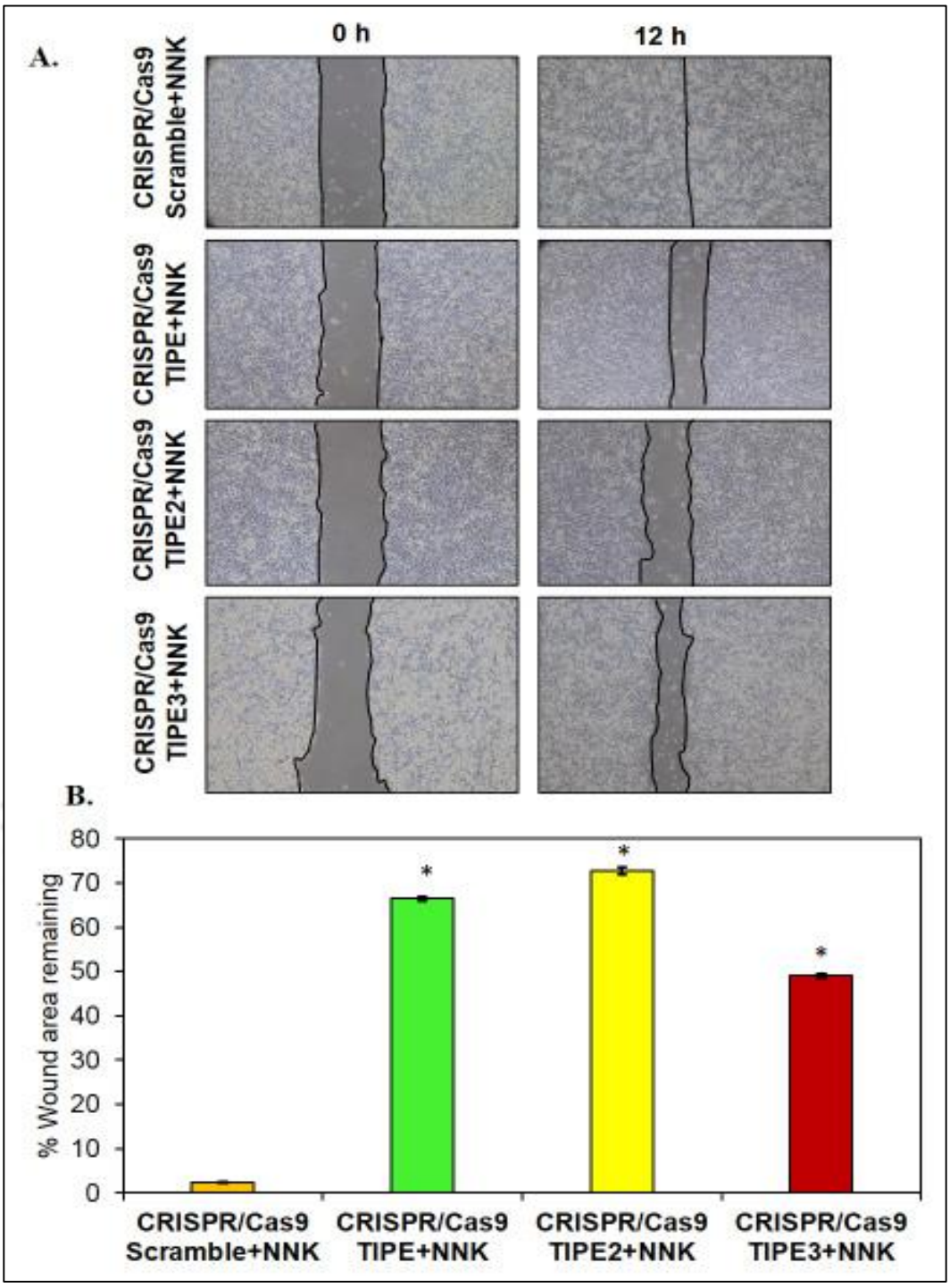

Figure 4.17. Effect of TIPE, TIPE2 and TIPE3 in NNK mediated migration of lung cancer cells. A. Representative images showing the effect of TIPE, TIPE2 and TIPE3 on the migration of NNK treated TIPE, TIPE2 and TIPE3 knockout cells along with NNK treated scrambled control; B. Graphical representation of percent area remaining in NNK treated TIPE, TIPE2 & TIPE3 knockout cells compared to NNK treated scrambled control. Data are represented as Mean±SE, * denotes $p<0.05$ compared to scrambled control.

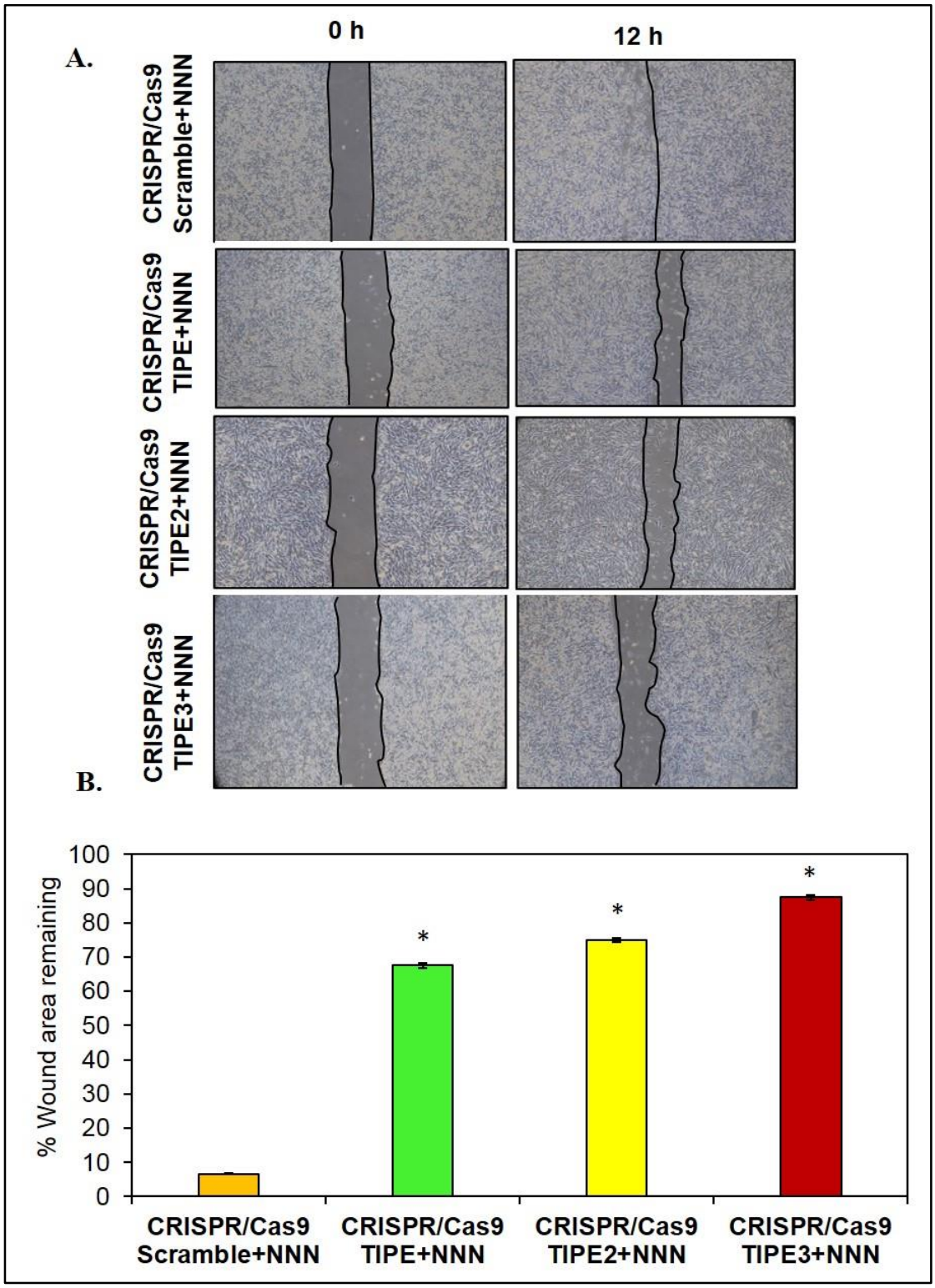

Figure 4.18. Effect of TIPE, TIPE2 and TIPE3 in NNN mediated migration of lung cancer cells. A. Representative images showing the effect of TIPE, TIPE2 and TIPE3 on the migration of NNN treated TIPE, TIPE2 and TIPE3 knockout cells along with NNN treated scrambled control, B. Graphical representation of percent area remaining in NNN treated TIPE, TIPE2 & TIPE3 knockout cells compared to NNN treated scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

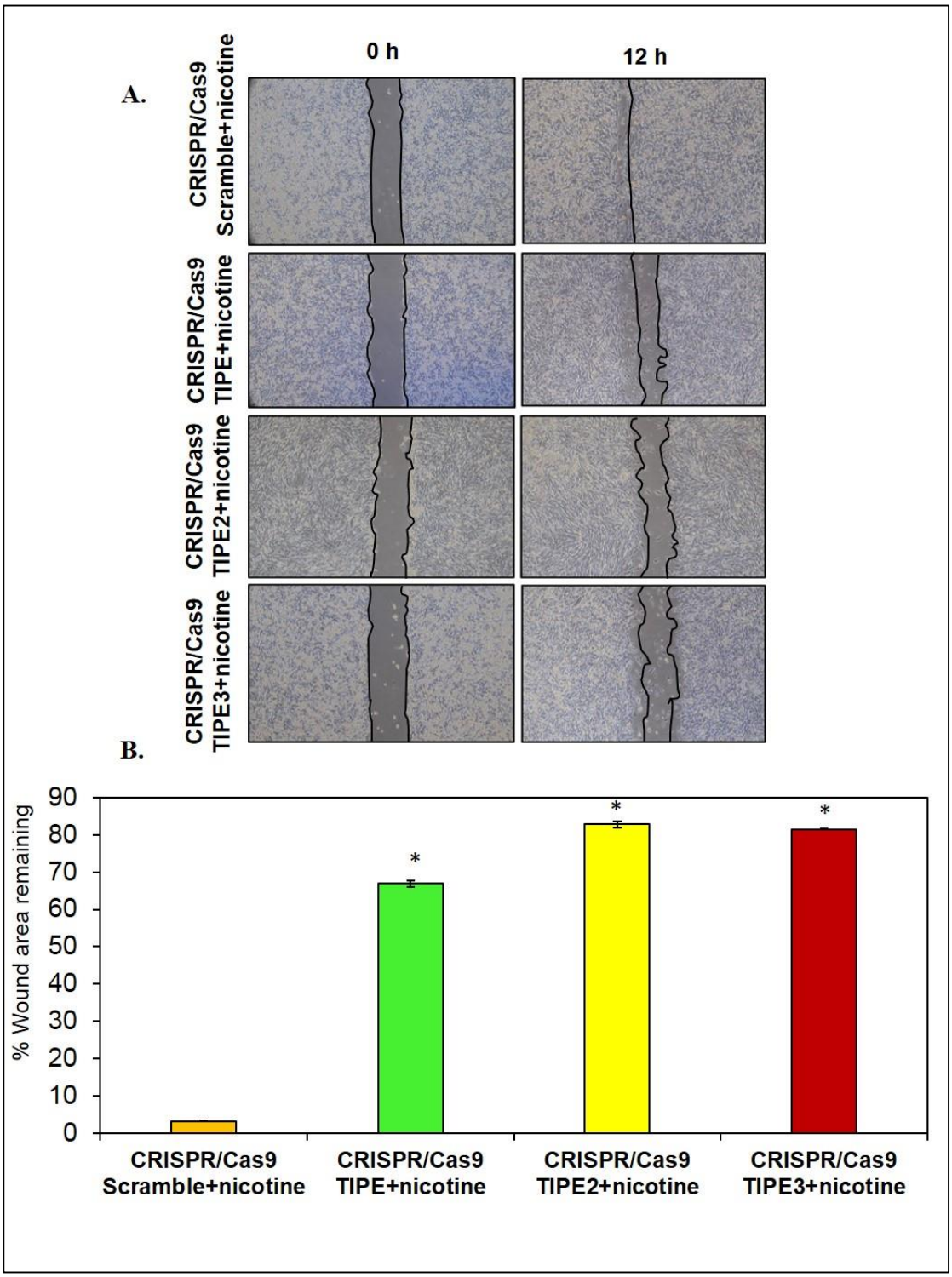

Figure 4.19. Effect of TIPE, TIPE2 and TIPE3 in nicotine mediated migration of lung cancer cells. A. Representative images showing the effect of TIPE, TIPE2 and TIPE3 on the migration of nicotine treated TIPE, TIPE2 and TIPE3 knockout cells along with nicotine treated scrambled control, B. Graphical representation of percent area remaining in nicotine treated TIPE, TIPE2 & TIPE3 knockout cells compared to nicotine treated scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

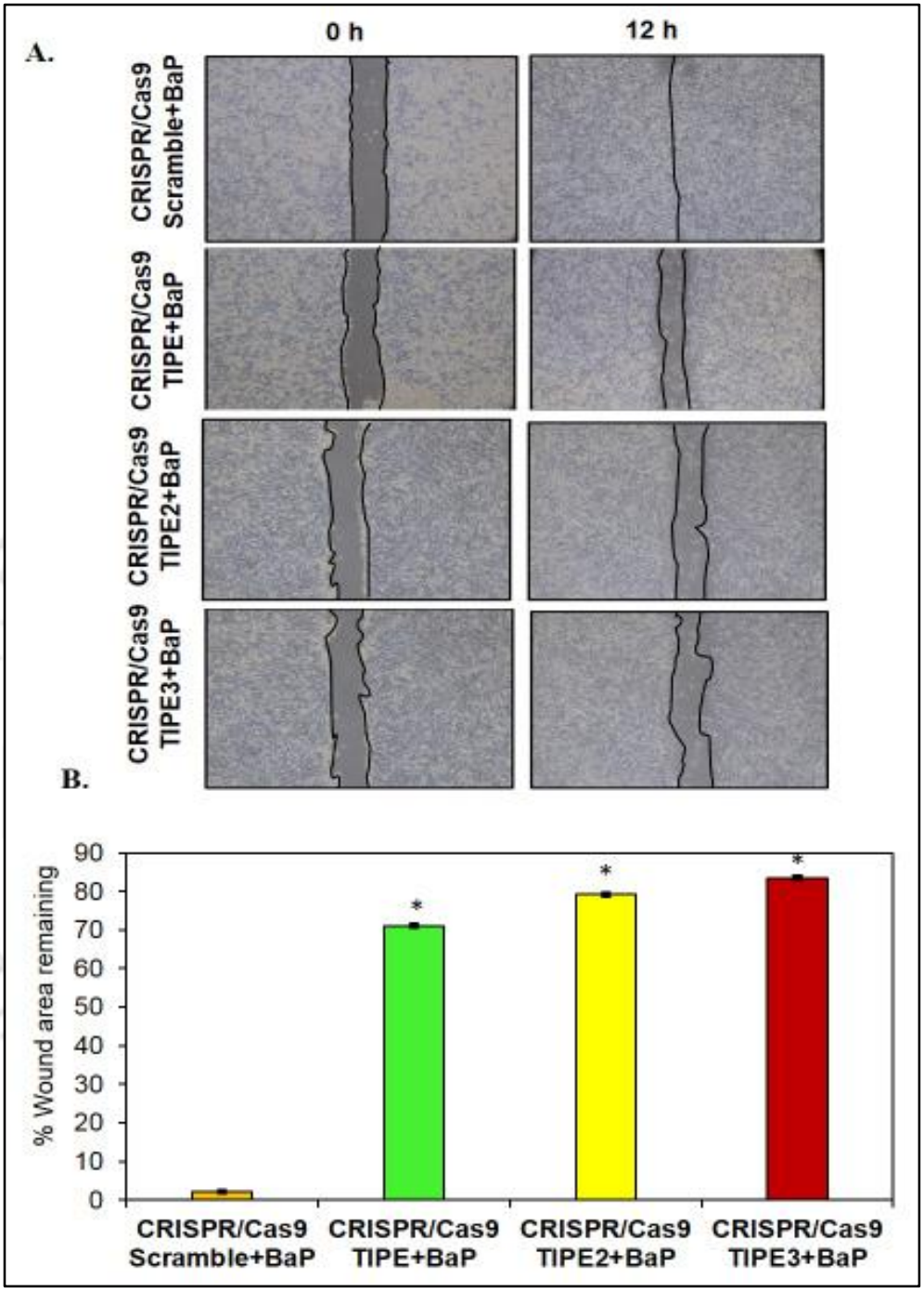

Figure 4.20. Effect of TIPE, TIPE2 and TIPE3 in BaP mediated migration of lung cancer cells. A. Representative images showing the effect of TIPE, TIPE2 and TIPE3 on the migration of BaP treated TIPE, TIPE2 and TIPE3 knockout cells along with BaP treated scrambled control, B. Graphical representation of percent area remaining in BaP treated TIPE, TIPE2 & TIPE3 knockout cells compared to BaP treated scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

measure the migration potential of tobacco components' treated TIPE, TIPE2 and TIPE3 knockout cells, migration assay was performed as mentioned in the 'Materials and methods' section. The results of this assay showed that in the case of tobacco components such as NNK, NNN, nicotine and BaP treated scrambled control cells, there was complete healing of wound at 12 h, whereas the untreated scrambled control as reported earlier was found to take 24 h for the healing of the wound implying the ability of these components to increase the invasion and migration potential of the tumor cells (Figure 4.17, 4.18, 4.19 & 4.20). Notably, in the case of NNK, NNN, nicotine and BaP treatment, CRISPR/Cas9 TIPE, TIPE2 and TIPE3 knockout cells exerted significant inhibition in the wound healing. In the case of NNN and BaP treated CRISPR/Cas9 TIPE3 cells, percent wound area was found to be the highest followed by CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE cells. Further, in NNK and nicotine treated CRISPR/Cas9 TIPE2 cells, percent wound area was found to be the maximum. Thus, these results clearly suggest that TIPE, TIPE2 and TIPE3 are strongly involved in the positive regulation of NNK, NNN, nicotine and BaP induced migration of lung cancer cells.

4.3.10.4. Effect of tobacco components on the modulation of different targets in TIPE, TIPE2 and TIPE3 knockout lung cancer cells

Tobacco induced lung carcinogenesis is driven by alterations in various signal transduction pathways. From the above findings, it can be observed that TIPE, TIPE2 and TIPE3 are involved in the positive regulation of NNK, NNN, nicotine and BaP induced proliferation, survival and migration of lung cancer cells. As mentioned earlier, there are different signaling molecules/pathways associated with these cancer hallmarks. Therefore, in order to decipher the molecular targets through which TIPEs

mediate their tumorigenic effect in tobacco induced lung cancer, we performed Western blot analysis to examine the expression of different gene products involved in diverse processes in cancer cells.

4.3.10.4.1. Effect of tobacco components on the modulation of Akt/S6/STAT-3 signaling in TIPE knockout lung cancer cells

In this study, we determined the mechanism of action of TIPE in NNK, NNN, nicotine and BaP treated TIPE knockout cells. NNK treated TIPE knockout cells showed down regulation in the expression of proteins involved in proliferation, survival and angiogenesis such as Cox-2, survivin and VEGF-A compared to scrambled control cells treated with NNK. Further, upregulation in the expression of p53 tumor suppressor protein was also observed. In addition, NNK treated TIPE knock out cells showed modulation in the important components of Akt signaling cascade as downregulation in Akt, p-Akt^{S473} and p-S6$^{S235/236}$ were observed. 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/S6 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 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 (West *et al.*, 2003). Further, down regulation in STAT-3 and p-STAT-3^{S727} were also observed in NNK treated TIPE knock out cells compared to NNK treated scrambled control. It is well established that

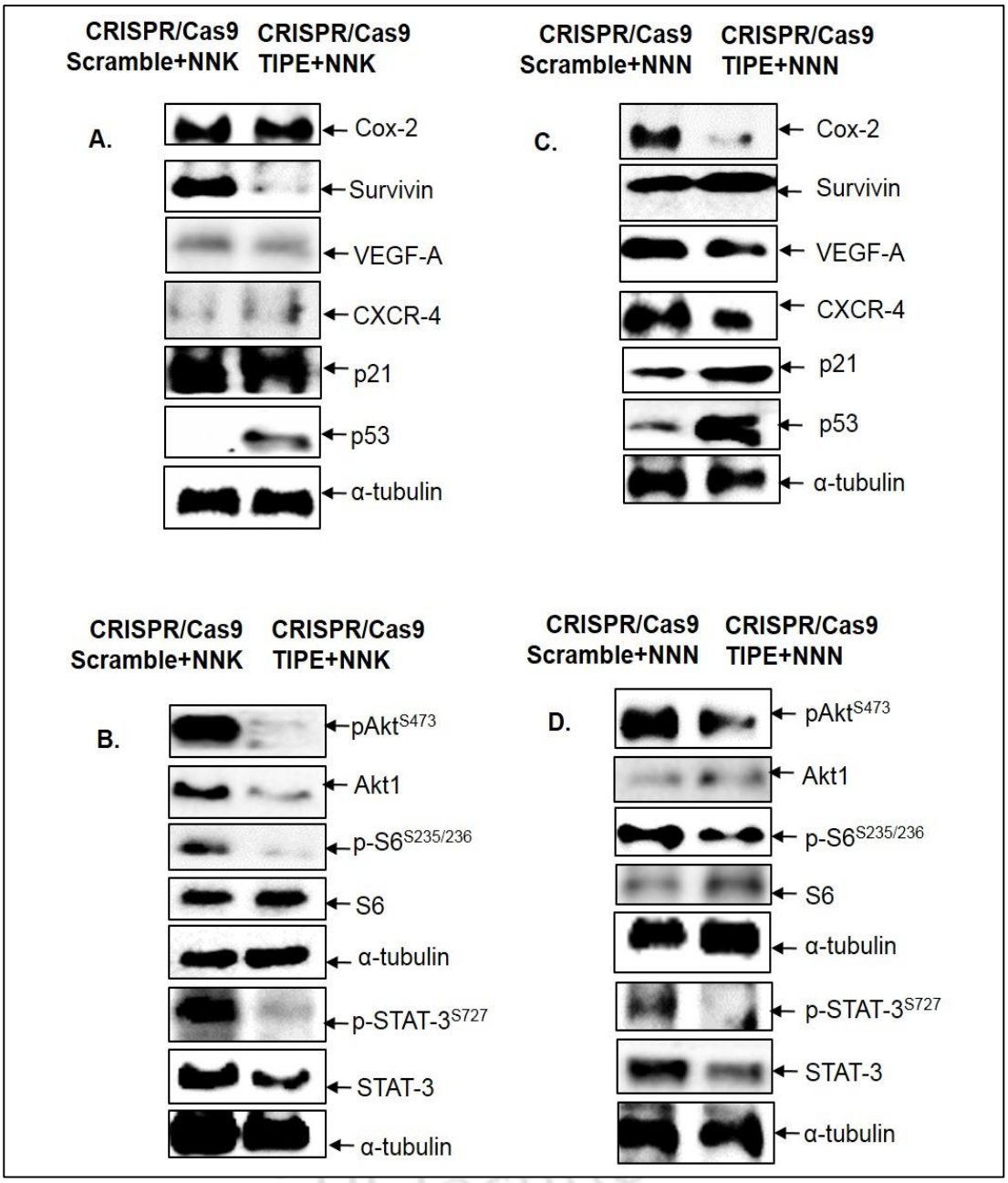

Figure 4.21. Effect on different signaling molecules/pathways in NNK and NNN treated TIPE knockout cells. A. Effect on the expression of proteins involved in cell growth and survival in NNK treated TIPE knockout cells, B. Effect on Akt/S6/STAT-3 signaling in NNK treated TIPE knockout cells, C. Effect on the expression of proteins involved in cell growth and survival in NNN treated TIPE knockout cells, D. Effect on Akt/S6/STAT-3 signaling in NNN treated TIPE knockout cells. α-tubulin was used as loading control.

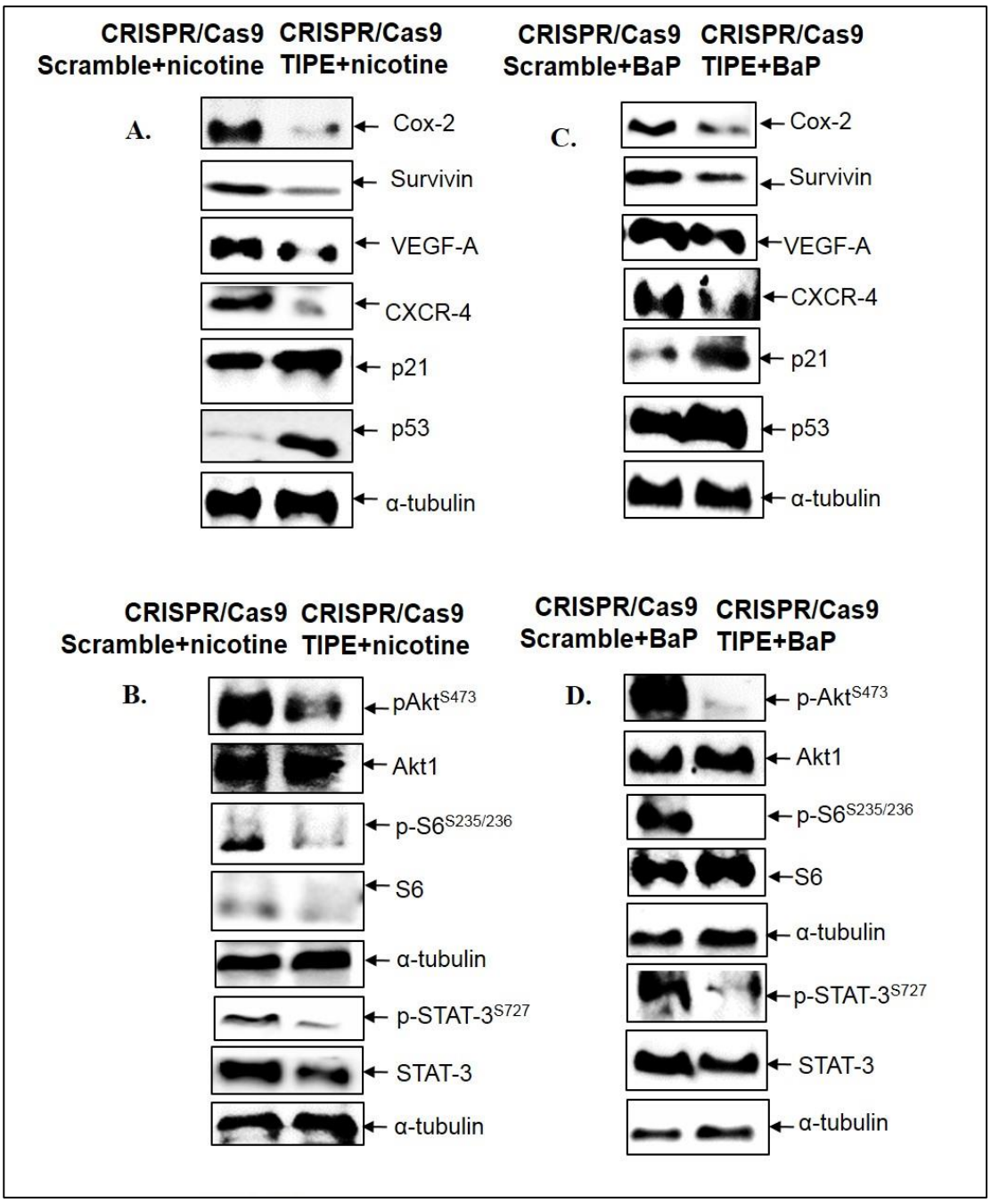

Figure 4.22. Effect on different signaling molecules/pathways in nicotine and BaP treated TIPE knockout cells. A. Effect on the expression of proteins involved in cell growth and survival in nicotine treated TIPE knockout cells, B. Effect on Akt/S6/STAT-3 signaling in nicotine treated TIPE knockout cells, C. Effect on the expression of proteins involved in cell growth and survival in BaP treated TIPE knockout cells, D. Effect on Akt/S6/STAT-3 signaling in BaP treated TIPE knockout cells. α-tubulin was used as loading control.

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 (Nagpal *et al.*, 2002). Additionally, NNN treated TIPE knock out cells were found to exhibit downregulation in the expression of proteins involved in the proliferation, invasion and angiogenesis such as Cox-2, CXCR-4 and VEGF-A. In addition, BaP and nicotine treated TIPE knockout cells also showed downregulation in the expression of Cox-2, survivin, CXCR-4 and VEGF-A. 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$^{S235/236}$, STAT-3 and p-STAT-3^{S727} was observed compared to respective treated scrambled control cells. Taken together, TIPE is involved in the positive regulation of tobacco induced lung carcinogenesis via Akt/S6/STAT-3 signaling axis and its downstream targets (Figure 4.21 &4.22).

4.3.10.6. Effect of tobacco components on the modulation of NF-κB and NF-κB regulated gene products in TIPE2 knockout lung cancer cells

NNK treated TIPE2 knockout cells downregulated the expression of proteins involved in cell proliferation, growth, survival, invasion, migration and metastasis such as Cox-2, Cyclin D1, survivin, CXCR4 and MMP-9. In addition, NNK treated TIPE2 knockout cells showed downregulation of both NF-κB and p-NF-κB^{S536}. NF-κB is a transcription factor that regulates the expression of genes involved in lung carcinogenesis (Cai *et al.*, 2011). Alvira and group reported high levels of nuclear NF-κB in lung cancer tissue, with increased NF-κB activity strongly correlating with more advanced disease in lung adenocarcinoma (Alvira *et al.*, 2014). Exposure to NNK was shown to activate NF-kB which subsequently up-regulates Cyclin D1 and promotes proliferation of both normal

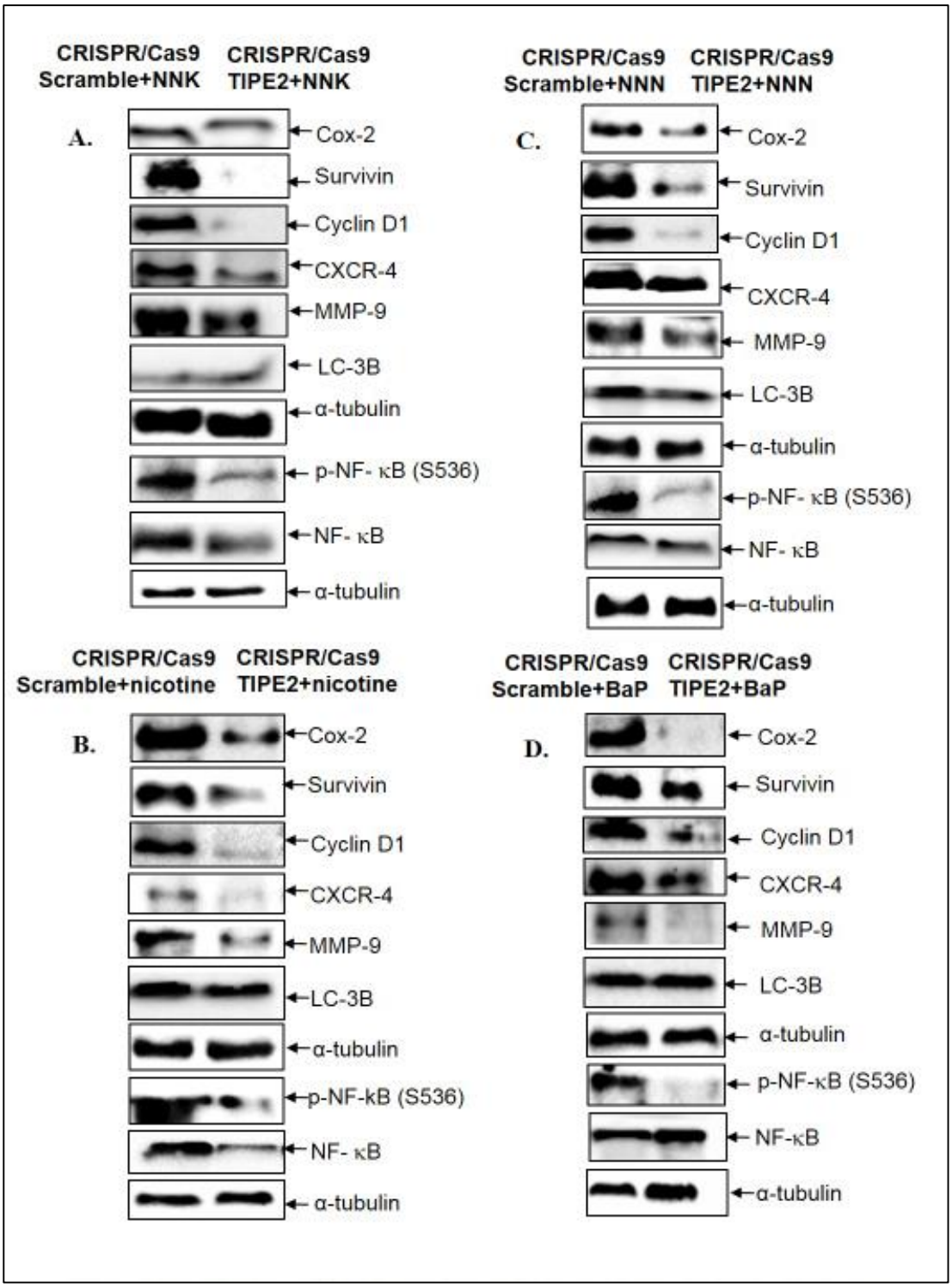

Figure 4.23. Effect on different signaling molecules/pathways in NNK, NNN, nicotine and BaP treated TIPE2 knock out cells. A-D. Effect on NF-κB and NF-κB regulated genes products in NNK, NNN, nicotine and BaP treated TIPE2 knockout cells. α-tubulin was used as loading control.

human bronchial epithelial and small airway epithelial cells (Cai *et al.*, 2011). Additionally, NF-κB can be activated by cigarette smoke and its components such as nicotine and NNK in different NSCLC cells as well (Chen *et al.*, 2011). Similar to NNK treatment, in case of NNN, nicotine and BaP treated TIPE2 knockout cells as well, downregulation in the expression of Cox-2, Cyclin D1, survivin, CXCR-4 and MMP-9 were observed. Further, downregulation in LC-3B, the marker for autophagosomes' formation was also obtained in all the three cases. In addition, NNN and nicotine treated TIPE2 knockout cells showed downregulation in NF-κB and p-NF-κB^{S536} compared to NNN and nicotine treated scrambled control cells. In case of BaP treatment, TIPE2 knockout NCIH460 lung cancer cells exerted downregulation of p-NF-κB^{S536}. Further, downregulation of LC-3B can be predicted to cause reduced cell proliferation and survival of NNN, nicotine and BaP treated TIPE2 knockout lung cancer cells. Taken together, TIPE2 is involved in the positive regulation of tobacco mediated lung carcinogenesis by increasing the proliferation, survival and migration of tobacco components' treated TIPE2 knockout lung cancer cells through modulation of NF-κB and NF-κB regulated gene products which are involved in growth, proliferation, survival, invasion, migration and metastasis of tumor cells (Figure 4.23).

4.3.10.7. Effect of tobacco components on the modulation of Akt/mTOR/S6/STAT-3 signaling in TIPE3 knockout lung cancer cells

TIPE3 knockout cells treated with NNK showed downregulation of c-Myc and LC-3B. c-Myc is involved in the regulation of apoptosis and LC-3B is a marker of autophagosomes formation. In addition, NNK treated TIPE3 knockout cells showed marked downregulation of Akt, p-Akt^{S473}, mTOR, p-mTOR^{S2448}, S6 and p-S6$^{S235/236}$. The P13K/Akt/mTOR signaling pathway is frequently activated in cancer cells which

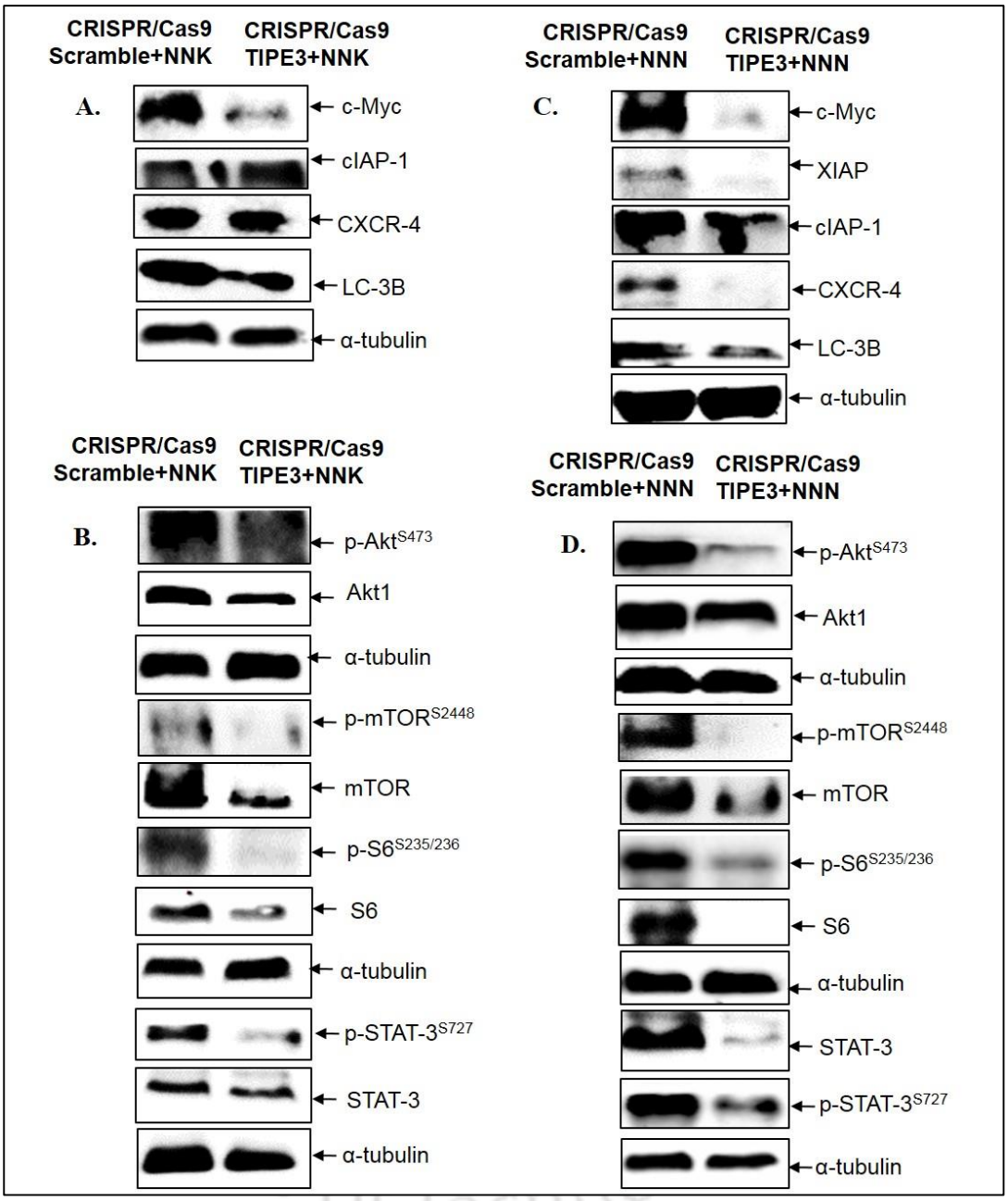

Figure 4.24. Effect on different signaling molecules/pathways in NNK and NNN treated TIPE3 knockout cells. A. Effect on the expression of proteins involved in cell growth and survival in NNK treated TIPE3 knockout cells, B. Effect on Akt/mTOR/S6/STAT-3 signaling in NNK treated TIPE3 knockout cells, C. Effect on the expression of proteins involved in cell growth and survival in NNN treated TIPE3 knockout cells, D. Effect on Akt/mTOR/S6/STAT-3 signaling in NNN treated TIPE3 knockout cells. α-tubulin was used as loading control.

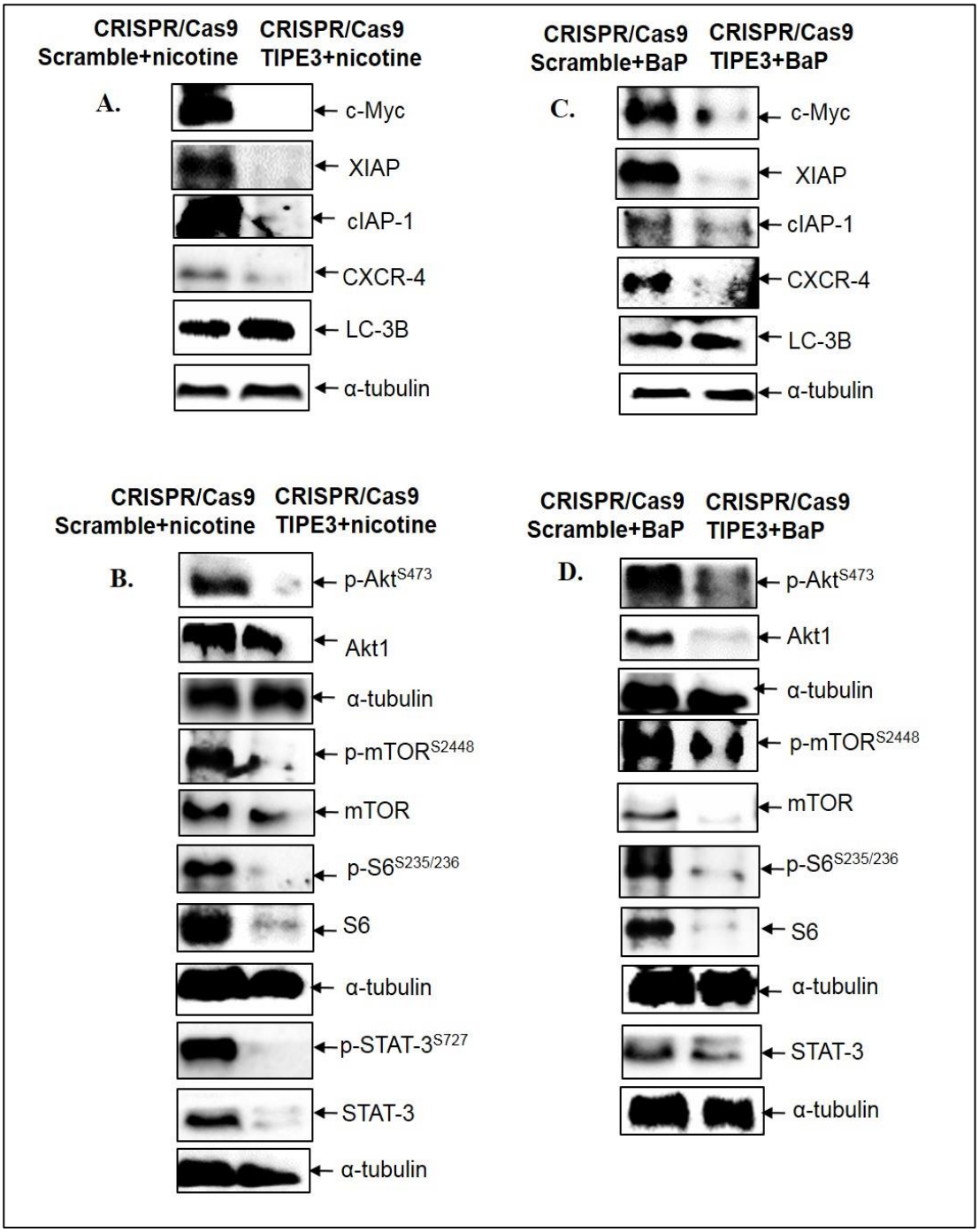

Figure 4.25. Effect on different signaling molecules/pathways in nicotine and BaP treated TIPE3 knockout cells. A. Effect on the expression of proteins involved in cell growth and survival in nicotine treated TIPE3 knockout cells, B. Effect on Akt/mTOR/S6/STAT-3 signaling in nicotine treated TIPE3 knockout cells, C. Effect on the expression of proteins involved in cell growth and survival in BaP treated TIPE3 knockout cells, D. Effect on Akt/mTOR/S6/STAT-3 signaling in BaP treated TIPE3 knockout cells. α-tubulin was used as loading control.

induces cell proliferation, tumor growth and production of growth factors such as VEGF. As mTOR plays a key role in the regulation of different cellular processes in cancer cells, it serves as an intriguing therapeutic target (Bradley and Watson, 2014). Notably, Akt/mTOR pathway is considered as an interesting target for tobacco-mediated lung tumorigenesis (Memmott and Dennis, 2010). Reports suggest that inhibitors of the Akt/mTOR pathway prevent tumor formation in mouse models of carcinogen-induced lung tumorigenesis (Memmott and Dennis, 2010). Another study suggests that the CCI-779, an inhibitor of mTOR; which is downstream of Akt, inhibited malignant progression of premalignant lesions, with activated mTOR in the alveoli of mice which develop lung cancer due to activated K-Ras (Scott *et al.*, 2015). Further, involvement of STAT-3 was also reported in NNK mediated lung cancer. For instance, Min and group showed that NNK induced an increase in *IGF2* transcription via β-adrenergic receptor**,** STAT3 and NF-κB activation in lung cancer **(**Min *et al.*, 2016). In case of our study as well, down regulation in the expression of STAT-3 and p-STAT-3^{S727} was observed in NNK treated TIPE3 knockout cells. In case of NNN, nicotine and BaP treated TIPE3 knockout NCIH460 cells, downregulation in the expression of proteins involved in the regulation of apoptosis and invasion such as cIAP-1, XIAP, c-Myc and CXCR-4 was observed when compared to scrambled control cells treated with NNN, nicotine and BaP. Further, downregulation of Akt, p-Akt^{S473}, mTOR, p-mTOR^{S2448}, S6, p-S6$^{S235/236}$, STAT-3 and p-STAT-3^{S727} were also observed in NNN and nicotine treated TIPE3 knockout cells. In case of NNN treated knockout cells, decrease in the expression of LC-3B was noticed. Additionally, in BaP treated TIPE3 knockout NCIH460 cells, there was decreased expression of Akt, p-Akt^{S473}, mTOR, p-mTOR^{S2448}, S6, p-S6$^{S235/236}$ and STAT-3. Collectively, TIPE3 is found be

involved in tobacco mediated lung carcinogenesis through modulation of Akt/mTOR/S6/STAT-3 signaling axis (Figure 4.24 & 4.25).

4.4. Conclusion

In this chapter, we studied the role of TIPEs in the regulation of different cancer hallmarks. Our results showed that knockout of TIPE, TIPE2 and TIPE3 reduced the proliferation, survival, invasion and migration of NCIH460 human lung cancer cells. On the other hand, knockout of TIPE1 increased the proliferation, survival, invasion and migration of human lung cancer cells. Further, mechanistic studies revealed that all the three oncogenic proteins i.e. TIPE, TIPE2 and TIPE3 exerted their effect through modulation of Akt/mTOR/S6/NF-κB/STAT-3 signaling axis, whereas in case of TIPE1, involvement of NF-κB was not observed and hence found to function via Akt/mTOR/S6/STAT-3 signaling. Further, TIPE, TIPE1, TIPE2 and TIPE3 were found to inflect the expression of different proteins involved in growth, proliferation, survival, apoptosis regulation, invasion, angiogenesis, migration and metastasis such as Cox-2, survivin, Cyclin D1, cIAP-1, XIAP, Bcl-2, c-Myc, Caspase 9, CXCR-4, VEGF-A and MMP-9. In addition, this TIPE family of proteins were also found to exert their function through regulation of autophagy, as LC-3B was found to be downregulated in TIPE, TIPE2 and TIPE3 knockout cells, whereas it showed upregulation in TIPE1 knockout lung cancer cells. Further, modulation in the expression of p53 and its target p21 by TIPE, TIPE1 and TIPE2 also signifies their role in regulating different hallmarks of cancer. Thus, this family of proteins was found to have crucial role in the pathogenesis of lung cancer. Upon confirming this, we have also validated the role of the oncogenic proteins i.e. TIPE, TIPE2 and TIPE3 in tobacco induced lung carcinogenesis. Our results showed for the first time that TIPE, TIPE2 and TIPE3 are involved in the

positive regulation of NNK, NNN, nicotine and BaP induced proliferation, survival and migration of lung cancer cells. Moreover, involvement of Akt/S6/STAT-3 signaling in TIPE knockout lung cancer cells treated with different tobacco components was observed. Additionally, while TIPE2 exhibited its function against tobacco induced lung carcinogenesis through modulation of NF-κB and NF-κB regulated gene products, TIPE3 was found to function via Akt/mTOR/S6/STAT-3 in tobacco mediated lung cancer. Taken together, TIPE family of proteins were found to have profound role in the development and progression of lung cancer and also in tobacco-induced lung carcinogenesis. Thus, targeting this family of proteins holds enormous prospect in therapeutic interventions for the successful management of lung cancer, the aggressive neoplasm. Nonetheless, these findings need to be further validated in the *in vivo* and clinical settings to wholly establish their diagnostic and prognostic importance.

Chapter 5

Discussion, Conclusion and Future prospective

5.1. Discussion and Conclusion

Lung cancer represents the most common cause of death due to cancer across the globe. As mentioned, it consists of two main histological types; NSCLC and SCLC. NSCLC constitutes around 80–85% of all lung cancer cases while 15-20% cases are reported to be SCLC. Smoking is considered to be the predominant risk factor for lung cancer as it is found to be linked with around 90% lung cancer cases. Tobacco contains a variety of carcinogens, which include nitrosamines such as NNK, PAHs, and aromatic amines etc. which classically function through formation of DNA adducts and ultimately lead to miscoding and mutations in different genes which are vital in the regulation of different cellular processes (Hecht, 2012; Ge *et al*., 2015). Apart from tobacco smoking, various other risk factors of lung cancer include exposure to varied occupational and environmental carcinogens, genetic susceptibility, consumption of alcohol, insufficient physical activity, diet and food supplements etc. (Larsen and Minna, 2011, Molina *et al*., 2008). The treatment strategies for lung cancer include surgery for early stages, chemotherapy with concurrent radiation for some locally advanced cancers and palliative chemotherapy for metastatic disease. The introduction of anti-angiogenic agents and TK inhibitors of the EGFR protein has been found to improve response rates for some NSCLC patients (Molina *et al.,* 2008). Despite the significant advancements in the field of therapy for management of lung cancer, the prognosis for patients with lung cancer remains extremely poor. The poor prognosis linked with this disease is due to late stage diagnosis as it exhibits negligible signs and symptoms at early stage (Lemjabbar-Alaoui *et al.,* 2015; Horeweg *et al.,* 2014). In addition, patients undergoing treatment for lung cancer also face manifold complications such as rapid postoperative recurrences and development of chemoresistance against a panel of agents such as doxorubicin, etoposide, gemcitabine, vinorelbine, paclitaxel, docetaxel, and topotecan;

currently available for the treatment of lung cancer (Chudacek *et al.*, 2014; Caulo *et al.*, 2012; Chang, 2011; Yang *et al.*, 2015). Taken together, the poor survival rates 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 (Hirsch *et al.*, 2002). Hence, there arises an urgent need to develop novel biomarkers for diagnosis and prognosis which can facilitate effective management of this aggressive neoplasm.

TIPE family of proteins which is a newly identified group of proteins consisting of four members namely TIPE, TIPE1, TIPE2 and TIPE3 holds immense prospect in this regard. This family is reported to be strongly associated with cell proliferation, inflammation and carcinogenesis. Interestingly, although the members of this family share a significant sequence homology but they are reported to be involved in different biological activities. For example, despite having a common fold between TIPE1 and TIPE2, TIPE2 plays an important role in immune homeostasis, whereas TIPE1 may not have a crucial role to play in immunity (Bordoloi *et al.*, 2018). TIPE is the most widely studied member of this family which facilitates prognosis of different malignancies. TIPE1 has been reported to have important role in the regulation of cell death. Though TIPE2 was first identified as an abnormally expressed gene in the inflamed spinal cord of mice having autoimmune encephalomyelitis, it was later found to be expressed in different cell types. The newest member of this family namely TIPE3 is reported to be involved in the transfer of lipid second messengers PIP2 and PIP3 to the plasma membrane (Bordoloi *et al.*, 2018). Notably, various *in vitro* and *in vivo* studies carried out to elucidate the role of these proteins have revealed their importance in the regulation of inflammatory responses and tumorigenesis. In addition, expression analyses in clinical studies also showed them to exert deregulation in different

malignancies (Bordoloi *et al.,* 2018). Therefore, we studied the role of this family of proteins in the development and progression of lung cancer through extensive analysis of their expression, function and associated mechanism of action not only individually but also on a comparative basis. Remarkably, expression of these proteins were found to show notable variability among themselves (Bordoloi *et al.,* 2018). Therefore, firstly, we analyzed the expression of all the four proteins in human tissue samples with the help of lung cancer tissue microarray containing tissues of different lung cancer types, pathologies, stages, grades, age groups and sexes along with normal lung tissues. Our results showed them to be differentially expressed in lung cancer tissues. For instance, TIPE, TIPE2 and TIPE3 exerted significant upregulation in malignant lung tissues when compared to normal lung tissues. TIPE protein is reported to be overexpressed in various human cancers such as breast cancer, cervical cancer, colon cancer, endometrial cancer, ESCC etc. (Bordoloi *et al.,* 2018; Padmavathi *et al.,* 2018). The expression of TIPE was found to be positively associated with lymph node metastasis, p-TNM and pT stage, Ki-67 expression and poor patient survival in lung cancer (Dong *et al.,* 2010; Xing Y *et al.,* 2018*).* Considering the case of TIPE2, it was found to exert remarkably less expression in breast cancer, glioma and gastric cancer (Wang *et al.,* 2017; Liu *et al.,* 2016; Wu *et al.,* 2016). Contrary to those studies, we found TIPE2 to be significantly overexpressed in lung cancer tissues. In line with our findings, a study conducted by Hao and colleagues also showed enhanced expression of TIPE2 in both diffuse large B-cell lymphoma and peripheral T-cell lymphoma (Hao *et al.,* 2016). Li and group also showed TIPE2 to be up-regulated in NSCLC tumor tissues than the adjacent normal tissues (Li *et al.,* 2016). Additionally, TIPE3 was also found to be significantly upregulated in lung cancer tissues than the normal tissues as evinced by our immunohistochemical analysis. Upregulation of TIPE3 was observed in

case of esophageal cancer, cervical cancer, colon cancer and lung cancer as per studies carried out by different groups (Lian *et al.,* 2017; Wang *et al.,* 2018). Further, we obtained reduced expression of TIPE1 in lung cancer tissues compared to normal tissues. Our finding is supported by a study conducted on gastric cancer which showed TIPE1 to be downregulated in gastric cancer tissues, correlating inversely with differentiation status and distant metastasis (Liu *et al.,* 2018). Additionally, Wu and group also reported TIPE1 to be downregulated in lung cancer tissues, which positively correlated with patient survival (Wu *et al.,* 2017). Thus, the findings of our analysis provided a preliminary basis for considering TIPE, TIPE2 and TIPE3 to have role in the positive regulation of lung cancer whereas involvement of TIPE in the negative regulation of lung cancer pathogenesis. We further analyzed the expression of this family of proteins in both SCLC and NSCLC lung cancer types where TIPE, TIPE2 and TIPE3 showed upregulation in both SCLC and NSCLC tissues compared to normal tissues, with more pronounced upregulation in NSCLC type. In case of TIPE1, in line with the previous results, a marked downregulation was observed in both the lung cancer types. In addition, we analyzed the expression of this family of proteins in different lung cancer pathologies such as adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma, small cell carcinoma and bronchioalveolar carcinoma. In all the pathological conditions, they were found to be differentially expressed. Upon analyzing their expression in tissue samples of patients of different age groups, an eventual decrease in the expression of TIPE, TIPE1, TIPE2 and TIPE3 in the tissue samples of older patients than the younger ones was observed. Smoking habits of people have been found to vary with the ages. As per reports cited earlier, percent smokers are relatively less among older age group people compared to the younger ones. Thus high expression of TIPE, TIPE2 and TIPE3 in the tissue

samples of lower age group patients compared to higher age grouped ones gives us an indication of the involvement of these three proteins in the regulation of tobacco induced lung carcinogenesis. Further, TIPE, TIPE2 and TIPE3 were found to exert high expression in malignant females and males compared to normal females and males respectively. In addition, we noticed that the expression of TIPE, TIPE2 and TIPE3 were relatively more in malignant males compared to malignant females. As mentioned earlier, the risk of lung cancer is relatively high in males than females (Bain *et al.*, 2004). Smoking might be one of the possible reasons for this increased susceptibility in males, as it is estimated that men smoke approximately five times as much as women worldwide (Hitchman and Fong, 2011). Therefore TIPE, TIPE2 and TIPE3 can be again predicted to have strong involvement with tobacco induced lung carcinogenesis. However, in the case of TIPE1, no such association was observed. Further, upon analyzing their expression on the basis of lung cancer grade and stage, TIPE, TIPE2 and TIPE3 were found to be upregulated whereas TIPE1 showed downregulation in different stages and grades of lung cancer than normal tissues. A study conducted by Dong and group showed TIPE to be positively correlated with p-TNM stage, lymph node metastasis, expression of Ki-67, and poor survival in lung cancer (Dong et al., 2010). Contrary to TIPE, overexpression of TIPE2 was found to be negatively correlated with advanced clinical stage of lung cancer (Li et al., 2016). Further, the association between tumor grade and TIPE expression was studied in endometrial cancer, ovarian cancer and prostate cancer. In all the three cancer types, TIPE was found to be positively correlated with tumor histologic grade (Liu *et al.,* 2013; 2014; Zhang *et al.,* 2013). However, in our finding, no significant difference in the expression of TIPE family of proteins among different stages and grades of tumor was observed indicating the involvement of this family of proteins in tumor initiation. However, their

role in tumor progression needs to be confirmed with more advanced stage conditions of the disease or metastatic tissues. Taken together, TIPE, TIPE2 and TIPE3 are strongly associated with the positive regulation of lung carcinogenesis whereas TIPE1 might negatively regulate lung cancer. Further, TIPE, TIPE2 and TIPE3 possess a strong correlation with tobacco induced lung carcinogenesis based on their expression profile in tissues of different age groups and sexes.

We predicted to have a strong association between TIPE, TIPE2 and TIPE3 with tobacco and its related carcinogens. No study to date has reported the effect of tobacco components in the regulation of the expression of TIPEs. Therefore, we evaluated the role of tobacco extract on the expression of TIPE, TIPE2 and TIPE3 in human lung epithelial cells. For this purpose, we have used tuibur, a quite typical tobacco smoke-saturated aqueous concentrate which is conventionally consumed in the North-Eastern part of India particularly in Mizoram. This addictive preparation is extremely toxic as heavy metals such as cadmium, lead, nickel, arsenic and triethylene glycol were found to be present in tobacco water. Besides, a high concentration of NNN is also present in tuibur (Sinha *et al.*, 2006). It is widely known that tobacco smoke is an extremely toxic and carcinogenic combination of more than 5,000 chemicals (Talhout *et al.*, 2011). It constitutes above 60 carcinogens whereas unburned tobacco has nearly 16 identified carcinogens. Among these different carcinogens, the most potent ones include tobacco-specific nitrosamines such as NNK and NNN, PAH etc. (Petti, 2009). The organospecificity of NNK is especially recognized for the lung (Hecht and Hoffmann, 1988). Further, among above five hundred different PAHs present in tobacco smoke, BaP is considered to be the most potent one with ability to induce lung tumors effectively (Vu *et al.*, 2015). Besides, nicotine, another constituent of tobacco which is primarily associated with addiction is also reported to play a crucial role in the

pathogenesis of different cancers through its ability to bind with nAChRs (Warren and Singh, 2013). Therefore, along with the tobacco extract, we also studied the effect of various tobacco components such as NNK, NNN, nicotine and BaP on the expression of *TIPE*, *TIPE2* and *TIPE3* in human lung epithelial cells. Our results showed that the mRNA expression of *TIPE*, *TIPE2* and *TIPE3* were upregulated in tuibur treated L132 cells significantly implying their involvement in tobacco mediated lung carcinogenesis. In addition, upon treatment with different concentrations of NNK, a complete dose dependent upregulation in the expression of *TIPE* and *TIPE2* was observed. In case of *TIPE3* as well, a significant upregulation after treating L132 cells with different concentrations of NNK was observed. Besides, NNN treated L132 cells also showed notable upregulation in *TIPE2* in a dose dependent fashion whereas *TIPE3* was found to be upregulated dose dependently up to 0.25μM NNN treatment. *TIPE* was also found to show upregulation in NNN treated L132 though a clear dose dependent pattern was not observed. Additionally, nicotine treatment resulted in a dose dependent upregulation of *TIPE* and *TIPE2* in L132 lung cells. In case of *TIPE3*, upregulation was observed at 0.5 and 0.75 μM treatment with nicotine. These tobacco components exert their carcinogenic effect through modulation of PI3K/Akt, PKC, NF-κB, c-Src, FAK, Wnt, MEK1/2-ERK1/2, NF-κB, JAK/STAT, p53, p21, Rac1, cyclins, Bcl-2, c-Myc and diverse other signaling molecules/pathways which are involved in the proliferation, survival and angiogenesis of different cancer cells (Ge *et al.*, 2015; Shen *et al.*, 2012; Hirata *et al.*, 2017; Yamakawa *et al.*, 2016; Zhang *et al.*, 2017; Schaal and Chellappan, 2014; Pei *et al.*, 1999; Cai *et al.*, 2011; Du *et al.*, 2018; Raja *et al.*, 2016). Interestingly, TIPE, TIPE2 and TIPE3 also function through regulation of different targets such as PI3K/Akt, MEK-ERK, Wnt/β-catenin, Rac1, Cyclin D1, Caspase 3, -8, -9 etc. (Bordoloi *et al.*, 2018). Thus, the involvement of the common targets strongly implies

their plausible involvement in tobacco mediated lung carcinogenesis. Altogether, deregulation of TIPE proteins might serve as one of the key molecular events in the development and progression of lung cancer, in particular tobacco induced lung carcinogenesis as tobacco, which is responsible for 90% of all lung cancer cases is strongly involved in the modulation of the expression of *TIPE*s.

In order to decipher the exact role of this family of proteins and their downstream targets, we disrupted the expression of TIPEs with the help of CRISPR/Cas9-mediated gene editing. Subsequently, we determined the effect of individual gene knockout in lung cancer cells on different hallmarks of cancer. Further, we determined their downstream targets which are involved in the pathogenesis of lung cancer. Notably, siRNA or shRNA mediated silencing of these proteins were already performed which resulted in the modulation of cell growth, proliferation, invasion and metastasis of lung cancer cells as evinced by a few studies (Bordoloi *et al.*, 2018, Padmavathi *et al.*, 2018). In this study, we reported the comparative analysis upon individual knockout of all the four proteins on the regulation of different processes in lung carcinogenesis. After generating successful knockout clones for TIPE, TIPE1, TIPE2 and TIPE3 and their subsequent confirmation through Western blot, we determined the effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the proliferation, survival, migration and invasion of lung cancer cells. Knockout of TIPE, TIPE2 and TIPE3 resulted in significantly decreased proliferation whereas knockout of TIPE1 led to the increased proliferation of NCIH460 cells compared to scramble control. The reduction in proliferation obtained for TIPE, TIPE2 and TIPE3 knockout cells were found to be different. The highest inhibition in the proliferation of NCIH460 cells was obtained upon knock out of TIPE (77% inhibition). Along with proliferation, enhanced survival

also presents a major feature of cancer cells. Upon determining the effect of the knockout of TIPEs on the survival of NCIH460 cells using colony formation assay, we observed that knockout of TIPE, TIPE2 and TIPE3 resulted in reduced clonogenic potential of NCIH460 cells compared to scramble control implying that TIPE, TIPE2 and TIPE3 are involved in increasing the survival fraction of lung cancer cells. Highest reduction in the colony formation was observed in TIPE knockout cells followed by TIPE2 and TIPE3 knockout NCIH460 cells respectively. In contrast, 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 scramble control. In line with our results, knockdown of TIPE reduced the proliferation and colony formation of colon cancer cells (Miao *et al.,* 2012). Further, TIPE depletion was found to be associated with reduced proliferation of esophageal and gastric cancer cells whereas its overexpression caused enhanced cell proliferation (Sun *et al.,* 2016; Hadisaputri *et al.,* 2012; Hu *et al.,* 2016; Han *et al.,* 2018; Dong *et al.,* 2017). In addition, TIPE1 inhibited the growth and colony formation of HCC cells in contrast to our finding (Zhang *et al.,* 2015). However, overexpression of TIPE1 reduced colony formation of lung cancer cells as reported by Wu and group (Wu *et al.,* 2017). In addition, overexpression of TIPE2 inhibited the proliferation and colony formation of breast and lung cancer cells respectively (Li *et al.,* 2016; Wang *et al.,* 2017). However, the findings obtained in our study shows a differed effect. In addition, in line with our findings, knockdown of TIPE3 was shown to inhibit the proliferation of breast cancer as well as lung cancer cells (Lian *et al.,* 2017; Wang *et al.,* 2018). Taken together, our results suggest that TIPE, TIPE2 and TIPE3 are responsible for the positive regulation of lung cancer cell proliferation and survival whereas TIPE1 negatively regulates the proliferation and survival of lung cancer cells. It is widely known that lung cancer is one of the most

aggressive neoplasm in which majority of the patients are diagnosed with advanced stage of the disease. The acquired ability of lung cancer cells to migrate and invade nearby cells is strongly linked with their extremely high metastatic ability (Tungsukruthai *et al.,* 2017; Millar *et al.,* 2017). Upon analyzing the effect of TIPEs on the migration and invasion of lung cancer cells, we found that loss of TIPE, TIPE2 and TIPE3 inhibited the migration potential of lung cancer cells effectively whereas loss of TIPE1 enhanced the migration of lung cancer cells. Further, TIPE and TIPE1 were found to inflect the invasiveness of lung cancer cells as well. Similar to our findings, decreased expression of TIPE was reported to inhibit the growth, migration and invasion of gastric cancer cells effectively (Li *et al.,* 2015). TIPE also promoted invasion potential of lung cancer cells through modulation of the Hippo pathway (Han *et al.,* 2018). The expression of TIPE in breast cancer cells was reported to have strong correlation with enhanced invasion as well as frequency of pulmonary colonization of tumor cells (Zhang *et al.,* 2006). Further in case of TIPE1, its overexpression was found to be associated with decreased invasion of lung cancer cells which again correlates with our findings clearly suggesting the anti-tumor role of TIPE1 in lung cancer (Wu *et al.,* 2017). Again, contrary to our results, overexpression of TIPE2 was reported to exert an inhibitory effect on the migration of lung and prostate cancer cells (Li *et al.,* 2016; Lu *et al.,* 2016). Additionally, stable overexpression of TIPE3 with a C-terminal flag, plausibly localized in the plasma membrane, induced the growth and migration of NSCLC cells through modulation of ERK and Akt pathway (Wang *et al.,* 2018). Collectively, TIPE and TIPE1 are found to be involved not only in the proliferation, survival and migration, but also in the invasion of lung cancer cells exemplifying their critical role in the progression and metastasis of lung cancer and hence their immense therapeutic implications. Notably, there are different signaling molecules/pathways

associated with different cancer hallmarks (Li and Mansmann, 2014). Therefore, to determine the mechanisms of TIPE, TIPE1, TIPE2 and TIPE3 mediated proliferation, survival, invasion and migration of the lung cancer cells, expression analysis of different target proteins was performed with the help of Western blot. Knockout of TIPE, TIPE2 and TIPE3 resulted in the downregulation of different proteins involved in the regulation of apoptosis such as survivin, cIAP-1, XIAP, Bcl-2 and upregulated the expression of Caspase 9. Survivin, cIAP-1 and XIAP which belong to the IAP family plays role in the inhibition of apoptosis (Erkanli S *et al.*, 2007). Further, Bcl-2 is an anti-apoptotic protein involved in the suppression of caspase-dependent as well as Caspase-independent apoptotic pathways. Additionally, Caspase-9 is responsible for cytokine processing and apoptosis (Perfettini *et al.*, 2002). We also observed that loss of TIPE, TIPE2 and TIPE3 led to the downregulation of Cox-2 and Cyclin D1, which plays important role in cellular growth and differentiation. Further loss of TIPE and TIPE3 also resulted in downregulation of the oncogene; c-Myc, which plays a vital role in cell growth and metabolism (Inoue and Fry, 2015; Miller *et al.*, 2012; Erkanli *et al.*, 2007). Additionally, in TIPE knockout cells, downregulation of LC-3B, the marker of autophagosomes' formation was also observed which in turn might contribute towards reduced the cell proliferation and survival of lung cancer cells. Autophagy is a catabolic cellular mechanism which involves degradation of dysfunctional components of cell through formation of autophagosomes' and thus maintains proper immune homeostasis (Mortezavi *et al.*, 2017). In addition, loss of TIPE2 and TIPE3 caused downregulation of CXCR-4 and MMP-9, which are involved in the invasion, migration and metastasis of tumor cells. Further, TIPE and TIPE3 knockout exerted downregulation of VEGF-A, which is involved in angiogenesis (Hao *et al.*, 2007). Downregulation in the expression of CXCR-4 was observed in case of TIPE knockout cells as well. Besides,

TIPE and TIPE2 knockout cells showed high expression of tumor suppressors namely p53 and p21. p53 regulates different cellular activities such as apoptosis, senescence, cell growth, migration, and invasion. p21 is a target of p53 which regulates cell growth and invasion (Kim *et al.*,2017). Increasing lines of evidence indicate that PI3K/ Akt/mTOR pathway, which plays a crucial role in oncogenesis is frequently activated in lung cancer (Cheng *et al.,* 2014). Upon determining the involvement of TIPEs in this signaling axis, we observed that knockout of TIPE, TIPE2 and TIPE3 affected the vital constituents of Akt/mTOR pathway as loss of them downregulated the expression of Akt1, p-Akt^{S473}, p-Akt^{T308}, mTOR, p-mTOR^{S2448}, S6 and p-S6$^{S235/236}$. Further, upregulation in the expression of PTEN, a negative regulator of Akt was observed in TIPE, TIPE2 and TIPE3 knockout cells (Sarris *et al.,* 2012). Thus, TIPE, TIPE2 and TIPE3 are found to activate Akt/mTOR/S6 signaling pathway which in turn contributes to the pathogenesis of lung cancer. Further, reports suggest that constitutive activation of the PI3K/Akt leads to aberrant activity of STAT-3 and NF-κB (Han *et al.,* 2010). Our results showed that knock out of TIPE and TIPE3 downregulated the expression of NF-κB, p-NF-κB^{S536}, STAT-3 and p-STAT-3^{S727} notably. TIPE2 knockout cells also led to the downregulation of NF-κB, p-NF-κB^{S536} and STAT-3. Altogether, TIPE, TIPE2 and TIPE3 induced lung carcinogenesis is mediated through modulation of Akt/mTOR/S6/NF-kB/STAT-3 signaling axis. Contrary to TIPE, TIPE2 and TIPE3, loss of TIPE1 was found to upregulate the expression of proteins involved in cell growth, survival, apoptosis regulation, migration, metastasis and angiogenesis such as Cox-2, survivin, Cyclin D1, cIAP-1, XIAP, c-Myc, MMP-9 and VEGF-A. In addition, decreased expression of tumor suppressors such as p53 and p21 were also observed in TIPE1 knockout cells. Besides, 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. Furthermore, downregulation of Redd1, the negative regulator of mTOR was also observed. Collectively, this is the first study which reports that loss of TIPE1 increases the proliferation, survival, invasion and migration of lung cancer cells through activation of Akt/mTOR/S6/STAT-3 signaling axis.

We have discussed earlier that TIPE, TIPE2 and TIPE3 might be involved in tobacco mediated lung carcinogenesis. To confirm the same, we treated TIPE, TIPE2 and TIPE3 knockout NCIH460 cells with four different tobacco components such as NNK, NNN, nicotine and BaP and their effect on the proliferation, survival and migration was studied and the associated downstream targets were identified as well. Tobacco-carcinogen transformed human bronchial epithelial cells have been reported to exert enhanced activation of the PI3K/Akt pathway leading to their increased proliferation and as well as survival (West *et al.,* 2004). Aforementioned, NNK and NNN are potent lung carcinogens. The binding of NNK and NNN to the nAChR induces cell proliferation, survival, migration, and invasion of tumor cells (Xue *et al.,* 2014). In addition, nicotine is also reported to induce proliferation and angiogenesis in varied cellular models (Dasgupta *et al.,* 2006). Further, BaP was also found to promote the proliferation of lung cancer cells effectively (Wang *et al.,* 2015). Tobacco and its components are well known to have significant involvement in cancer cell migration as well. Chronic exposure to cigarette smoke resulted in the activation of PAK6 in NSCLC cells, which in turn regulates different processes in cancer including metastasis (Raja *et al.,* 2016). Further, components present in tobacco such as nicotine and NNK are also reported to influence the migration of lung cancer cells (Dasgupta *et al.,* 2009; Wang et al., 2015). Therefore, we evaluated the effect of NNK, NNN, nicotine and BaP on the proliferation, survival and migration of NCIH460 human lung cancer cells after

individual knockout of TIPE, TIPE2 and TIPE3. Upon treatment with all the four tobacco components, there was a decrease in the proliferation of CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells. In the case of CRISPR/Cas9 TIPE3 cells treated with NNK, NNN, nicotine and BaP, highest reduction in proliferation was observed. Further, significant reduction in the proliferation in tobacco components' treated CRISPR/Cas9 TIPE and CRISPR/Cas9 TIPE2 cells was also noted. Similar to the proliferation assay, in the colony formation assay as well, decreased survival fraction of CRISPR/Cas9 TIPE, CRISPR/Cas9 TIPE2 and CRISPR/Cas9 TIPE3 cells treated with NNK, NNN, nicotine and BaP were observed compared to scramble control cells treated with the same. CRISPR/Cas9 TIPE2 cells showed the highest reduction in survival fraction as denoted by remarkably decreased number of colonies formed upon treatment with NNK, NNN, nicotine and BaP. Further, NNK, NNN, nicotine and BaP treated CRISPR/Cas9 TIPE, TIPE2 and TIPE3 knockout cells were found to exert decreased migration potential as well. In the case of NNN and BaP treatment, CRISPR/Cas9 TIPE3 cells showed highest reduction in migration potential whereas in NNK and nicotine treatment, CRISPR/Cas9 TIPE2 cells presented the highest wound area remained. Thus, these results clearly suggest that TIPE, TIPE2 and TIPE3 are strongly involved in the positive regulation of NNK, NNN, nicotine and BaP induced proliferation, survival and migration of lung cancer cells.

Tobacco induced lung carcinogenesis is driven by alterations in various signal transduction pathways. Therefore, we further identified those molecular targets through which TIPEs plausibly mediate their tumorigenic effect in tobacco induced lung cancer. NNK, NNN, nicotine and BaP treated TIPE knockout cells showed down regulation in the expression of proteins involved in the proliferation, survival and angiogenesis such

as Cox-2, survivin and VEGF-A than the srambled control cells treated with the respective components. Further, upregulation in the expression of p53 and p21 was also observed. In addition, TIPE knockout cells treated with tobacco components caused downregulation of p-Akt^{S473} and p-S6$^{S235/236}$, STAT-3 and p-STAT-3^{S727}. Taken together, TIPE is found to have involvement in the positive regulation of TIPE mediated lung carcinogenesis via Akt/S6/STAT-3 signaling. In case of TIPE3 knockout cells treated with tobacco components, downregulation of proteins involved in the regulation of apoptosis, invasion and autophagosomes formation such as cIAP-1, XIAP, c-Myc, CXCR-4 and LC-3B were observed. Further, tobacco components' treated TIPE3 knockout cells showed marked downregulation of Akt, p-Akt^{S473}, mTOR, p-mTOR^{S2448}, S6, p-S6$^{S235/236}$, STAT-3 and p-STAT-3^{S727}. The PI3K/Akt/mTOR signaling pathway is frequently activated in cancer cells which induces cell proliferation, tumor growth and production of growth factors such as VEGF (Bradley and Watson, 2014). Notably, in 90% of the NSCLC cells, constitutive activation of PI3K/Akt pathway has been noticed which promoted cellular survival as well as resistance to γ-radiation or chemotherapy. Further, Akt/mTOR pathway is also considered as an interesting target for tobacco-mediated lung tumorigenesis (Memmott and Dennis, 2010). Additionally, involvement of STAT-3 was also reported in tobacco components' mediated lung cancer (Min *et al.*, 2016). Thus, TIPE3 functions via Akt/mTOR/S6/STAT-3 signaling axis in tobacco induced lung carcinogenesis. Additionally, TIPE2 is found to be involved in the positive regulation of tobacco mediated lung carcinogenesis through increased proliferation, survival and migration of tobacco components' treated TIPE2 knockout lung cancer cells by modulating the expression of NF-κB and NF-κB regulated gene products such as Cox-2, survivin, Cyclin D1, MMP-9 and CXCR-4. NF-κB regulates the expression of various genes

involved in lung carcinogenesis (Cai *et al.*, 2011). Notably, NF-κB has been found to be activated by cigarette smoke and its components such as nicotine and NNK in different NSCLC cells (Chen W *et al.*, 2011). Altogether, TIPE family of proteins were found to have profound role in the development and progression of lung cancer and particularly in tobacco-induced lung cancer and hence specific targeting of them holds enormous prospect in newer therapeutic interventions in lung cancer.

5.2. Limitations and future prospective of the study

The present study establishes a strong correlation between TIPE family of proteins and lung carcinogenesis, specifically tobacco induced lung carcinogenesis. Nonetheless, there are a few limitations associated with the study and hence needs further validation. The expression of TIPE family of proteins in human lung cancer tissues was investigated using TMA slides which were devoid of tissues from Indian population. Lung cancer is a major global burden. It is also a leading cause of death due to cancer in India in both males and females. Although development of lung cancer is a multifactorial process driven by various factors such as exogenous exposures, genetic variations, somatic genetic events, it appears to have several racial and ethnic differences which not only impacts the incidence, survival and mortality in lung cancer but also diverse molecular events associated with it. Therefore, analysing the expression of this important family of proteins in lung cancer tissues of different types, anatomic locations, stages and grades from Indian population will help immensely to strengthen the study. This study also lacks the expression analysis of TIPE family of proteins in highly advanced stages of lung cancer such as Stage IIIa and IV. Therefore assessing the expression of TIPEs in tissue samples of highly advanced and metastatic disease stage would help us to know their role in lung cancer progression in a much meticulous

and precise manner. Further, inclusion of different pre-neoplastic lesions of lung cancer for analysing the expression of TIPEs would help us in a much more defined understanding of the involvement of these proteins in the development of lung cancer. Additionally, 90% of the lung cancer cases are due to tobacco smoking. The TMA slides used in this study did not include a detailed description of the tobacco exposure and smoking status along with other related parameters of the patients provided for analysis. Information on those factors if provided, would have helped us to obtain a much confirmatory idea on the involvement of TIPEs in tobacco induced lung carcinogenesis. Along with that, inclusion of several other parameters such as details of response of the patients to treatment, overall survival, progression free survival, chemo and radiation resistance and status of tumor recurrence etc. would aid in unravelling the clinical values of TIPEs in lung cancer.

Secondly, we reported the involvement of *TIPE*s in tuibur, a water extract of tobacco and various tobacco derived components' such as NNK, NNN, BaP and nicotine in lung epithelial cells. Tobacco, the major risk factor of lung cancer, is a carcinogenic mixture of more than 5000 chemicals and 20 known lung carcinogens. As in this study we evaluated the effect of only three potent carcinogens namely, NNK, NNN and BaP, therefore further studies can be performed with other lung carcinogens present in tobacco. Besides, effect of various other constituents present in tobacco will provide more insights on the involvement of TIPEs in lung cacrcinogenesis mediated via tobacco. In addition, lung cancer incidence is found to be very high in North-Eastern part of India with highest reported incidences from Mizoram in the country in both males and females. We evaluated the effect of tuibur treated lung epithelial cells on the expression of *TIPE*s in this study. However, the effect of other traditional and region

specific preparations of tobacco consumed by the local people of North-Eastern states like hidakphu on the expression of *TIPE*s can be studied further. In addition, the non-involvement of TIPE1, the negative regulator of lung cancer in tobacco mediated lung carcinogenesis was predicted based on immunohistochemical analysis. However, validation of the same in the subsequent analysis would have been more substantial.

In addition, we disrupted the expression of TIPE, TIPE1, TIPE2 and TIPE3 with the help of CRISPR/Cas9 gene knockout tool and studied their role in lung carcinogenesis, in particular tobacco induced lung carcinogenesis. Our study was confined to the elucidation of various downstream targets of TIPEs. Determining the upstream mediators responsible for the modulation of TIPEs in lung cancer, in particular tobacco induced lung cancer seems to be of vital importance. In addition, restoration of TIPEs can be performed via conditional gene knockout method for further investigation and validation on the role of TIPEs. Further, potential crosstalk among the members of this family can further be studied as they were found to be involved in the regulation of lung cancer through involvement of various common molecular targets apart from the ones which were specific for the definite proteins of this family. We have carried out single deletion of TIPE proteins in lung cancer cells. Though they are reported to exhibit diverse biological activities despite their structural similarity, still it is requisite to evaluate the effect of the deletion of more than one TIPE family of proteins in the development and progression of lung cancer. In addition, as mentioned earlier, tumor recurrence and development of chemoresistance present a major obstacle in the effective treatment of lung cancer. Therefore understanding the role of TIPEs in the regulation of resistance against existing lung cancer therapies such as gefitinib, erlotinib, afatinib, pembrolizumab etc. is highly critical which needs to be investigated

in detail. Furthermore, global gene and protein expression profiling using microarray of cells with disrupted expression of TIPEs would enrich our knowledge on the complicated relations among diverse genes or proteins implicated in lung cancer pathogenesis. Furthermore, the crystal structures of two members of the TIPE family, namely, TIPE2 and TIPE3 from *Homo sapiens* and TIPE from *Mus musculus* (mTIPE) have been determined. Therefore determining the crystal structute of TIPE1, the least studied member of this family would help to understand its role and function in different malignancies including lung cancer much precisely. In addition, analysis of the function and role of TIPEs in lung cancer was performed in single cell line. However, to strengthen our study, it needs to be validated in multiple NSCLC and also if possible in SCLC cells. Noteworthy, the findings obtained in the *in vitro* settings definitely need to be validated in the *in vivo* and clinical settings as well to fully establish the diagnostic and prognostic values of TIPE family of proteins which in turn will facilitate development of novel therapeutic strategies for the successful clinico- management of this aggressive neoplasm.

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List of Abbreviations

AdVTIPE2	: Adenovirus-mediated human TIPE2 gene transfer
AhR	: Aryl hydrocarbon receptor
ALDH	: Aaldehyde dehydrogenase
BaP	: Benzo[a]pyrene
Bcl-2	: B-cell lymphoma 2
Bcl-XL	: B-cell lymphoma 2- extra large
BPDE	: Benzo(a)pyrene-7,8-diol-9,10-epoxide
BPDE-dG	: Benzo(a)pyrene-7,8-diol-9,10-epoxide-N(2)-deoxyguanosine
BSA	: Bovine serum albumin
CCL20	: Chemokine (C-C motif) ligand 20
CDK	: Cyclin-dependent kinase
cDNA	: Complementary DNA
CFTR	: Cystic fibrosis transmembrane conductance regulator
cIAP-1	: Cellular inhibitor of apoptosis protein-1
CIS	: Carcinoma *in situ*
CNV	: Choroidal neovascularization
Cox-2	: Cyclooxygenase-2
CRISPR	: Clustered regularly interspaced short palindromic repeats
CTLA-4	: Cytotoxic T-lymphocyte-associated antigen
CXCR-4	: C-X-C chemokine receptor type 4
CYP	: Cytochrome P450
DAB	: 3,3'-Diaminobenzidine
DED	: Death effector domain
DLBCL	: Diffuse large B-cell lymphoma
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DTT	: Dithiothreitol
EC	: Endometrial carcinoma
ECL	: Enhanced chemiluminescence
EDTA	: Ethylenediaminetetraacetic acid
EGFR	: Epidermal growth factor receptor
EMT	: Epithelial-to-mesenchymal transition
EOC	: Epithelial ovarian cancer
ERK	: Extracellular signal-regulated kinase
ER-α	: Estrogen receptor-alpha
ER-β	: Estrogen receptor -beta
ESCC	: Esophageal squamous cell carcinoma
ES-SCLC	: Extensive-stage disease -small cell lung cancer
FAK	: Focal adhesion kinase
FBS	: Fetal bovine serum
FLIP	: Fas-associated death domain-like interleukin-1beta-converting

	enzyme- inhibitory protein
FP	: Forward primer
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
GCB	: Germinal center B-cell
GWASs	: Genome-wide association studies
HCC	: Hepatocellular carcinoma
HDAC	: Histone deacetylase
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIF‑1α	: Hypoxia-inducible factor 1-alpha
HO-1	: Heme oxygenase-1
HPV	: Human papilloma virus
HRP	: Horseradish peroxidase
IAP	: Inhibitor of apoptosis protein
IARC	: International agency for research on cancer
IASLC	: International association for the study of lung cancer
IDC	: Invasive ductal breast carcinoma
IGF2	: Insulin-like growth factor 2
IHC	: Immunohistochemistry
IL	: Interleukin
JAK	: Janus-activated kinase
LC-3B	: Light chain-3B
LE-AS5	: Liposomal formulation of TIPE antisense oligonucleotide
LS-SCLC	: Limited-stage disease -small cell lung cancer
MAGE-A3	: Melanoma-associated antigen A3 vaccine
MAPK	: Mitogen-activated protein kinase
MAP2K1	: Mitogen-activated protein kinase kinase 1
MEM	: Minimum essential medium
mRNA	: Messenger RNA
miRNA	: microRNA
MMP	: Matrix metalloproteinase
Mtor	: Mammalian target of rapamycin
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUC1	: Mucin-1
nAChR	: Nicotinic acetylcholine receptor
NF-κB	: Nuclear factor-kappa B
NHL	: Non-Hodgkin's lymphoma
NKX2-1	: NK2 homeobox 1
NNK	: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	: N-nitrosonornicotine
NSCLC	: Non-small cell lung cancer
OS	: Osteosarcoma
PAH	: Polycyclic aromatic hydrocarbons
PAK6	: p21 -activated kinase 6
PARP	: Poly ADP ribose polymerase
PBS	: Phosphate buffer saline
PE	: Plating efficiency

Pen-Strep	: Penicillin-Streptomycin
PIP2	: Phosphatidylinositol 4,5-bisphosphate
PIP3	: Phosphatidylinositol 3,4,5-trisphosphate
PAC	: Pulmonary adenocarcinoma
PKC	: Protein kinase C
PMSF	: Phenylmethylsulfonyl fluoride
PTCL	: Peripheral T-cell lymphoma
Rac1	: Ras-related C3 botulinum toxin substrate 1
Rb	: Retinoblastoma
REDD1	: Regulated in development and DNA damage response 1
RNA	: Ribonucleic acid
ROS	: Reactive oxygen species
RP	: Reverse primer
RT	: Room temperature
RT-PCR	: Reverse transcription-polymerase chain reaction
SCLC	: Small cell lung cancer
SF	: Survival fraction
SNP	: Single nucleotide polymorphism
SOCC	: Store‑operated calcium channel
STAT	: Signal transducer and activator of transcription
TBST	: Tris-buffered saline with 1% tween 20
TGIF	: TG-interacting factor
TIPE/TNFAIP8	: Tumor necrosis factor-α-induced protein eight
TIPE1/TNFAIP8L1	: Tumor necrosis factor-α-induced protein eight like 1
TIPE2/TNFAIP8L2	: Tumor necrosis factor-α-induced protein eight like 2
TIPE3/TNFAIP8L3	: Tumor necrosis factor-α-induced protein eight like 3
TKI	: Tyrosine kinase inhibitor
TMA	: Tissue microarray
TNF-α	: Tumor necrosis factor-alpha
TNM	: Tumor node metastasis
uPA	: Urokinase-type plasminogen activator
VEGF	: Vascular endothelial growth factor
VEGFR	: Vascular endothelial growth factor receptor
XIAP	: X-linked inhibitor of apoptosis
YAP	: Yes-associated protein
α7nAChR	: alpha7-subtype of nAChR
µg	: Micogram
µM	: Micromolar
mM	: Milimolar
h	: Hour

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Articles and Book chapters

1. **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. TIPE Family of Proteins and Its Implications in Different Chronic Diseases. Int J Mol Sci. 2018;19(10). pii: E2974.

2. Padmavathi G, Banik K, Monisha J, **Bordoloi D**, Shabnam B, Arfuso F, Sethi G, Fan L, Kunnumakkara AB. Novel tumor necrosis factor-α induced protein eight (TNFAIP8/TIPE) family: Functions and downstream targets involved in cancer progression. Cancer Lett. 2018;432:260-271

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53. Kunnumakkara AB, **Bordoloi D**, Sailo BL, Roy NK, Thakur KK, Banik K, Shakibaei M, Gupta SC, Aggarwal BB. Cancer Drug Development: Missing Links. (Manuscript accepted in Experimental Biology and Medicine).

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55. Banik K, Ranaware AM, Deshpande V, **Bordoloi D**, Nalawade SP, Padmavathi G, Sailo BL, Shanmugam MK, Fan L, Arfuso F, Kunnumakkara AB. Honokiol for cancer therapeutics: A traditional medicine that can modulate multiple oncogenic targets (Manuscript under revision).

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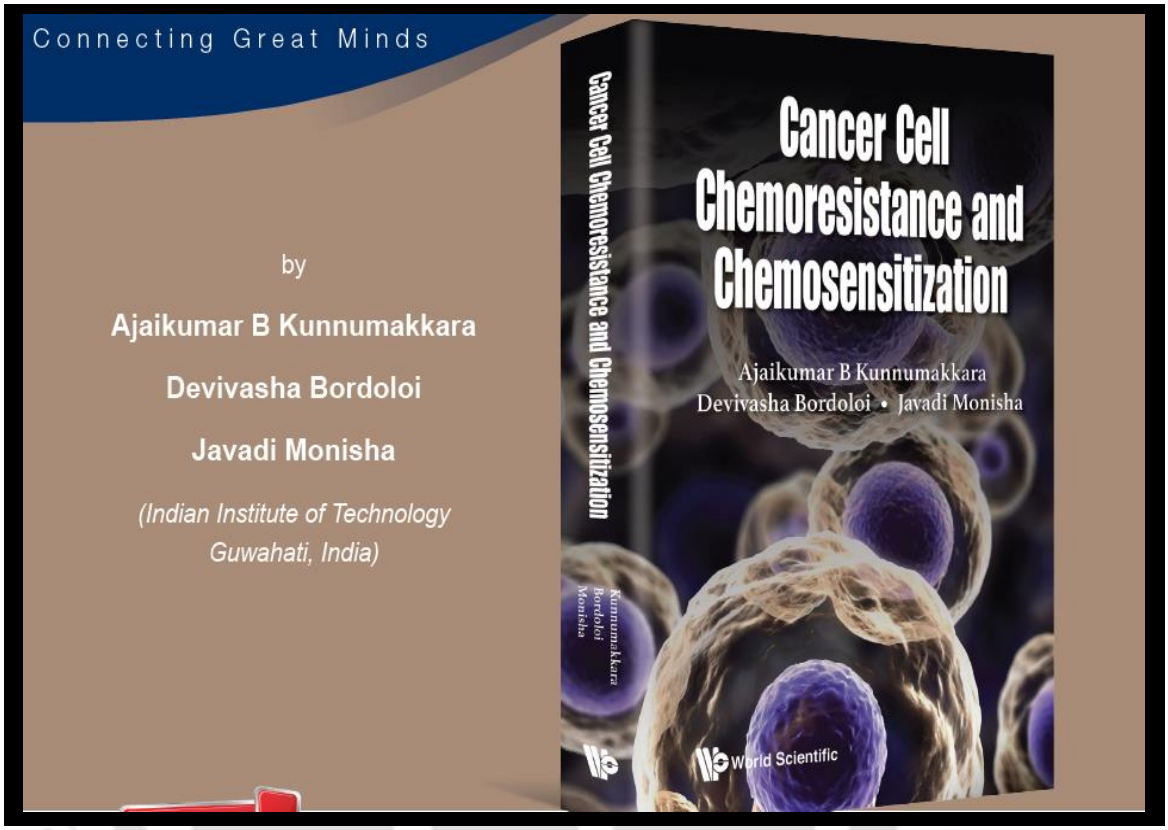
58. **Bordoloi D**, Kunnumakkara AB. TIPE1 exerts anti-tumor effect through modulation of Akt/mTOR/S6/STAT-3 signaling axis in human lung cancer (Manuscript Submitted).

Book

Cancer Cell Chemoresistance and Chemosensitization, edited by: Ajaikumar B Kunnumakkara, **Devivasha Bordoloi**, Javadi Monisha, Publisher: World Scientific Publications (2018).



Abstracts presented in conferences

1. **Bordoloi D**, Monisha J, Roy NK, Padmavathi G, Kunnumakkara AB. 'Evaluation of a chalcone derivative as natural alternative for oral cancer therapy', Third 'International Conference on Nutraceuticals and Chronic Diseases', Rishikesh-Dehradun, India, 2018.

2. **Bordoloi D**, Monisha J, Roy NK, Padmavathi G, Kunnumakkara AB. 'Exploration of the anti-neoplastic potential of a flavonoid from Varnish tree against human head and neck Cancer', National Conference on Ethno-medicine and traditional health practices in North-East region of India, National Institute of Pharmaceutical Education and Research Guwahati, Assam, India, 2018.

3. **Bordoloi D**, Monisha J, Roy NK, Padmavathi G and Kunnumakkara AB. 'The plant polyphenol from *Toxicodendron verniciflum* exhibits anti-cancer potential against human squamous cell carcinoma', Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.

4. **Bordoloi D,** Banik K, Harsha C, Devi KA, Kunnumakkara AB. 'Curcumin, the golden nutraeutical: multitargeting for multiple malignancies', Research Conclave,

Indian Institute of Technology Guwahati, Assam, India, 2017.

5. **Bordoloi D,** Sailo BL, Padmavathi G, Thakur KK, Kunnumakkara AB. **'**The potential influence of a tetrahydroxychalcone "Butein" to obliterate cancer', Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.

6. Padmavathi G**,** Simona SP, Roy NK, **Bordoloi D**, Monisha J, Padikkala J, Kunnumakkara AB. 'An Investigation on the Cancer Preventive Potential of the Spice *Carum copticum* (Ajwain) Against Azoxymethane Induced Colon Carcinogenesis', First 'International Conference on Nutraceuticals and Chronic Diseases', Kochi, India, 2016.

7. Padmavathi G**,** Simona SP,Roy NK, **Bordoloi D**, Monisha J,Padikkala J, Kunnumakkara AB. 'Prevention of Azoxymethane Induced Colon Carcinogenesis by the Spice *Carum copticum* (Ajwain)', 'Translational Cancer Research', Ahmedabad, India, 2016.

8. **Bordoloi D**, Monisha J, Padmavathi G, Singh MS, Kunnumakkara AB. An investigation on the anti-cancer mechanism of a chalconoid isolated from *Toxicodendron vernicifluum* against human oral squamous cell carcinoma, International conference on Nutraceuticals and chronic diseases 2016 (INCD-2016), Kerala, India, 2016.

9. Roy NK, Monisha J, Padmavathi G, **Bordoloi D,** Kunnumakkara AB, Precise distinction of general Akt Inhibitors into Akt Isoform specific inhibitors, Indian Association of Cancer Research-2016 (IACR-2016), New Delhi, India, 2016.

10. **Bordoloi D,** Monisha J, Padmavathi G, Singh MS, Kunnumakkara AB. Butein Suppresses the Proliferation, Survival, Invasion and Metastasis of Head and Neck Squamous Carcinoma Cells, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2016.

11. **Bordoloi D,** Monisha J, Padmavathi G, Singh MS, Kunnumakkara AB. Therapeutic Potential of Butein in Head and Neck Cancer, Translational Cancer Research-2016 (TCR-2016), Ahmedabad, India, 2016.

Abstracts in conference proceedings

1. Kunnumakkara AB, **Bordoloi D**, Sailo BL, Padmavathi G, Monisha J. 'Fruits, Vegetables and Their Components in Cancer Prevention: What We Learned Thus Far?', 8th International Aromatherapy Conference, San Fransisco, November 6-8, 2015, 145-88.

2. Kunnumakkara AB, Sailo BL and **Bordoloi D**. Molecular Biology Tools in Development of Novel Cancer Diagnosis Methods and Personal Cancer Medicine, National conference on Advances in cancer genomics, Mizoram University, 2014: 17.

3. **Bordoloi D**, Sailo BL, Kunnumakkara AB. 'Nutraceuticals: Safe, efficacious and affordable agents for cancer prevention and treatment', Asian Aroma Ingredients Congress-2016, Delhi, April 22-24, 2016, 37-50.

Awards and achievements

1. Selected to participate at the 5th AIST International imaging workshop at Biomedical Research Institute, AIST, Tsukuba, Japan, January 21-30, 2018 as **one of the 20 candidates** from 210 international applications.

2. Received '**Best Presentation award**' for the paper entitled "Investigation on the anti-cancer potential of 'Butein', a tetrahydroxychalcone against head and neck squamous cell carcinoma" at the 5th AIST International imaging workshop held at Biomedical Research Institute, Tsukuba Science city, Japan, January 21-30, 2018.

3. Received '**Best Oral Presentation Award**' for the paper entitled "The anti-neoplastic potential of a chalconoid isolated from Chinese lacquer tree against human oral cancer" at the International conference on Trends in Biochemical and Biomedical Research, Varanasi, India, February 13-15, 2018.

4. Received '**Best Oral Presentation Award'** for the paper entitled "Anti-cancer potential of a bio-active flavonoid through the suppression of genes involved in proliferation, survival, invasion and metastasis of HNSCC cells" at the International Conference on Nutraceuticals and Chronic diseases 2016 (INCD-2016), Goa, India, September 1-3, 2017.

5. Received '**Young Scientist Award**' for the paper entitled "An investigation on the anti-cancer mechanism of a chalconoid isolated from *Toxicodendron vernicifluum* against human oral squamous cell carcinoma" at the International conference on nutraceuticals and chronic diseases 2016 (INCD-2016), Kerala, India, 2016.

Conferences, workshops and trainings attended

1. Participated in Indo-japan symposium on "**Hope from Herbs: research based Care and cure Potentials**" jointly organized by IIT Guwahati and AIST, Japan, held on 8th May, 2017.

2. Participated in the National conference on **'Recent Developments in Medical Biotechnology and Structure Based Drug Designing'** organized by Department of Biosciences and Bioengineering, IIT Guwahati, India, held on 6th & 7th December, 2015.

3. Participated in a 9 day advanced research training workshop on **'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approach'** sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kalyani, Kolkata held during October 5-13, 2015.

4. Participated in a 6 day research training workshop on **'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approach'** sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kolkata and Department of Biotechnology, Assam University, Silchar held during April 6-11, 2015 and **Selected** for the advanced level workshop.

5. Participated in a 5 day national course on **'Theoretical and Practical aspects of Cancer Research'** conducted under the Technical Education Quality Improvement Programme sponsored by the Ministry of Human Resource Development, Govt. of India, from February 4th – 8th, 2015.

6. Participated in a 2 day national workshop on **'Flow Cytometry Data Analysis'** organized by Department of Biotechnology, Indian Institute of Technology Guwahati from 23rd – 24th January, 2015.

7. Participated in the National Conference on **'Recent Advances in Cancer Biology and Therapeutics'** organized by Department of Biotechnology, IIT Guwahati, India, held on 5th December, 2014.

8. Participated in a 4 day national workshop on **'Next Generation Sequencing and Data Analysis'** organized by Biotech Hub, Centre for the Environment, Indian Institute of Technology Guwahati held during May 14-17, 2014.

9. Participated in the International Conference on '**Disease Biology and Therapeutics'**, organized by IASST Guwahati held during December 3-5, 2014.

10. Attended DBT sponsored 3 day workshop on "**Recent trends in biosciences**" organized by Institutional Biotech Hub (IBT, HUB), Centre for Biosciences, Debraj Roy College, Golaghat, Assam, 2013.