AN INVESTIGATION ON THE ROLE OF LIPOCALIN RECEPTOR IN THE DEVELOPMENT 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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Dedicated to

My beautiful family

For their unceasing love, endless prayers and unwavering support

DEPARTMENT OF BIOSCIENCES 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 **"An investigation on the role of lipocalin receptor in the development 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 Prof. Ajaikumar B. Kunnumakkara.

Sincere efforts have been made to duly acknowledge the contributions from others for their ideas, technical help, references or any 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 **"An investigation on the role of lipocalin receptor in the development of lung cancer"**, submitted by Bethsebie Lalduhsaki Sailo (Roll No: 136106027) 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.

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Bethsebie Lalduhsaki Sailo

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List of Abbreviations

5-FU: 5-fluorouracil
AAH: Atypical adenomatous hyperplasia
ABL: Abelson oncogene
ACT: Anthracycline-induced cardiotoxicity
AhR: Aryl hydrocarbon receptor
AIS: Adenocarcinoma in situ
ALK: Anaplastic lymphoma kinase
AP4: Activating enhancer-binding protein-4
BaP: Benzo[a]pyrene
BCR: Breakpoint cluster region
BOCT: Brain-type organic cation transporter
BPDE: Benzo(a)pyrene-7,8-diol-9,10-epoxide
BSA: Bovine serum albumin
ccRCC: clear cell Renal Cell Carcinoma
CDC: Centers for Disease Control and Prevention
CDK: Cyclin-dependent kinase
CDKI: Cyclin-dependent kinase inhibitor
cDNA: Complementary DNA
cIAP-1: Cellular inhibitor of apoptosis protein-1
CML: Chronic myelogenous leukemia
COX-2: Cyclooxygenase-2
CRC: Colorectal Cancer
CREB: cAMP-responsive element binding protein
CRISPR: Clustered regularly interspaced short palindromic repeats
CSF: Cerebrospinal fluid
CXCR4: C-X-C chemokine receptor type 4
CYP: cytochrome P450
DAB: 3,3'-Diaminobenzidine
DMEM: Dulbecco's Modified Eagle Medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
EC: Endometrial carcinoma
ECL: Enhanced chemiluminescence
EGFR: Epidermal growth factor receptor
EML4: Echinoderm microtubule-associated protein-like 4
EMT: Epithelial-to-mesenchymal transition
ERK: Extracellular signal-regulated kinase
ER-α: Estrogen receptor alpha
ER-β: Estrogen receptor beta
ESCC: Esophageal squamous cell carcinoma
FADD: Fas-associated death domain
FAK: Focal adhesion kinase
FBS: Fetal bovine serum
FDA: Food and Drug Administration

FEN1: Flap endonuclease 1
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GBM: Glioblastoma multiforme
GWA: Genome-wide association
HCC: Hepatocellular carcinoma
HDR: High dose rate
HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HER2: Human epidermal growth factor receptor 2
HGF: Hepatocyte growth factor
HIF-1α: Hypoxia-inducible factor 1-alpha
HMC: Human mesangial cells
HPV: Human papillomavirus
HRP: Horseradish peroxidase
IAP: Inhibitor of apoptosis protein
IARC: International Agency for Research on Cancer
IHC: Immunohistochemistry
IKK: IκB kinase
IL: Interleukin
JAK: Janus-activated kinase
KLF: Kruppel-like factor
KRAS: Kirsten-Ras sarcoma viral oncogene homolog
LC3B: Light chain 3B
LCC: Large cell carcinoma
LCN2: Lipocalin-2
LCN2R: Lipocalin receptor
LDR: Low dose rate
MAPK: Mitogen-activated protein kinase
MIA: Minimally invasive adenocarcinoma
miRNA: MicroRNA
MMP: Matrix metalloproteinase
MT: Metallothionein
mTOR: Mammalian target of rapamycin
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myt1: Myelin transcription factor 1
nAChR: Nicotinic acetylcholine receptor
NFAT: Nuclear factor of activated T-cells
NF-κB: Nuclear factor-kappa B
NGAL: Neutrophil gelatinase-associated lipocalin
NGALR: Neutrophil gelatinase-associated lipocalin receptor
NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN: N-nitrosonornicotine
NSCLC: Non-small cell lung cancer
NTD: N-terminal domain
OS: Overall survival
PAH: Polycyclic aromatic hydrocarbons
PAK6: p21 (RAC1)-activated kinase 6

PBS: Phosphate buffer saline
PDAC: Pancreatic ductal adenocarcinoma
PE: Plating efficiency
Pen-Strep: Penicillin-Streptomycin
p-HH3: Phosphorylated histone H3
PI3K: Phosphoinositide 3-kinase
PKA: Protein kinase A
PKB: Protein kinase B
PKC: Protein kinase C
PMSF: Phenylmethylsulfonyl fluoride
PSC: pancreatic cancer stellate cells
PTEN: phosphatase with tensin homolog
qPCR: Quantitative Real-time PCR
RAP1: Repressor/activator protein-1
Rb: Retinoblastoma
RCC: Renal cell carcinoma
RIP: Receptor-interacting protein
RME: Receptor-mediated endocytosis
ROS: Reactive oxygen species
RT: Room temperature
RTK: Receptor tyrosine kinase
RT-PCR: Reverse transcription-polymerase chain reaction
SABR: Stereotactic ablative radiotherapy
SBRT: Stereotactic Body Radiation Therapy
SCC: Squamous-cell carcinoma
SCLC: Small cell lung cancer
SDS: Sodium dodecyl sulfate
SF: Survival fraction
SLC: Solute carrier
SLC22A17: Solute carrier family 22, member 17
SNP: Single nucleotide polymorphism
SOX: Sex-determining region Y (Sry) box
STAT: Signal transducer and activator of transcription
TBA: Tubulin-binding agent
TBST: Tris-buffered saline with 1% tween 20
TCGA: The Cancer Genome Atlas
TF: Transferrin
TFBS: Transcription factor binding sites
TGF-β: Transforming growth factor-beta
TIPE: Tumor necrosis factor-α-induced protein 8
TIPE2: Tumor necrosis factor-α-induced protein 8 like 2
TKI: Tyrosine kinase inhibitor
TKs: Tyrosine kinases
TM: Transmembrane
TMA: Tissue microarray
TNFR: Tumor necrosis factor receptor

TNF-α: Tumor necrosis factor-alpha
TNF-β: Tumor necrosis factor-beta
TNM: Tumor node metastasis
TRADD: TNFR-associated death domain
TRAF-2: TNFR-associated factor-2
VATS: Video-assisted thoracoscopic surgery
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor
µg: Microgram
µM: Micromolar
h: Hour
mM: Milimolar
nM: Nanomolar

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1

Introduction and Review of Literature

1.1. Introduction

Lung cancer remains the most commonly diagnosed malignancy worldwide. It is the leading cause of cancer-related mortality in males and the second leading cause of cancer-related deaths in females globally (Torre *et al*., 2016; Bray *et al*., 2018). According to GLOBOCAN 2018, lung cancer burden accounts for approximately 11.6% of all the new cancer cases and 18.4% of total cancer-related deaths making it the most prevalent cancer and cause of cancer-related deaths in both men and women combined (GLOBOCAN, 2018; Bade *et al*., 2020) (Figure 1.1). The incidence and mortality rate of lung cancer is closely linked to tobacco smoking patterns (Barta *et al*., 2019). The incidence of lung cancer cases markedly varies by sex, age, race, socioeconomic status, as well as geography due to the dissimilarities in historical smoking patterns. As tobacco smoking is the predominant etiological factor for lung cancer, evidently the highest lung cancer prevalence is in North America and Europe where smoking uptake originated the earliest (Torre *et al*., 2016). However, Chinese women were found to have a higher lung cancer rate than women of European countries despite a relatively lower prevalence of smoking, which is chiefly due to exposure to air pollution arising from unventilated combustion of charcoal for cooking and heating purposes (Torre *et al*., 2015; Bray *et al*., 2018). Other risk factors of lung cancer include outdoor air pollution, passive smoking, exposure to numerous occupational and environmental carcinogens such as arsenic, asbestos, radon, silica dust, polycyclic aromatic hydrocarbons, etc. (Torre *et al*., 2015; Jemal *et al*., 2011; Islami *et al*., 2015).

In India, with the increasing trend of smoking, the lung cancer epidemic accounts for 5.9% of all the new cancer cases (GLOBOCAN, 2018; Malhotra *et al*., 2018; Behera

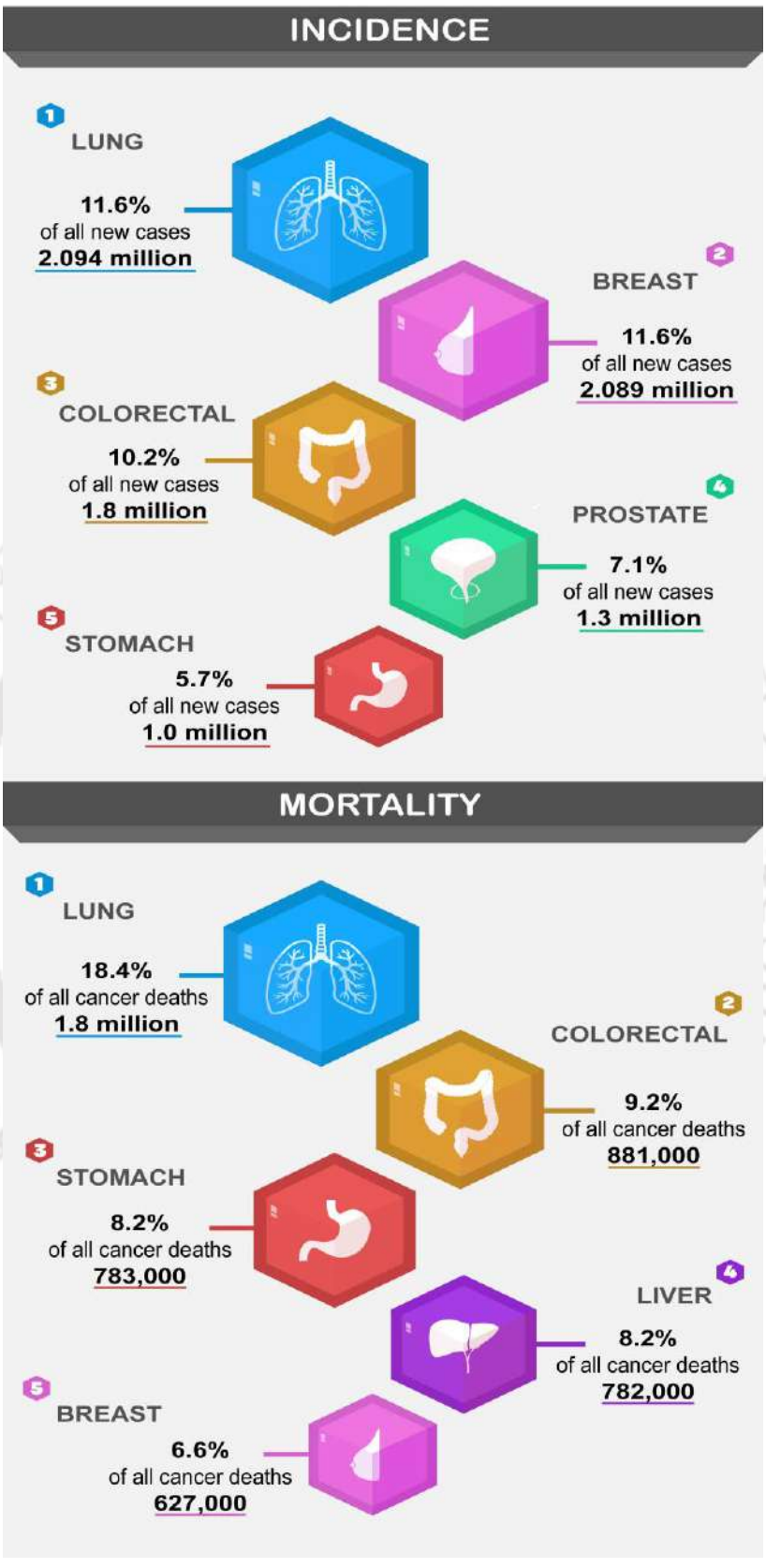

Figure 1.1. Percentages of new cancer incidence and cancer deaths worldwide in 2018 for both sexes combined (GLOBOCAN 2018)

and Balamugesh, 2004). It was found to be the most common malignancy among males. Among the states of India, the lung cancer rate was reported to be the highest in Mizoram (age-adjusted rate 28.3 and 28.7 per 100,000 populations in males and females respectively). Moreover, the time trends of this malignancy displayed a substantial increase in urban cities like Delhi, Chennai, and Bengaluru in both sexes (Malik and Raina, 2015). The recent exponential growth of economic and industrial activities in Delhi urban attributed to the elevated lung cancer incidence (Malhotra *et al*., 2018). In terms of histology of lung cancer, squamous cell carcinoma has been the most prevalent type in India whereas, in Western countries, adenocarcinoma has become more prevalent (Behera and Balamugesh, 2004).

Despite the groundbreaking advancements in diagnosis and therapeutic strategies, this aggressive disease has a dismal overall 5-year survival rate with approximately 5% in developing countries and 15% in developed countries (Malik and Raina, 2015). Late/advanced stage diagnosis due to negligible clinical symptoms at early stages, tumor relapses, lack of precise molecular marker and acquisition of chemoresistance attribute to the elevated mortality rate and poor clinical outcomes (Heuvers *et al*., 2012; Indovina *et al*., 2011; Pendharkar *et al*., 2013). Hence, the quest for a suitable diagnostic marker for early detection and molecular target for developing an effective treatment regimen has considerably intensified for the effective management of this deadly malignancy.

Recently, the solute carrier (SLC) transporter family of protein has gained increased attention as potential therapeutic targets (Lin *et al*., 2015). One member of this transporter family is a cell surface receptor known as neutrophil gelatinase-associated lipocalin receptor (NGALR) or SLC22A17/ Lipocalin-2 receptor. Studies have shown that NGALR has immense potential as a biomarker and molecular target for cancer

therapy as it has been found to be overexpressed in various human malignancies (Liu *et al*., 2018). Increasing lines of evidence have suggested that this solute carrier is upregulated in esophageal squamous cell carcinoma (ESCC), hepatocellular cancer, glioma, colorectal cancer and endometrial cancer. It has been reported that upregulation of NGALR promoted the progression of ESCC (Du *et al*., 2011); caused unfavorable clinicopathologic features with shorter overall survival in hepatocellular carcinoma (Zhang *et al*., 2012b); resulted in poor clinical outcome in glioma (Liu *et al*., 2011); induced deeper invasion and poor cellular differentiation of colorectal cancer (Lv *et al*., 2010); increased aggressiveness in endometrial cancer cells and poor survival of the patients (Miyamoto *et al*., 2011). Conversely, it has been reported that NGALR expression is downregulated in chronic myelogenous leukemia (CML) and clear cell Renal Cell Carcinoma (ccRCC) (Richardson *et al*., 2005; Liu *et al*., 2018). However, the expression of NGALR and its role in lung cancer is yet to be determined. Interestingly, in lung cancer, NGALR ligand, neutrophil gelatinase-associated lipocalin (NGAL) was found to be elevated and contributed to erlotinib resistance in non-small cell lung cancer (Krysan *et al*., 2013). Moreover, downregulation of NGAL was reported to suppress the growth of human lung adenocarcinoma (Song *et al*., 2015). However, for NGAL to exert its function, it requires the presence of its receptor, NGALR. Hence, the present study focuses on unraveling the expression and role of NGALR in the development and progression of lung cancer, which would open new opportunities for finding novel biomarkers and promising therapeutic targets to ameliorate the treatment of this aggressive malignancy.

1.2. Types of Lung Cancer

Lung cancer may originate from the respiratory epithelial cells and is an extremely invasive, rapidly metastasizing, and highly heterogeneous disease at a cellular as well as histological level. Lung carcinogenesis involves a series of morphological changes that take several years to progress from normal epithelium to an invasive one. About 70% of lung cancer cases are diagnosed at an advanced stage (stage III or IV) (Dela Cruz *et al*., 2011; Lemjabbar-Alaoui *et al*., 2015; de Sousa and Carvalho, 2018).

In order to make better therapy and prognostic decisions, lung cancer is categorized into two types based on histology i.e., small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC comprises approximately 15% of all the cases and NSCLC encompasses 85% of all the cases (Indovina *et al*., 2011; Lemjabbar-Alaoui *et al*., 2015).

1.2.1. Small Cell Lung Cancer (SCLC)

SCLC is an aggressive form of lung cancer that exhibits a dismal prognosis (Arcaro, 2015). This subtype of lung cancer cells is a small, round, or fusiform shape, scarce cytoplasm, and finely granular nuclear chromatin, and inconspicuous or absent nucleoli (Travis, 2011). SCLC is derived from the hormonal cells of the lung, existing as a central mass, and has the ability to aggressively metastasize into submucosal lymphatic vessels as well as regional lymph nodes, without invading the bronchus (Travis, 2011; Lemjabbar-Alaoui *et al*., 2015). SCLC is predominantly caused by cigarette smoking thus, SCLC incidence has tremendously declined in the past decade due to the cessation of smoking (Arcaro, 2015). SCLC patients are further grouped into limited disease and extensive disease. Patients diagnosed with limited diseases have a median survival of

16-24 months and are treated with a combination of chemotherapy and radiation therapy. Most SCLC patients are diagnosed with the extensive disease having a median survival of 7-12 months only and are treated with chemotherapy alone to which they often relapse despite initial response to it (Arcaro, 2015). However, survival is extremely poor even for the limited disease. Age at the time of diagnosis in SCLC is higher compared to never-smokers with adenocarcinoma (Torres-Durán *et al*., 2016). Comparatively, SCLC has been found to have a faster doubling time, a higher growth fraction, and faster development of widespread metastases than that of NSCLC (Kalemkerian *et al*., 2013).

1.2.2. Non-Small Cell Lung Cancer (NSCLC)

NSCLC is further subcategorized into adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma (Molina *et al*., 2008; Indovina *et al*., 2011; Lemjabbar-Alaoui *et al*., 2015). Adenocarcinoma is the most diagnosed subtype and accounts for 38.5 % of all cases of lung cancer. It arises in peripheral bronchi as a malignant epithelial tumor having glandular differentiation with production of mucin (Rodriguez-Canales *et al*., 2016). The subtypes adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA), instigate from alveoli and expands through the interalveolar connections. AIS and MIA have excellent disease-free survival post complete resection (5-year rate reaches 100%) (Lemjabbar-Alaoui *et al*., 2015). Adenocarcinoma is the most prevalent type in never-smoker patients (Rodriguez-Canales *et al*., 2016). Squamous-cell carcinoma is considered as a malignant epithelial tumor that displays morphological features such as keratinization, intercellular bridges, or squamous pearl formation (Travis, 2011; Rodriguez-Canales *et al*., 2016). SCC accounts for roughly 20 % of all the lung cancer cases and typically located in the central region, arising in a

main or lobar bronchus (Rodriguez-Canales *et al*., 2016). Large cell carcinoma (LCC) figures roughly 2.9 % of the total lung cancer cases. It is an undifferentiated carcinoma devoid of the classic glandular or squamous morphology (Rodriguez-Canales *et al*., 2016; Lemjabbar-Alaoui *et al*., 2015). LCC is not a single entity but are poorly differentiated forms of the other types and, perhaps, some truly undifferentiated "stem cell-like" tumors (Gazdar and Brambilla, 2010). LCC exists as large peripheral masses and exhibit early metastases (Collins *et al*., 2007).

1.3. Risk Factors of Lung Cancer

Lung cancer is one of the most preventable diseases as majority of this malignancy can be avoided massively by abstinence from cigarette smoking (Torre *et al*., 2015). It is well established that tobacco smoking is responsible for approximately 90% of the total lung cancer cases (Lemjabbar-Alaoui *et al*., 2015; Wang *et al*., 2015). Besides tobacco, other well-known risk factors include unhealthy diet, exposure to air pollution and occupational carcinogens, intrinsic factor such as genetic susceptibility, etc. (Figure 1.2.) and are discussed below in detail.

1.3.1. Tobacco Smoke

Tobacco smoking in all forms including cigarette, cigar, pipe smoking is the major contributor to the prevailing lung cancer epidemics (Molina *et al*., 2008; Alberg *et al*., 2007). Approximately 90% of lung cancer cases are ascribed to inhalation of cigarette smoke (Hammerschmidt and Wirtz, 2009). A study has shown that smoking amplified lung cancer risk by 5 to 10-fold with a clear dose-response relationship (Schwartz and Cote, 2016). The magnitude of cigarette smoking effect tremendously outweighed all other lung cancer etiological factors (Dela Cruz *et al*., 2011). The risk for lung cancer

elevates with the extent of smoking and the number of cigarettes consumed per day. It has been projected that an average male smoker had nearly 9 to 10-fold risk for developing lung cancer, while heavy smokers showed at least a 20-fold risk. Passive or second-hand smokers are also at great risk. It has been reported that passive smoking during childhood significantly elevates the possibility of suffering lung cancer in later life by 3.6-fold (Molina *et al*., 2008). Further, studies have also shown that tobacco metabolites are detectable in 90% of urine samples of children having smokers' parents (Pallis *et al*., 2013). Moreover, lung cancer risk augmented by approximately 20% in non-smokers upon exposure to environmental tobacco smoke (Schwartz and Cote, 2016). The smoke emerging from a cigarette is an aerosol that comprises roughly 5000 compounds (Pfeifer *et al*., 2002; Wang *et al*., 2015b). Out of these compounds, the International Agency for Research on Cancer has considered 73 compounds to be carcinogenic. There are more than 20 compounds that are specifically carcinogenic to the lung including tobacco-specific nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), polycyclic aromatic hydrocarbons (PAH), 3-butadiene, arsenic, ethyl carbamate, ethylene oxide, chromium, cadmium, nickel, polonium-210, and aromatic amines (Pfeifer *et al*., 2002; Ge *et al*., 2015; Wang *et al*., 2015b). Among these toxic carcinogens, NNK is considered the most causative agent for lung cancer (Ge *et al*., 2015). Studies have shown that cigarette smoke stimulates epithelial cells and macrophages and elicits pulmonary inflammation and secretion of inflammatory cytokines and chemokines including transforming growth factor-β (TGF-β), tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, etc. (Wang *et al*., 2015b). Moreover, these carcinogens are covalently bound with DNA and form DNA adducts consequently causing mutation leading to lung carcinogenesis (Hecht, 2012; Amos *et al*., 1999).

1.3.2. Diet

It has been found that consumption of fruits and vegetables, especially cruciferous vegetables may exhibit a protective role against lung cancer. Isothiocyanates are a group of natural compounds found in cruciferous vegetables that has a significant anti-cancer property. Contrariwise, poor diets such as high intake of processed meat, deep-fried food, and chili are known to elevate the risk of lung carcinoma (Molina *et al*., 2008). In addition, consumption of alcohol increases the susceptibility to this disease (Freudenheim *et al*., 2005). However, due to the tight correlation between consumption of alcohol and tobacco smoking in several populations, it is challenging to clarify the exact contribution of alcohol to lung cancer alone while controlling for the possible confounding tobacco effect (Malhotra *et al*., 2016).

1.3.3. Exposure to Air Pollution and Occupational Carcinogens

Exposure to air polluted with carcinogenic compounds such as various polycyclic aromatic hydrocarbon compounds, indoor radon, vapor from cooking oil, and unventilated combustion of coal for domestic are associated with the development of lung cancer purposes (Molina *et al.*, 2008; Jemal *et al*., 2011; Pallis *et al*., 2013). It has been estimated that approximately 17% of annual premature lung cancer mortality in adults is attributed to exposure to carcinogens arising from cooking with solid fuels such as charcoal, coal, wood, etc. (Hashim and Boffetta, 2014). Many work environments have exposed workers to numerous carcinogens causing higher risks of lung cancers. It has been found that exposure to occupational carcinogens such as silica dust, asbestos fiber, uranium, arsenic, chromium, nickel, radon wood dust, diesel exhaust are also risk factors for lung cancer (Ruano-Ravina *et al*., 2003; Alberg *et al*., 2005; Molina *et al.*, 2008). Moreover, workers at uranium mines and nuclear plants

have an increased lung cancer risk due to constant exposure to radioactive particulate mass including radon and its decay products (Molina *et al*., 2008; Malhotra *et al*., 2016). Besides, increasing epidemiological studies have demonstrated a strong association of exposure of inorganic and organic arsenic to lung cancer risk (Hashim and Boffetta, 2014).

1.3.4. Intrinsic Factors

Intrinsic factors such as age, family history, and genetic susceptibility also contribute to the development of this disease (Young *et al*., 2009). The highest incidence of lung cancer peaks at around 65 years of age and declines after the age of 80 years. This decline with age after 80 may be due to less smoking habit or due to the development of resistance (Ruano-Ravina *et al*., 2003). Epidemiological studies showed that there are approximately 2.5 folds higher chances of lung cancer in families with a history of the disease after controlling for tobacco smoke (Amos *et al*., 1999; Pallis *et al*., 2013). A meta-analysis has demonstrated a 2-fold elevated lung cancer risk for persons having a family history of lung cancer with a higher risk in non-smokers as well (Dela Cruz *et al*., 2011). A genome-wide association (GWA) studies have identified multiple genetic polymorphisms underlying lung cancer risk (Malhotra *et al*., 2016). The major susceptibility locus that influences lung cancer risk is found to be at 6q23–25p (Molina *et al*., 2008). In 2008, three independent GWA studies reported that single nucleotide polymorphism (SNP) variations at chromosome locus 15q24-q25.1 were linked with an elevated risk of nicotine dependence as well as lung cancer development. Further, two meta-analyses have shown that variations at 15q25.1, 5p15.33, and 6p21.33 are linked with lung cancer risk (Larsen and Minna, 2011). Besides, the correlation of lung cancer with mendelian cancer syndromes, including Bloom and Werner syndromes were also

reported. Additionally, polymorphisms in genes associated with DNA repair enzymes active in nucleotide excision repair (ERCC1, XPD, and XPA), base excision repair (XRCC1 and OGG1), and double-strand break repair (XRCC3), and diverse mismatch repair pathways are also reported to be associated with the risk of lung cancer (Dela Cruz *et al*., 2011).

1.3.5. Other Factors

Infection with human papillomavirus (HPV)16/18 and previous respiratory diseases like chronic obstructive pulmonary disease (COPD) and tuberculosis are associated with increased lung cancer risk. In females, expression of hormone estrogen receptor-α (ER-α) expression and lack of estrogen receptor -β (ER-β) are reported to link with the poor prognosis in NSCLC (Kawai *et al*., 2005). HIV/AIDS patients also have a high risk of this disease (McErlean *et al*., 2011).

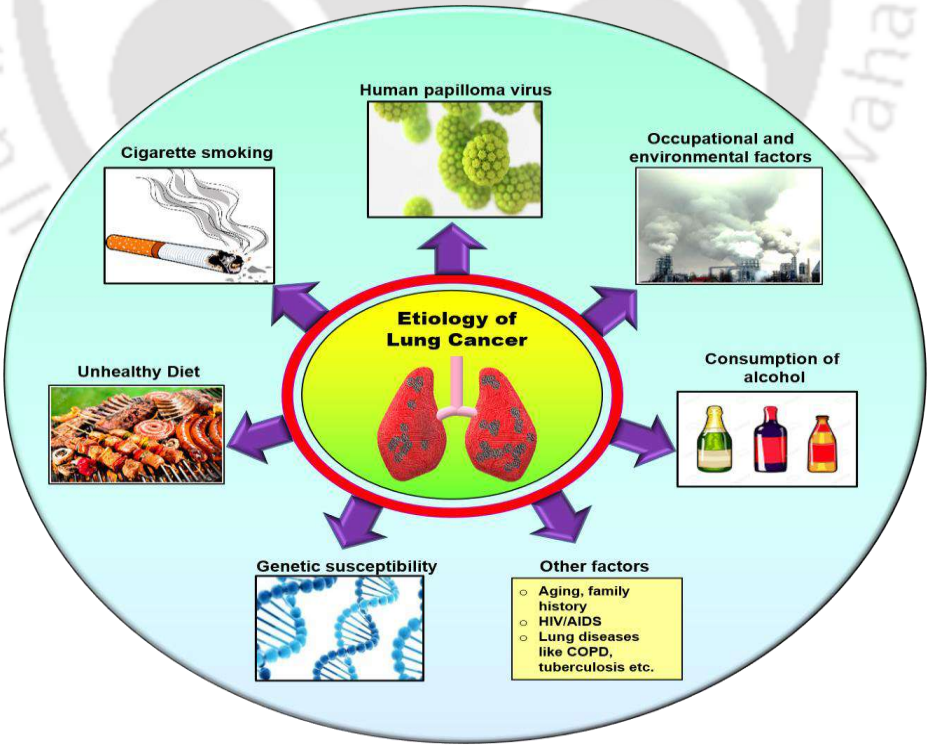

Figure 1.2. Risk factors that contribute to lung cancer development

1.4. TNM Classification of Lung Cancer

Accurate staging of lung cancer is imperative for delivering efficacious therapy and improve the outcome of lung cancer patients (Carter *et al*., 2018). TNM staging remains the most essential prognostic factor for predicting recurrence rates as well as survival times (Woodard *et al*., 2016). Lung cancer staging based on a TNM staging system (T stands for characteristics of the primary tumor, N for nodal involvement, and M for distant metastasis) developed by the International Association for the Study of Lung Cancer (IASLC) relied on a statistical analysis of an international database comprising of more than 100,000 lung cancer patients (Detterbeck, 2018; Carter *et al*., 2018). The staging system precisely addressed the T, N, and M components; the stage groups; and the methodology and validation used for lung cancer (Detterbeck, 2018) (Table 1.1).

1.5. Preneoplastic Lesions of Lung Cancer

Lung cancer is an extremely intricate neoplasm both biologically and histologically that takes several years or decades to develop, preceded by series of sequential preneoplastic changes (Wistuba *et al*., 2006; Gazdar *et al*., 2010). The development of invasive lung cancer may comprise of two different but complementary processes. On one hand, exposure of the airway epithelium to carcinogens from smoking induces extensive cellular and molecular changes that lead to the accumulation of mutations and epigenetic alterations resulting in the formation of dysplasia and eventually invasive carcinoma, a process known as “multistep carcinogenesis”. Also, it has been proposed that airway epithelial lesions may be achieved from a single progenitor cell that has gone through either mutation or epigenetic alterations and subsequent clonal expansion

Table 1.1. TNM Stage 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 + 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.

wherein a new premalignant lesion is formed at a site away from its origin (Hirsch *et al*., 2002). The three major morphologic forms of preneoplastic lesions observed in the lung are atypical adenomatous hyperplasia (AAH), squamous dysplasia, and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia, which account for the development of certain lung cancers subtypes (Wistuba *et al*., 2006). Bronchial dysplasia and AAH are considered as the preneoplastic lesions for squamous cell carcinoma and adenocarcinoma respectively (Hirsch *et al*., 2002). In the case of adenocarcinoma, AAH progresses to preinvasive AIS which subsequently leads to invasive adenocarcinoma (Yatabe *et al*., 2011; McWilliams *et al*., 2002). In the case of the bronchial epithelium of smokers, the preinvasive changes in the bronchial epithelium include i) mild dysplasia which has moderately increased risk ii) severe dysplasia which has high-risk lesions leading to iii) carcinoma *in situ* (McWilliams *et al*., 2002; Gazdar and Brambilla, 2010) (Figure 1.3).

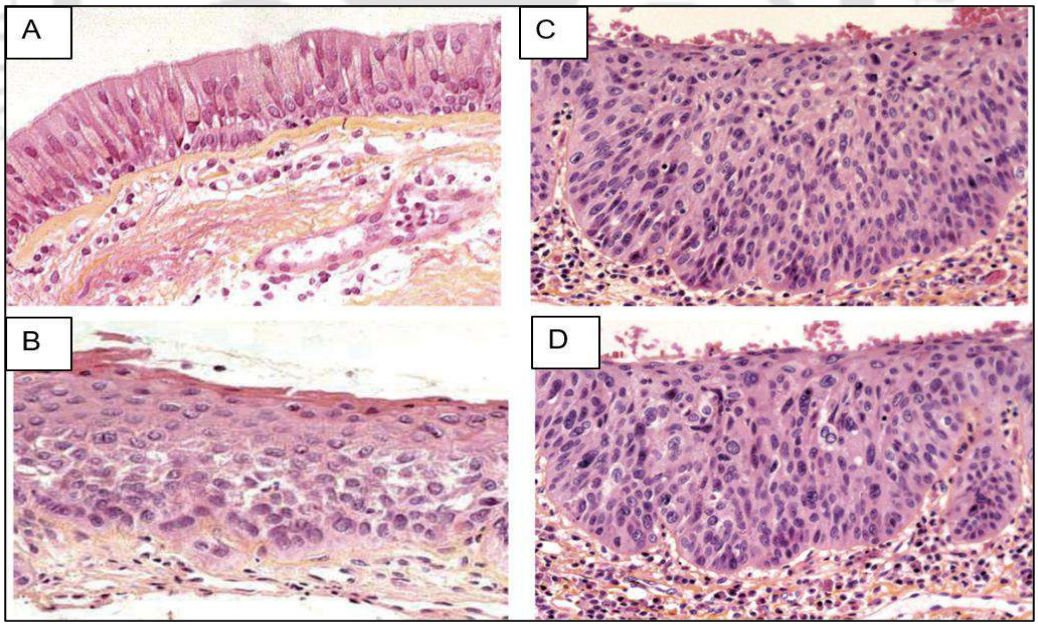

Figure 1.3. Preinvasive changes in the bronchial epithelium of smokers: (A) Normal bronchial epithelium comprised of basal cells, ciliated cells and goblet cells. (B) Mild dysplasia: cell atypia and architectural alteration at the lower one third of a squamous metaplastic epithelium. (C) Severe dysplasia: increased thickness and cytological atypia on epithelium. (D) Carcinoma in situ: cyto-architectural atypia and lack of progressive maturation and orientation from basal to upper layer of epithelium (Gazdar and Brambilla, 2010).

1.6. Molecular Alterations in Lung Cancer

Lung cancer pathogenesis involves a series of molecular alterations at numerous levels (genetic, epigenetic, protein expression) that take place over several years (Massion and Carbone, 2003; Cooper *et al*., 2013). Several mutations in various oncogenes cause alterations in their protein structures, copy number, and abnormal combination of proteins which ultimately lead to tumor development (Cooper *et al*., 2013). Significant targetable mutations in lung cancer include overexpression of growth-promoting proteins such as BRAF, Kirsten-Ras sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), and human epidermal growth factor receptor 2 (HER2), and phosphatidylinositol 3-kinase (PI3K) pathway, ALK rearrangement as well as inactivation or aberration of tumor suppressor genes such as phosphatase and tensin homolog (PTEN), p53, etc. (Cooper *et al*., 2013; Devarakonda *et al*., 2015). Particularly in lung adenocarcinoma pathogenesis, smoking-related activation of RAS signaling, and nonsmoking-related activation of EGFR signaling are the two distinct molecular pathways detected (Wistuba *et al*., 2006). EGFR is a cell-surface receptor tyrosine kinase (RTK) that activates the RAS/RAF/MEK/ERK pathway and initiates enhancement of angiogenesis, invasion, and metastasis. Roughly, 62% of NSCLC patients are characterized by overexpression of the *EGFR* gene and often correlated with poor prognosis (Webb *et al*., 2010). *EGFR* mutations occur within exon 21 and deletions of exon 19 cover more than 90% of lung cancer-specific changes. Studies have also shown that EGFR-mutation is frequent in females, and prevalent in adenocarcinoma type of lung cancer, in non-smokers, and in Asian ethnicity (Wood *et al*., 2015). Gefitinib and Erlotinib are the two Food and Drug Administration (FDA) approved tyrosine kinase inhibitors (TKIs) that target EGFR, and have been used for treating advanced cases of

NSCLC resistant to conventional chemotherapeutic drugs (Webb *et al*., 2010). Activation of the RAS/RAF/MEK/MAPK pathway occurs regularly in lung cancer through *KRAS* gene mutation which is observed exclusively in 10– 50 % of lung adenocarcinoma and seldom observed in squamous cell carcinoma and SCLC (Webb *et al*., 2010). *KRAS* mutation is limited to NSCLC (mostly in adenocarcinoma) and is frequently seen in neoplasms arising from smokers and scarcely seen in patients of East Asian origin (Webb *et al*., 2010; Larsen *et al*., 2011). Mutation in *KRAS* occurred in codons 12, 13, or 61. Amongst them, codon 12 mutation is the most prevalent one accounting for 80% and resulted in the substitution of glycine with either cytosine (Gly12Cys) or valine (Gly12Val) (Larsen *et al*., 2011; Devarakonda *et al*., 2015). This mutation causes constitutive upregulation of downstream molecules including PI3K and MAPK, making KRAS mutant lung tumors independent of EGFR signaling and thus resistant to EGFR-TKIs and chemotherapy (Larsen *et al*., 2011). Mutation of the p53 gene occurs in over two-thirds of the lung and is mostly observed in squamous cell carcinoma and SCLC, and is also seen in 70% of NSCLC (Yang *et al*., 2015; Massion and Carbone, 2003). It is also reported that the *hTERT* gene is overexpressed in 57% of NSCLC (Lemjabbar-Alaoui *et al*., 2015). Apart from these, fusion proteins have also been reported in NSCLC including EML4-ALK fusion (Wood *et al*., 2015). This novel fusion protein is formed on chromosome 2p by the inversion of two closely located genes, i.e., a fusion between echinoderm microtubule-associated protein like-4 (EML4) and anaplastic lymphoma kinase (ALK). This fusion leads to constitutive oligomerization resulting in malignant transformation and most predominant in adenocarcinomas and non or light smokers (Larsen *et al*., 2011). Besides these, the chromosomal aberration is also seen in lung cancer including aneuploidy, specific allelic loss at 3p, 4q, 9p, and 17p, and gain at 1q, 3q, 5p, and 17q63 (Larsen *et al*.,

2011). Particularly in NSCLC, loss of genomic regions of chromosomes 3p and 9p is observed in preinvasive lesions and the normal epithelium of smokers. In the case of squamous cell carcinoma, amplification of chromosomal region 3q26 and deletion of chromosome 3p have been found to be prevalent (Massion and Carbone, 2003).

1.7. Treatment Modalities for Lung Cancer

With the alarming upsurge in lung cancer-related mortality worldwide, treatment of lung cancer remains a challenge as the benefit of conventional treatment approaches has reached a plateau thus necessitating new and efficacious treatment regime (Steven *et al*., 2016).

1.7.1. Surgery

In the epoch of personalized treatment, surgery has remained the most consistent and curative therapy to combat lung cancer and has a high success rate for patients with lung cancer provided the tumor is resectable (early stage, stages I and II) (Molina *et al*., 2008). The 5-year survival is approximately 77–92% for clinical stage IA, 68% for stage IB, 60% for stage IIA, and 53% for stage IIB (Hirsch *et al*., 2017). Surgery followed by chemotherapy is the standard treatment approach for patients with resectable stage IIIA NSCLC (Lemjabbar-Alaoui *et al*., 2015). Surgery is also used for lung cancer patients with locally advanced disease, recurrent oligo-metastasis after pulmonary resection, N2 lymph node metastases, or relapsed tumors after chemotherapy (Suda *et al*., 2014). In NSCLC, lobectomy is the standard surgical approach with an operative mortality of 1.3% (Molina *et al*., 2008). Five-year survival rates post-surgical resection are between 60% to 80% for stage I NSCLC and for stage II NSCLC patients it ranges from 30% to 50% (Lemjabbar-Alaoui *et al*., 2015).

Minimally invasive surgical techniques, including video-assisted thoracoscopic surgery (VATS) and robotic surgery and sub-lobar resection including wedge resection and segmentectomy, have been recently utilized for lung cancer management (Suda *et al*., 2014). The advantage of surgical treatment is that it provides adequate tumor samples for molecular analysis, which aid in drug selection in the adjuvant therapeutic strategy or after disease recurrence (Suda *et al*., 2014).

1.7.2. Chemotherapy

Most lung cancer patients are usually diagnosed at a locally advanced or metastatic stage, thus making surgery, not a suitable treatment option. In such cases, chemotherapeutic treatment becomes highly effective to destroy the cancer cells and prolong the patients' life and thus has become the mainstay of care for advanced lung cancer cases (Molina *et al*., 2008; Yang *et al*., 2015; Steven *et al*., 2016). Adjuvant chemotherapy is generally applied for resected stages IIA- IIIA NSCLC patients which are known to prolong the overall survival rate in clinical settings (Lemjabbar-Alaoui *et al*., 2015). Over the past several decades, cytotoxic agents with platinum (cisplatin or carboplatin)-based therapy remains the standard-of-care for NSCLC patients as it yields better response (Lemjabbar-Alaoui *et al*., 2015; Noonan *et al*., 2015; Zhang *et al*., 2019). Cisplatin is considered the most effective drug for all lung cancer patients (Willers *et al*., 2013). First-line platinum-based therapy may comprise of doublets of cisplatin or carboplatin combined with antimetabolites (gemcitabine, pemetrexed), tubulin-binding agent (TBA) (paclitaxel, docetaxel) or vinca alkaloids (vinblastine) to alleviate lung cancer (Chang *et al*., 2011; Lemjabbar-Alaoui *et al*., 2015). Moreover, the addition of antiangiogenic drugs such as nintedanib to chemotherapeutic agents has been found to significantly improve the survival rate in NSCLC patients (Noonan *et al*.,

2015). In the case of SCLC, combined therapy consisting of platinum and etoposide is predominantly used. Studies showed that despite 80–90% of SCLC patients being responsive to chemotherapy, the rapid development of chemoresistance makes them uncurable (Xiao *et al*., 2014). Novel chemotherapeutics are also being developed, such as platinum analogs including picoplatin (a cisplatin analog), ABT-751 (sulfonamide), etc. (Chang, 2011).

1.7.3. Radiation Therapy

The first major trial of radiation therapy for the management of unresectable lung cancer was executed by the Veterans Administration Lung Cancer Study Group wherein both SCLC and NSCLC patients were randomly subjected to thoracic radiation therapy or a placebo. The survival was considerably higher with radiation therapy than with placebo (Molina *et al*., 2008). Radiation therapy has become the fundamental treatment approach for patients with SCLC and promising care for the management of early stages of lung cancer (Videtic, 2013; Parashar *et al*., 2013). It has been conventionally used for patients who are declined for standard surgical resection. Stereotactic radiation therapy exhibits remarkable local control rates with favorable survival for patients in the early stage with less toxicity. Brachytherapy is another technique that enables high local control rates in operable patients. It includes a high dose rate (HDR) and low dose rate (LDR) brachytherapy (Parashar *et al*., 2013). Isotopes that have been used for radiation therapy include iodine-125, palladium-103, and cesium-131. Stereotactic Body Radiation Therapy (SBRT) is used for exposure of tumors to a high dose of radiation without harming the normal tissues. It is mostly employed for tumors size greater than 5 cm without lymph node involvement (Parashar *et al*., 2013). Stereotactic ablative radiotherapy (SABR) has been recognized as a

curative therapy for peripheral early-stage NSCLC patients who are medically inoperable or at high susceptibility for complications during surgery (Baker *et al*., 2016). Notably, a combination of cisplatin-based chemotherapy with radiation therapy yield a better prognosis than radiation therapy alone (Molina *et al*., 2008). Recently, it has been shown that SABR followed by chemotherapy has become a promising treatment regimen for stage I SCLC (Gensheimer and Loo, 2017).

1.7.4. Targeted Therapy

The emergence of therapies targeting specific oncogenic-driver proteins has revolutionized the management of cancer. Targeted therapies elicit substantial responses in several cancer subtypes, thereby improve the outcome of patients (Mayekar and Bivona, 2017). It is well established that lung cancer involves alteration in multiple cellular signaling and regulatory pathways including RTKs such as EGFR, and modulation of angiogenesis pathways, apoptosis, etc. In 40-80% of NSCLC patients, EGFR has been found to be overexpressed and is directly associated with poor prognosis (Krysan *et al*., 2013). TKIs specific to the activated EGFR such as erlotinib and gefitinib are highly effective targeted therapy used for NSCLC (Krysan *et al*., 2013; Steuer *et al*., 2014). Also, a combination of the monoclonal antibody, bevacizumab which targets vascular endothelial growth factor (VEGF), with chemotherapy has been used for the treatment of NSCLC (Molina *et al*., 2008). Crizotinib therapy has been used for the management of patients with advanced, ALK-positive NSCLC (Lemjabbar-Alaoui *et al*., 2015). Other ALK inhibitors such as ceritinib and alectinib are considered more potent than crizotinib and inhibit on-target mechanisms of resistance to crizotinib. Ceritinib is approved by the FDA for metastatic ALK-positive NSCLC patients who are intolerant to crizotinib or had advanced post-treatment with

crizotinib (Mayekar and Bivona, 2017). In the case of KRAS mutated lung adenocarcinoma, MEK inhibitors such as trametinib and selumetinib have been shown to be effective when combined with chemotherapy (Hirsch *et al*., 2017). In the case of SCLC, topotecan has been used for second-line treatment. Due to the rapid development of chemoresistance in SCLC, all patients of any stage receive a doublet combined treatment of etoposide (or irinotecan) plus cisplatin or carboplatin (Hirsch *et al*., 2017).

1.8. Problems Associated with Lung Cancer Therapies

Despite the advancements in the treatment in the last several decades, the prognosis of lung cancer patients remains unsatisfactory (Lemjabbar-Alaoui *et al*., 2015). Various factors such as the development of chemoresistance, tumor relapse, late-stage diagnosis have significantly contributed to the poor clinical outcome of lung cancer patients**.**

1.8.1. Chemoresistance

Management of lung cancer remains a challenge today due to the acquisition of chemoresistance. Most lung cancer patients receive chemotherapy post-surgery and for those with the metastatic stage of lung cancer, chemotherapy is the only effective treatment option. However, these chemotherapies failed to elicit their effect due to the development of the resistance by cancer cells/tissues against apoptosis imposed by these genotoxic agents (Yang *et al*., 2015). It has also been revealed that NSCLC patients will eventually develop chemoresistance during their course of treatment. Resistance against chemotherapeutic agents like doxorubicin, docetaxel, etoposide, gemcitabine, paclitaxel, topotecan and vinorelbine have been reported in NSCLC cases (Chang, 2011). It is reported that mutation in p53 and KRAS led to the activation of

NF-κB that promotes chemoresistance in lung cancer cells via dysregulation of cell cycle and inhibition of apoptosis (Yang *et al*., 2015). Aforementioned, SCLC has a low curative rate due to rapid chemoresistance development which may be mediated by the HOXA1 gene that is regulated by microRNA-100 (Xiao *et al*., 2014). Additionally, resistance to EGFR-TKIs such as erlotinib arises due to the T790M mutation in the EGFR which resulted in modulation of the binding kinetic of erlotinib to EGFR (Sangodkar *et al*., 2010). Other mechanisms that render resistance to EGFR inhibitors include hyperactivation of downstream signaling such as activating mutations of proto-oncogene RAS, which subsequently activated MAPK/ERK signaling pathway, thereby enhancing cancer cell proliferation and survival (Sangodkar *et al*., 2010).

1.8.2. Tumor recurrence

Despite successful radical treatment, lung cancer patients frequently suffer fatal tumor recurrence. Tumor relapses occur after surgery in 30-70% of lung cancer patients in stages I and II after suitable treatment mainly due to systemic disease, which despite intensive treatment leads to death (Chudacek *et al*., 2014; Caulo *et al*., 2012). As SCLC highly is highly aggressive, it is substantially susceptible to relapse (Xiao *et al*., 2014). In NSCLC, most of the postoperative recurrence involves distant metastasis with or without locoregional recurrence (Yano *et al*., 2014). Local recurrence after surgery increases with the stages of primary cancer, however, even stage I patients suffer local recurrence up to 19% of the time. Such postoperative recurrence occurs very rapidly approximately 50–90% occurs within 2 years post initial operation and 90–95% occurs within 5 years (Fedor *et al*., 2013).

1.8.3. Late-stage Diagnosis

Although lung cancer therapies may have high curative potential in the early stages, approximately 70% of lung cancer patients are diagnosed at an incurable advanced or metastatic stage, mostly at stage III or IV due to negligible symptoms at an early stage (Lemjabbar-Alaoui *et al*., 2015; Horeweg and de Koning, 2014). Thus, overall survival remains extremely low. In the case of SCLC, it is reported that the 5-year survival rate remains less than 7% and most patients survive for only 1 year or less after diagnosis (Byers *et al*., 2015).

Hence, to overcome these drawbacks, it is imperative to identify novel biomarkers for early diagnosis and promising molecular targets which will pave way for the development of highly efficacious therapeutic approaches for better management of this aggressive malignancy. Interestingly, the lipocalin receptor, NGALR holds profound prospects as a promising biomarker and molecular target for cancer.

1.9. Neutrophil Gelatinase Associated Lipocalin Receptor (NGALR)

NGALR, also known as solute carrier family 22, member 17 (SLC22A17), 24p3 receptor (24p3R), brain-type organic cation transporter (BOCT), and lipocalin receptor-2 (LCN2R) is a 57.68 kDa protein belonging to the organic cation transporter family (Chia *et al*., 2015). In humans, the *NGALR* gene is located in chromosome 14q11.2 with 10 exon counts. It is a cell surface receptor for NGAL/Lipocalin-2 (LCN2) and belongs to a major facilitator superfamily (MFS) having transmembrane domains and facilitates the transport of NGAL/LCN2 across cytoplasmic or internal membranes by the process of endocytosis (Miyamoto *et al*., 2011; Fang *et al*., 2007). Its ligand NGAL is a glycoprotein found in granules of neutrophils and is involved in the transport and

delivery of iron and also plays a crucial role in the innate immune response during bacterial infection by sequestering iron (Chakraborty *et al*., 2012; Flo *et al*., 2004). The mouse homolog of NGAL is 24p3 and the mouse homolog of NGALR is identified as 24p3R (Zhang *et al*., 2012a). In 2005, Devireddy *et al*. isolated the first 24p3R from mouse FL5.12 cell. The deduced 24p3R has a single open reading frame of 520-amino acid containing 12 transmembrane domains (Devireddy *et al*., 2005). In 2007, Fang *et al*. reported that NGALR has three splices variants NGALR1, NGALR2, and NGALR3. It was found that NGALR3 and NGAL interact *in vivo* and co-localize mainly at the cell membrane (Fang *et al*., 2007). In the rodent kidney, this receptor for LCN2 was detected apically in distal convoluted tubules and collecting duct and most abundantly in the medulla (Betten *et al*., 2018). It was demonstrated that in normal human tissues, expression of NGAL and NGALR were found in the epithelium of the adrenal gland, renal tubule, kidney, and thymus, but were not found to be present in the heart, liver, and thyroid gland (Zhang *et al*., 2012a). Further, it was also reported that NGAL and NGALR exhibit different expression patterns in human embryonic, fetal and normal adult tissues, and their expressions were tissue and cell-specific throughout human development (Zhang *et al*., 2012a). Studies have shown that the N-terminal domain (NTD) of NGALR/LCN2-R is an intrinsically disordered, soluble extracellular domain that interacts with apoNGAL/LNC2, forming a fuzzy complex. The affinity between the human LCN2-R-NTD and apoNGAL is found to be a relatively weak affinity (≈10 μm) which explains that the N terminus of NGALR alone cannot be held accountable for the internalization of NGAL. Nonetheless, human LCN2-R-NTD may help in the fine-tuning of the interaction of NGALR with its ligand or assist NGALR in discriminating between apo- and holo-NGAL (Cabedo Martinez *et al*., 2016).

1.9.1. Topology of NGALR

Aforementioned, NGALR belongs to the SLC22 family of organic ion transporters, which are transmembrane proteins that usually facilitates the transport of small charged or polar molecules and typically comprises of 12 transmembranes (TMs) helical segments that are arranged in two bundles of six TMs each (TM1 and TM2) joined by a large intracellular loop. SLC22 transporters also possess a large soluble extracellular domain situated between TM1 and TM2 that usually includes two-three *N*-glycosylation sites and an intracellular C-terminal domain (Figure 1.4 A). However, sequence alignments revealed that NGALR displayed an atypical feature that differs from the canonical SLC22 sequence. The NGALR exhibits a unique putative topology wherein the first 100 residues form an extracellular soluble domain that consists of two *N*-glycosylation sites followed by the first bundle of five TMs, a large intracellular loop, and the final bundle of six TMs, and an intracellular C-terminal domain (Figure 1.4 B). However, in the topology initially proposed by Devireddy *et al*., the soluble extracellular N-terminal region spreads up to residue 33 followed by TM1 (projected as a soluble region in the other topology) and a relatively large intracellular loop. This alternative topology also exhibits a significant extracellular large loop between the two TM domain bundles and an extracellular C-terminal domain (Cabedo Martinez *et al*., 2016) (Figure 1.4 C).

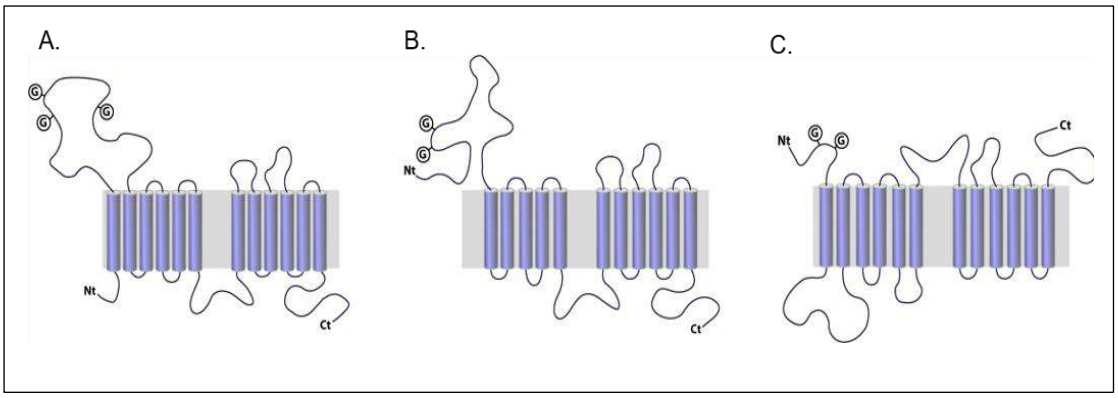

Figure 1.4. Topology of NGALR, A. Canonic topology of the SLC22 family of organic cation transporters, B. Proposed topology of NGALR, C. Alternative topology proposed by Devireddy *et al*. (Cabedo Martinez *et al*., 2016).

1.9.2. Function of NGALR

NGALR function is dependent on the status of its ligand NGAL. NGALR along with NGAL represent a novel iron transporting pathway that is imperative for the survival, growth and maturation of cells (Zhang *et al*., 2012a). It was demonstrated that depending on the iron content of its ligand 24p3, ectopic 24p3R expression governs whether the cell will undergo either iron uptake and inhibit apoptosis or induce apoptosis (Devireddy *et al*., 2005). 24p3R can bind both to holo form (loaded with iron) and apo form of 24p3 (without iron) and internalize by endocytosis (Monisha *et al*., 2014). The iron complex of the bacterial siderophore, enterobactin bound to the holo 24p3, whereas the apo form lacks this complex. When holo-24p3 binds to 24p3R and gets internalized, and detaches its bound iron, thus augmenting the intracellular iron level and evades apoptosis by hindering proapoptotic protein Bim (Richardson, 2005; Devireddy *et al*., 2005). In contrast, internalization of apo-24p3 subsequently gets associated with an intracellular mammalian siderophore, chelates the intracellular iron and transports it to the extracellular medium by exocytosis, thus intracellular iron level reduces, stimulating apoptosis via inducing the expression of Bim (Devireddy *et al*., 2005; Richardson, 2005). However, it is unclear whether apo- and holo-24p3 attaching

to the 24p3R follow similar intracellular trafficking routes, thus rendering further studies. Moreover, when holo-24p3 donates its iron for metabolism inside the cell, it is presumed that the apo-24p3 generated would undergo exocytosis. Nonetheless, this process remains ambiguous as the apo form can receive iron from putative intracellular mammalian siderophore. Thus, in-depth studies are prerequisites to elucidate whether holo- and apo-24p3 compete for 24p3R binding (Richardson, 2005).

It was reported that NGALR/24p3R is found to be expressed in the distal nephron of the kidney and facilitates protein endocytosis. 24p3R binds with high affinity to proteins filtered by the kidney and may facilitate receptor-mediated endocytosis (RME) of the proteins. As NGAL/24p3 is known to play an important role against bacterial infection by sequestering iron siderophores from the bacteria, the NGALR/24p3R in the distal nephron mediated this iron sequestration into epithelia and hence eliciting bacteriostasis (Langelueddecke *et al*., 2012). Additionally, it has been reported that in human mesangial cells (HMC), NGALR is involved in inflammation of HMC wherein NGALR expressions are enhanced by proinflammatory cytokine IL-1β *in vitro* via MAPK/ERK activation (Mao *et al*., 2011). Thus, NGALR expression was found to be upregulated in renal glomeruli of lupus nephritis and acute proliferative glomerulonephritis compared to normal kidney tissue (Mao *et al*., 2011). Further, 24p3R was also found to facilitate endocytosis of albumin in the collecting duct and activate NF-κB and TGF-β1 signaling pathways in murine CCD (mCCDcl1) cells *in vitro* (Dizin *et al*., 2013). Besides, *in vivo* studies showed the expression of NGALR in the luminal membranes of the mammalian intestinal mucosa, with higher expression in the ileum and colon and possibly assist in regulating host-microbiota homeostasis (Langelueddecke *et al*., 2013). It is also reported that NGALR may function as a high-affinity multi-ligand receptor in the lower intestine for apical internalization and

transcytosis of intact proteins/peptides such as transferrin (Tf), metallothionein (MT), etc. (Langelueddecke *et al*., 2013).

1.10. NGALR in Cancers

Cancer encompasses a group of neoplastic diseases characterized by perturbations in several molecular signaling pathways and alterations of more than 500 genes (Khatoon *et al*., 2020). Increasing lines of evidence strongly suggested that NGALR and its ligand NGAL play invaluable roles in the development and progression of various human malignancies. Importantly, NGALR expression is reported to be altered in various cancers including colon cancer, CML, esophagus cancer, endometrial cancer, glioma, renal cancer, liver cancer, etc. (Figure 1.5).

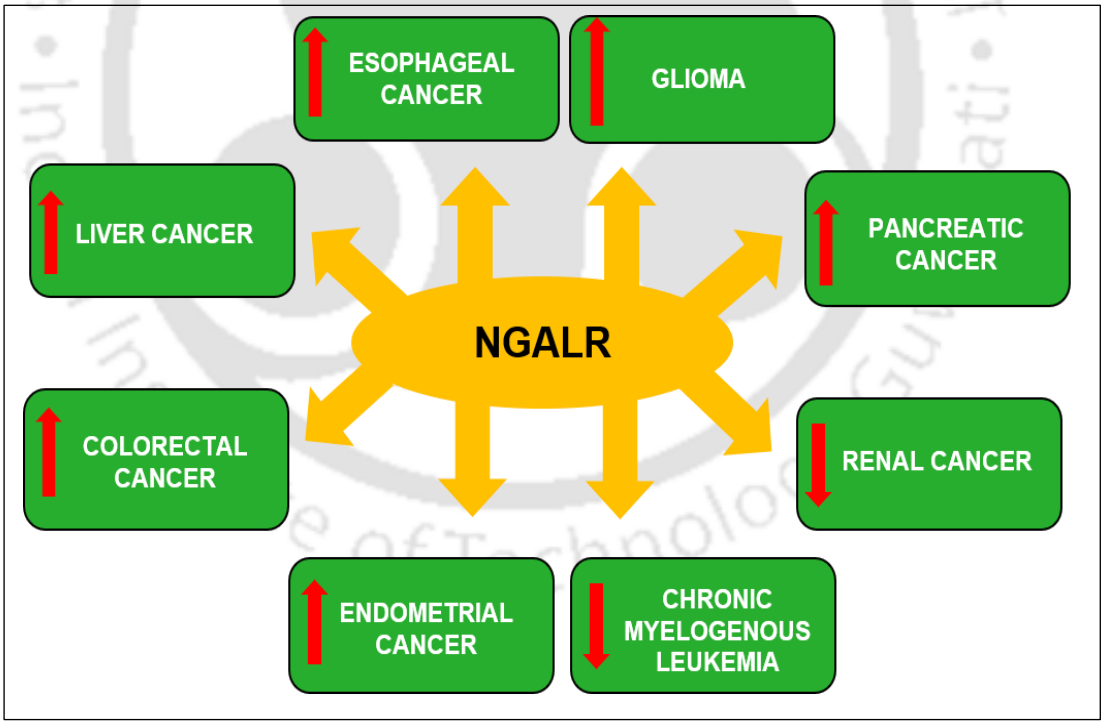

Figure 1.5. Expression profile of NGALR in various cancers

1.10.1. NGALR in Chronic Myelogenous Leukemia

Chronic myeloid leukemia (CML) is a cancer of the white blood cells that is characterized by a genetic translocation that involves a fusion of Abelson oncogene (ABL) from chromosome 9q34 with the breakpoint cluster region (BCR) gene from chromosome 22q11.2 thereby forming BCR-ABL fusion oncogene (Monisha *et al*., 2014; Jabbour and Kantarjian, 2018). Devireddy *et al*. reported that BCR-ABL misregulated the expression of both NGALR and NGAL in CML. It was found that in CML patients, NGAL was massively upregulated whereas NGALR was significantly downregulated by BCR-ABL. Forced expression of NGALR was found to promote apoptosis *in vivo*, indicating that downregulation of NGALR is imperative for survival along with the presence of the NGAL secreted in the conditioned medium (Devireddy *et al*., 2005). This misregulation by BCR-ABL may contribute to the severity and progression of CML by abrogating normal hematopoiesis (Richardson, 2005; Devireddy *et al*., 2005). Interestingly, treatment with the BCR-ABL inhibitor, imatinib, which is the first-line treatment for BCR–ABL+ CML patients, resulted in upregulation of the NGALR, thereby inducing apo-NGAL-mediated apoptosis (Richardson, 2005). It was later revealed that 24p3R/NGALR expression is regulated by the transcription factors, Runx and that BCR-ABL instigated a switch in binding from Runx3, an activator of NGALR, to Runx1, a repressor of NGALR, via Ras signaling pathway (Sheng *et al*. 2009).

1.10.2. NGALR in Colorectal Cancer

Colorectal cancer (CRC) is the second most prevalent cause of cancer-related mortality worldwide in both sexes combined hence, there is an urgent need for more efficacious therapy for CRC patients (Bray *et al*., 2018). It was demonstrated that NGALR

expression was significantly upregulated in CRC tissues than in normal colorectal tissues. This overexpression of NGALR was directly linked with deeper invasion and a high degree of Tumor, Node and Metastasis (TNM) stages in CRC patients. Additionally, co-expression of NGALR/ NGAL was found to be associated with poor cellular differentiation. These indicate that NGALR is a potential therapeutic target for the management of CRC patients (Lv *et al*., 2010).

1.10.3. NGALR in Endometrial Cancer

Endometrial cancer (EC) is the most prevalent gynecologic malignancy that elicits favorable outcome in the early stages of the disease while patients diagnosed at advanced stage undergo a dismal 5-year survival of about 47% - 58% in stage III and 15% -17% stage IV of the disease (Passarello *et al*., 2019). Immunohistochemical analysis showed that NGALR was markedly overexpressed EC cases, while in the normal endometrial tissue, no expression was found. This upregulation of NGALR was associated with higher tumor grade, more advanced tumors, and lower survival rate in EC patients. These findings suggested that NGALR may partake in the development and progression of this malignancy, thus making it a suitable target for the treatment of EC patients (Miyamoto *et al*., 2011).

1.10.4. NGALR in Esophageal Cancer

Esophageal cancer is an aggressive gastrointestinal malignancy with a low 5-year survival rate ranging from 15 - 25% globally (Watanabe *et al*., 2020). In esophageal squamous cell carcinoma (ESCC), it was reported that NGALR mRNA and protein levels were significantly elevated in the tumor cell membrane and cytoplasm compared to the normal esophageal epithelium. This overexpression of NGALR was mediated by

the hypomethylation of the CpG island in the promoter region of NGALR, thereby contributing to the progression of ESCC (Cui *et al*., 2008). Other studies showed that overexpression of NGALR and NGAL in ESCC patients was positively correlated with a poorer prognosis than those with low expression of both. It was further revealed that the high NGALR expression was closely associated with a decreased 5-year survival rate in patients with ESCC, thus making a novel prognostic factor as well as a promising target for the treatment of ESCC patients (Du *et al*., 2011). Further, it was proposed that NGALR is involved in NGAL-mediated iron transport in esophageal carcinoma. Fang *et al*. reported that NGALR has three splices variants namely, NGALR1, NGALR2, and NGALR3. Notably, NGALR3 was upregulated in 70% of cases of esophageal carcinoma compared to the adjacent normal epithelium, whereas NGALR2/1 was overexpressed in only 55% of the cases (Fang *et al*., 2007). Additionally, it was found that NGALR3 and NGAL interact *in vivo* and co-localize mainly at the cell membrane in esophageal carcinoma cells. Thus, this result suggested that the NGALR3 variant is the potential NGAL receptor and facilitates a unilateral intracellular iron-delivery pathway, which further supports tumor growth, thereby promoting malignancy in esophageal carcinoma (Fang *et al*., 2007; Candido *et al*., 2014).

1.10.5. NGALR in Glioma

Gliomas are the most commonly diagnosed malignant primary brain tumors which are largely incurable (Cahill *et al*., 2018). Extensive local invasion and metastasis contribute to the poor prognosis, thus necessitating a better understanding of the biology and molecular mechanism of this neoplasm. Studies have found overexpression of NGALR along with NGAL in human glioma tissues but absent or weakly expressed in

non-neoplastic brain and these overexpressions were found to be directly correlated with poor prognosis and low survival of the glioma patients. Additionally, enhanced NGAL/NGALR co-expression may indicate a more malignant phenotype in human gliomas, thus making NGALR a potential biomarker and therapeutic target for the management of gliomas (Liu *et al*., 2011).

1.10.6. NGALR in Hepatocellular Cancer

Hepatocellular cancer (HCC) constitutes about 90% of all primary liver cancer cases, with a high mortality rate as most HCC patients are diagnosed at advanced stages making only 15% of patients eligible for curative treatment (Ghavimi *et al*., 2020). A study has shown that HCC patients with upregulated expressions of both NGALR and NGAL were associated with poor prognosis and the overall survival was significantly shorter in patients with high NGALR and NGAL expressions compared to those with low expression, thus revealing the involvement of NGALR in the advancement of HCC and making NGALR a potential molecular target for HCC patients (Zhang *et al*., 2012b).

1.10.7. NGALR in Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most commonly diagnosed primary malignancy of the pancreas and is considered the most lethal cancer with a 5-year survival rate of less than 5% (Lu *et al*., 2017; Gandhi *et al*., 2018). Inflammation plays an essential role in the PDAC initiation and progression and enhances the aggressiveness of PDAC leading to increased chemoresistance and low survival rates (Khalafalla *et al*., 2017). It has been reported that NGAL/LCN2 is upregulated in PDAC patients and elevated the production of proinflammatory cytokines such as interleukin

(IL)-6, IL-1B, etc. in human pancreatic cancer stellate cells (PSC) of the PDAC tumor microenvironment. However, downregulation of its receptor NGALR significantly inhibited these effects, thereby depicting the crucial role of NGALR in NGAL-mediated pro-inflammatory responses in PSCs. Hence, NGALR and its ligand mediated the production of molecules essential for remodeling the PDAC stroma consequently resulting in enhanced tumor growth and decreased survival in PDAC cases (Gomez-Chou *et al*., 2017).

1.10.8. NGALR in Renal Cell Carcinoma

The prevalence of renal cell carcinoma (RCC) has been increasing over the past several years and clear cell renal cell carcinoma (ccRCC) is the most prevalent subtype accounting for about 70%–80% of all RCC cases (Rini *et al*., 2009). A systematic analysis on the expression patterns of NGAL and NGALR in 22 ccRCC data sets showed a considerable decreased in the expression NGAL and NGALR which provides the foundation for further studies on their biological roles in ccRCC (Liu *et al*., 2018).

1.10.9. NGALR in Other Cancers

Anthracyclines are a group of anti-cancer chemotherapeutic drugs used in approximately nearly 60% of childhood cancer patients. Reports showed that anthracycline-induced cardiotoxicity (ACT) is one of the most fatal drug reactions in children receiving cancer therapy with impending life-long consequences leading to substantial morbidity and mortality. ACT can result in severe clinical heart failure, hence, identifying patients with higher susceptibility for ACT is imperative for risk stratification that can ameliorate treatment and monitoring possibilities. Genotyping studies showed that NGALR/SLC22A17 was significantly associated with ACT and

help in improving the risk prediction model, thereby help in the patient risk stratification (Visscher *et al*., 2015). The spreading of cancer cells into the cerebrospinal fluid (CSF)–filled leptomeninges is referred to as leptomeningeal metastasis (LM). Cancer cells within the cerebrospinal fluid (CSF)-filled leptomeninges encountered microenvironmental challenges such as inflammation and scant micronutrients. It was found that these cancer cells expressed NGALR and its ligand NGAL that efficiently outcompete macrophages in the leptomeninges for iron thereby enhancing cancer cell growth within the leptomeningeal space *in vivo* (Chi *et al*., 2020). Moreover, in gastric cancer, NGALR was found to modulate immune cell infiltration and was correlated with low overall survival (OS) in gastric cancer patients (Wang *et al*., 2020a).

1.11. Importance of the Study

Despite the remarkable advancements and progress accomplished in treatment strategies, lung cancer remains the number one cause of cancer-related death. The survival rate for lung carcinoma remains dismal which is attributed to diagnosis at an advanced or metastatic stage of the disease due to lack of suitable biomarkers for early diagnosis. Moreover, the acquisition of chemoresistance and tumor relapse tremendously contribute to the poor clinical outcome of lung cancer patients. Therefore, there exists an unmet need to develop suitable biomarkers for early diagnosis and molecular targets which in turn will facilitate the development of a highly efficacious therapeutic regime for the better management of this deadly malignancy. It has been well established that multiple molecular alterations take place in the development and progression of lung cancer. Several studies have revealed that the protein NGALR is overexpressed in various cancers and is found to be closely associated with poor clinical outcome. However, the expression and the exact function of NGALR in lung cancer

has yet to be deciphered. Interestingly, its ligand NGAL is significantly upregulated in lung cancer and is contributing to the development of chemoresistance. Hence, determining the expression and role of NGALR in lung cancer may provide a novel biomarker and promising molecular target for lung cancer management. As it is a well-known fact that tobacco is the major risk factor for lung cancer, evaluating the role of tobacco components in modulating the expression of NGALR would facilitate new understandings of the development and progression of lung cancer. Additionally, as tumor necrosis factor (TNF) is implicated in inflammation-related carcinogenesis including lung cancer, determining the effect of TNF on NGALR expression would cater to a comprehensive understanding of NGALR function in lung carcinogenesis. Moreover, elucidating the role of NGALR in different hallmarks of lung cancer would help us identify a new therapeutic target for efficacious treatment of this fatal disease.

1.12. Objectives

The main objectives of this study are framed as follows:

1. To determine the differential expression of NGALR protein in normal human lung tissues and lung cancer tissues

2. To examine the effect of various tobacco components and tumor necrosis factors on the expression of NGALR in human lung epithelial cells and lung cancer cells and to analyze the upstream regulators of NGALR

3. To elucidate the role of NGALR in different processes involved in the development and progression of lung cancer

Chapter 2

Differential Expression of NGALR Protein in Normal Human Lung Tissues and Lung Cancer Tissues

2.1. Introduction

Globally, lung cancer continues to be the most prevalent malignancy and the foremost cause of cancer-related mortality. Despite the advancements achieved in the detection and treatment of lung cancer, the 5-year survival rate for lung cancer patients remains extremely dismal. The poor prognosis is mostly associated with the diagnosis of the disease at a late or advanced stage as well as the development of chemoresistance to the existing anti-cancer drugs, hence rendering the available treatment ineffective. Consequently, a comprehensive understanding of its molecular pathogenesis as well as identification of new biomarkers and molecular targets are crucial to facilitate better diagnosis and management of this deadly malignancy. Aforementioned, several studies have revealed that NGALR protein plays a significant role in the pathogenesis of various cancers including colorectal cancer, esophageal cancer, glioma, hepatocellular carcinoma, etc. However, its expression and role in lung cancer have not been unraveled. It is well established that lung cancer is characterized by immense molecular heterogeneity and encompasses different subtypes, pathological conditions, stages, and grades. Understanding the expression of NGALR in the above-mentioned categories of lung cancer is highly essential to unravel its role in the development and progression of lung cancer. Hence, this present study was aimed at determining the differential expression of NGALR in the lung cancer tissues of various subtypes, pathologies, stages, grades, age groups, and sexes. This study would help us elucidate the potential of NGALR as a diagnostic biomarker and novel therapeutic target for the treatment of this deadly disease.

2.2. Materials and Methods

2.2.1. The Cancer Genome Atlas (TCGA) Dataset Analysis

To access the genetic alteration of NGALR/SLC22A17 in lung cancer patients from genomic data, an open-access data portal, The Cancer Genome Atlas (TCGA) and cbioportal (http://www.cbioportal.org) were used. For this purpose, lung cancer was selected in the query section for visualization and analysis and filtered down to a particular study from the list of studies available for analysis. In this study, we have chosen the study "Lung Adenocarcinoma (TCGA, Nature 2014)" that consists of 230 samples and submitted the query by the gene name SLC22A17. The outcome of the patients having alterations of SLC22A17 was estimated in terms of overall survival by the Kaplan–Meier survival curve (Cerami *et al*., 2012; Gao *et al*., 2013).

2.2.2. Tissue Microarray

To determine the expression of NGALR protein in the normal lung tissues and different stages of lung cancer tissues, immunohistochemical analysis was performed on a tissue microarray (TMA) slide. For this purpose, the TMA slide containing paraffin-embedded normal and malignant lung tissues (US Biomax, Inc., Cat. No. LC1503) was purchased from US Biomax, Derwood, USA. The TMA slide contains a total of 150 tissues from 75 lung cancer patients i.e., duplicate cores per case (duplicated cores from the same patient were kept in two rows). The TMA slide consists of 29 adenocarcinoma, 29 squamous cell carcinoma, 3 adenosquamous carcinoma, 2 bronchioalveolar carcinoma, 4 small cell undifferentiated carcinoma, 2 large cell carcinoma, 1 neuroendocrine carcinoma, and 5 normal lung tissues (Table 2.1). Each tissue core is 1 mm in diameter and 5μm in thickness.

2.2.2.1. Tissue Microarray details

Lung cancer TMA, includes TNM, clinical-stage, grade and pathology from 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 specifications (LC1503):

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	T1N0M0	-	I	Malignant

				carcinoma with necrosis				
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

2.2.3. Immunohistochemistry (IHC)

For immunostaining, the tissue microarray, Histostain-Plus IHC Kit, HRP, broad-spectrum (Invitrogen, Cat. No. 859043; CA, USA), and Metal enhanced DAB Substrate Kit (Invitrogen, Cat No. 34065; CA, USA) were used as per the manufacturer's protocol, i.e.: deparaffinization, rehydration, peroxidase quenching, blocking, primary antibody incubation, secondary antibody-peroxidase conjugate incubation, the addition of DAB chromogen and counterstaining with hematoxylin. Lastly, the slide was dehydrated and mounted with a coverslip using D.P.X. mountant (Cat No DC4DF64352; Merck, New Jersey, USA) for microscopy. The primary antibody anti-24p3R antibody (Abcam, Cat. No. ab124506) was obtained from abcam®, Cambridge, USA, and used in the dilution of 1:20 for immunohistochemical analysis.

2.2.4. Scoring

The immunostained microarray slide was evaluated under an Olympus light microscope. Tissues that are stained brown are regarded as positive for the presence of antigen of interest and a score is given based on the intensity of staining and the number of positively stained cells. The score for the percentage of positively stained cells was measured from 0 to 4+ and staining intensity is scaled from 1 to 3 (McDonald and Pilgram 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		

2.2.5. Statistical Analysis

A student's *t*-test was performed to analyze the statistical significance. *p*-value <0.05 was considered statistically significant.

2.3. Results and Discussion

2.3.1. The TCGA Dataset Analysis

The mutational status of NGALR in lung cancer patients' tissue samples was studied. An oncoprint on the expression of *NGALR* gene (SLC22A17) from whole-exome sequencing of 230 lung adenocarcinoma cases, retrieved from cBioPortal showed that SLC22A17 is amplified in 10% of the cases i.e., it is found to be upregulated in 22 cases out of 230 sequenced cases/ patients analyzed (Figure 2.1 A). Upon considering the univariate analysis for survival data of the patients from TCGA datasets, it was found that the altered group has lower overall survival compared to the unaltered group (Figure 2.1 B). Precisely, the altered group showed 32.69 months of median overall survival (OS) whereas the unaltered group exhibit 45.31 months of median OS (Table 2.3). Thus, amplification of NGALR was associated with lower survival of lung cancer patients. From this preliminary finding, it can be predicted that NGALR is involved in the pathogenesis of lung cancer and may be associated with poor clinical outcome of lung cancer patients.

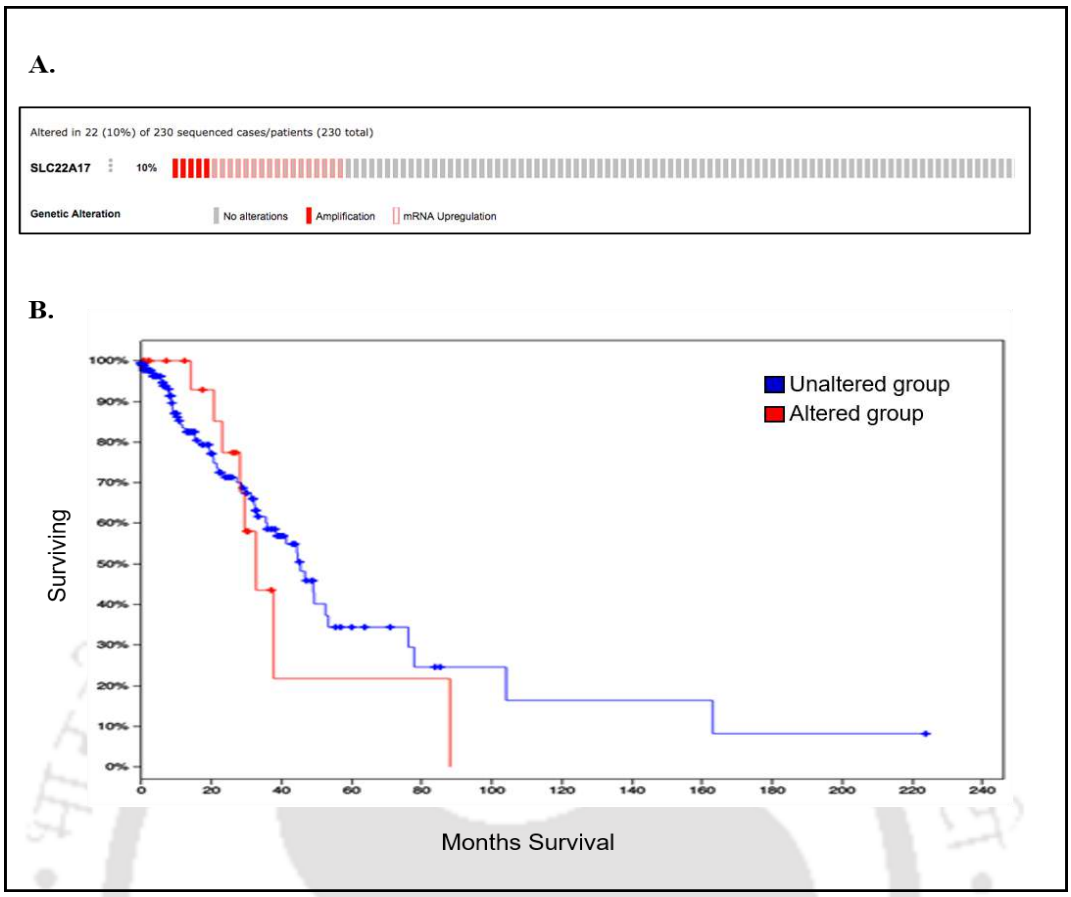

Figure 2.1. Expression of SLC22A17 from whole-exome sequencing of 230 lung adenocarcinoma cases, retrieved from cBioPortal, A. An oncoprint showing percentage of SLC22A17 alteration, B. Overall patients' survival status in altered SLC22A17 versus unaltered SLC22A17 group.

Table 2.3. Table showing median months overall survival in lung cancer patients with altered SLC22A17 versus unaltered SLC22A17 group:

Group	Median Months Overall
Altered	32.69
Unaltered	45.31

2.3.2. Immunohistochemistry

The TMA slide consists of tissues of both types of lung cancer i.e., SCLC and NSCLC.

They are characterized by different pathologies including small-cell undifferentiated

carcinoma, adenocarcinoma, bronchioalveolar carcinoma, squamous cell carcinoma, adenosquamous carcinoma, neuroendocrine carcinoma, and large cell carcinoma. Moreover, they are of various clinical stages (stage I, II, and IIIa), grades (grade 1, 2 and 3), and TNM status (T1N0M0, T2N0M0, T2N1M0, T3N0M0, T3N1M0). Also, the tissue samples are from lung cancer patients belonging to different age groups and include both genders. Expression studies have revealed that NGALR exhibits distinguished variability in different cancers however its expression has not yet been deciphered in lung cancer tissues. Hence, herein we have attempted to analyze the expression of NGALR based on different lung cancer types, pathological conditions, age, sex, grade, and stages of lung cancer compared to normal lung tissues. The total score (Q) of IHC is regarded as the expression score of NGALR protein for a tissue.

2.3.2.1. Expression Analysis of NGALR in Human Lung Cancer Tissues

The foremost aim of this study was to examine the expression of the NGALR protein in lung cancer tissues compared to normal lung tissues. A total of 5 normal tissues and 70 malignant tissues were considered. Our results showed that NGALR protein was significantly upregulated in human lung cancer tissues compared to the normal lung tissues (Figure 2.2). Previous studies have shown NGALR to be overexpressed in various human malignancies such as colon cancer, endometrial cancer, glioma, ESCC, liver cancer, etc. and involved in the progression of these cancers, thereby making it a potential prognostic indicator (Cui *et al*., 2008; Lv *et al*., 2010; Miyamoto *et al*., 2011; Liu *et al*., 2011; Zhang *et al*., 2012b). In line with these previous findings, we have also found that the expression level of NGALR is substantially higher in lung cancer tissues exerting around a 3-fold increase in expression compared to the normal lung tissues. Moreover, it has already been reported that NGAL binds to its receptor NGALR and

facilitates NGAL transport across the cytoplasmic or internal membranes by the process of endocytosis (Devireddy *et al*., 2005). Additionally, studies have shown that NGAL expression is upregulated in human lung cancer tissues, thus making it a potential therapeutic target (Song *et al*., 2015; Tang *et al*., 2015). Hence, this result suggests that NGALR is a positive regulator of lung cancer and perhaps partake in the malignant transformation of lung tissues.

2.3.2.2. Expression Analysis of NGALR in NSCLC and SCLC

Aforesaid, the two types of lung cancer are NSCLC and SCLC. NSCLC comprises approximately 80-85% of all lung cancer cases and SCLC constitutes about 15% of all newly diagnosed cases. NSCLC is subcategorized into adenocarcinoma, squamous cell carcinoma and large-cell carcinoma (Meerbeeck *et al.*, 2011; Lemjabbar-Alaoui *et al*., 2015). The expression of NGALR protein in both SCLC and NSCLC compared to the normal lung tissues was examined. Our result revealed that NGALR is upregulated in both SCLC and NSCLC tissues compared to the normal tissues, with significantly higher upregulation in NSCLC tissues. Moreover, between NSCLC and SCLC tissues, the increased fold change in expression of NGALR was higher in the case of NSCLC tissues (Figure 2.3 A). It is well evinced that tobacco smoking is the main risk factor for both lung cancer types. Studies showed that NSCLC displays a high etiologic association with smoking (Zheng *et al.*, 2007). Furthermore, the majority of SCLC cases are attributable to cigarette smoking and are quite rare in never-smokers (Kalemkerian *et al*., 2013; Torres-Durán *et al.*, 2016). Therefore, this enhanced expression of NGALR in both the subtypes of lung cancer might have a direct link with tobacco usage which requires further analysis.

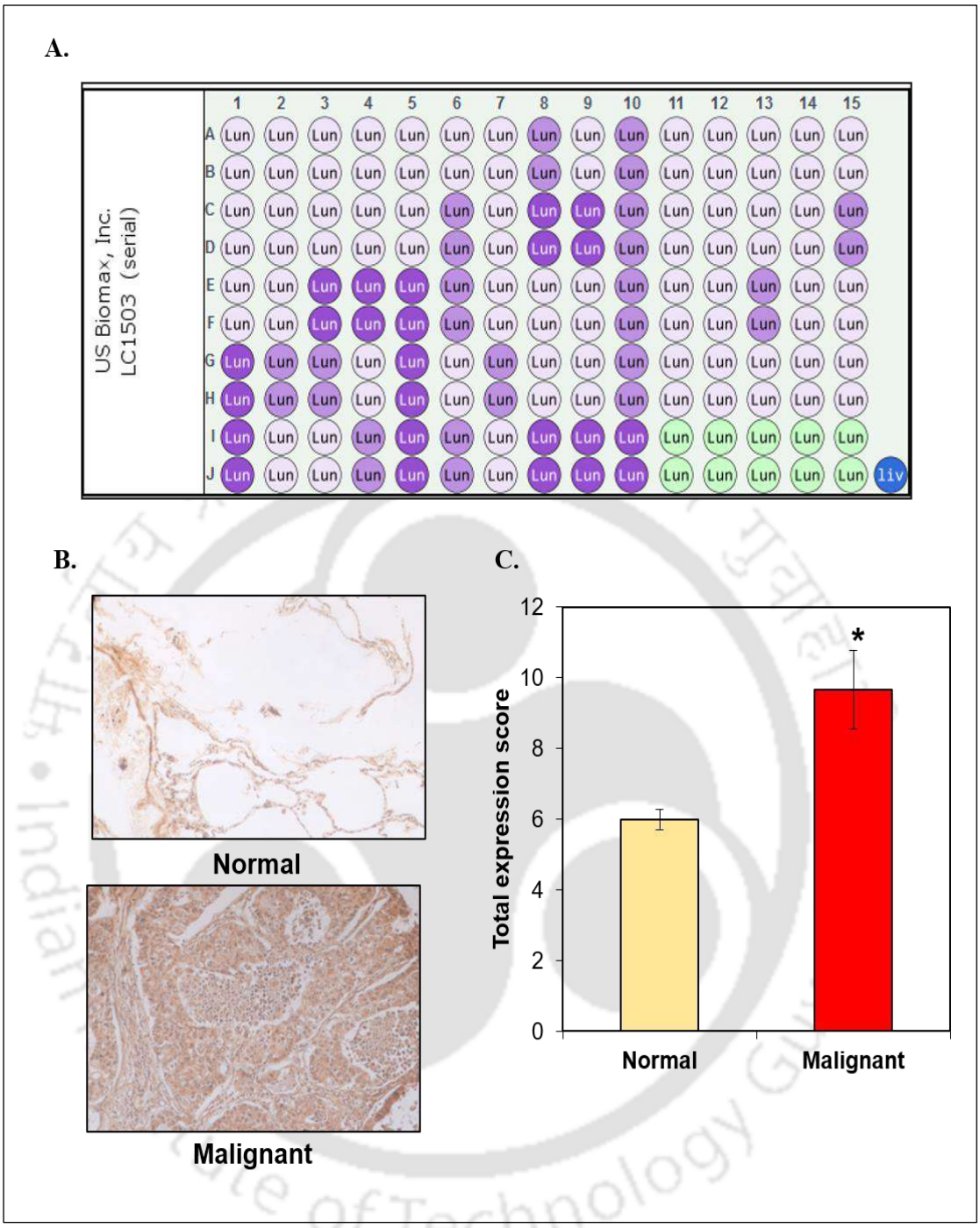

Figure 2.2. Expression of NGALR in lung cancer. A. Schematic representation of lung cancer tissue microarray slide, B. Representative images of the expression of NGALR in normal and malignant tissues, C. Expression of NGALR in lung cancer tissues in terms of expression score. Data are represented as Mean ± SE, * denotes $p < 0.05$ compared to normal tissues.

2.3.2.3. Expression Analysis of NGALR in Different Lung Cancer Pathologies

Lung cancer pathology covers both tissue diagnosis and an assortment of specific subtypes of lung cancers for additional molecular testing (Inamura, 2017). Based on pathology, lung cancer can be sectioned into several groups including adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma, small cell carcinoma, bronchioalveolar carcinoma, etc. In this study, we have compared the differential expression of NGALR protein with respect to these various lung cancer pathologies. Our results revealed that the expression of NGALR was significantly upregulated in adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma tissues compared to the normal tissues. Precisely, NGALR exhibited 3.5-folds increased expression in adenocarcinoma, 5-folds increased expression in adenosquamous carcinoma and 4-folds increased expression in squamous cell carcinoma, compared to the normal tissue (Figure 2.3 B). Although NGALR expression was upregulated in other disease conditions including bronchoalveolar carcinoma, large cell carcinoma, and small cell carcinoma, the difference in the expression compared to normal tissues was not significant, possibly due to the small sample size. More importantly, overall, the NGALR expression was upregulated in different pathological conditions of lung cancer compared to normal lung tissues. Thus, our data indicate the involvement of NGALR in lung cancer pathogenesis. However, additional studies with a larger number of patient samples are essential to confirm these findings.

2.3.2.4. Expression Analysis of NGALR in Lung Cancer Tissues of Different Age Groups

Next, the expression of NGALR protein in patients of different age groups between 31 to 80 years was evaluated. It was observed that NGALR did not show any significant

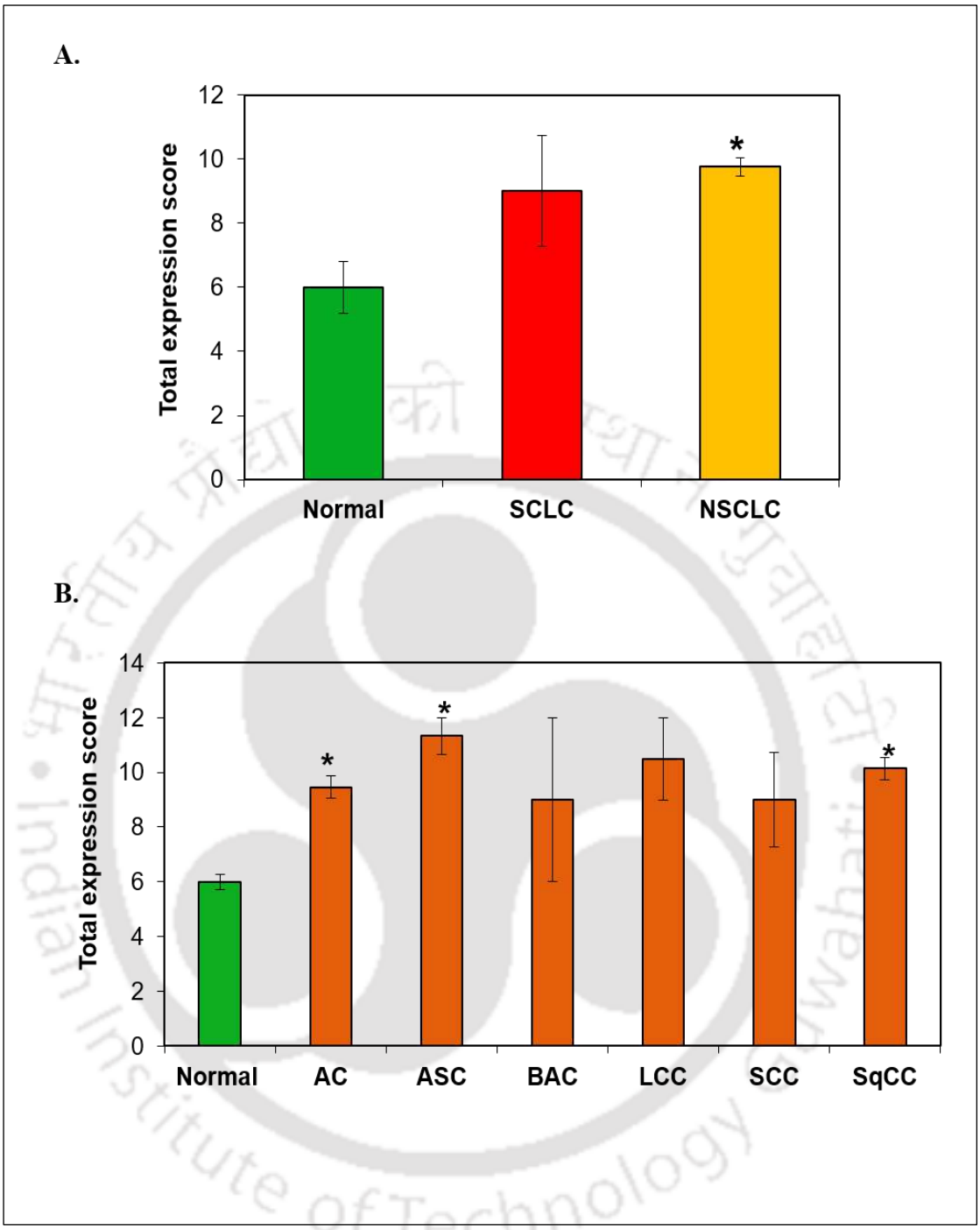

Figure 2.3. Differential expression of NGALR protein in different lung cancer types and pathologies, A. Expression of NGALR protein in different lung cancer types i.e., SCLC and NSCLC, B. Expression of NGALR protein in different lung cancer pathologies such as AC: adenocarcinoma; ASC: adenosquamous carcinoma; BAC: bronchioalveloar carcinoma; LCC: large cell carcinoma; SCC: small cell carcinoma and SqCC: squamous cell carcinoma. Data are represented as Mean ± SE, * denotes $p < 0.05$ compared to normal tissues.

difference in expression with respect to age, probably due to varying sample sizes among the different age groups. Nevertheless, we have found that the NGALR expression was highest among the 41-50 years age group followed by 61-70 years age group followed by 31-40- and 70-80-years' age group (Figure 2.4. A). This may be due to the fact that the prevalence of tobacco smoking, the predominant risk factor for lung cancer, varies with age amongst people. For example, according to the 2010 U.S. National Health Interview Survey by Centers for Disease Control and Prevention (CDC), age has been found to be associated with current smoking status, with just 5.1% of individuals aged 75 or older and 21.5 % of individuals aged 18–44 years were reported smoking (Schwartz and Cote, 2016). Thus, evaluating NGALR expression in a larger number of patients from different age groups would ascertain the NGALR expression pattern among different age groups.

2.3.2.5. Expression Analysis of NGALR based on Sex

Accumulating data suggests that smoking is much more prevalent among men than women, with an estimation of men smoking almost five times more than women globally (Hitchman and Fong, 2011; Schwartz and Cote, 2016). Hence, the risk of lung cancer is relatively higher in men compared to females (Bain *et al.,* 2004). Moreover, significant gender-based variations in epidemiology, molecular biology, hormonal influence, and treatment responses may also attribute to the different risks of lung cancer. The gender-based difference in the expression and mutation rates of numerous regulating gene pathways and differences in DNA repair capacity has also been implicated in the clinical disparity between men and women with lung cancer. Thus, a better insight into gender-based alterations in molecular changes may benefit lung cancer care (Donington and Colson, 2011). Furthermore, there is a strong correlation

between exposure to carcinogens from smoking and modulation of key molecular pathways. Hence, herein we attempted to analyze the expression of NGALR protein in both sexes (Figure 2.4 B). Our results showed that NGALR was upregulated in both malignant females and males' tissues compared to normal females and males' tissues respectively. The upregulation of NGALR was found to be significant in malignant male tissues compared to normal male tissues. Additionally, the enhanced expression of NGALR in malignant males' tissues was found to be higher compared to malignant females' tissues. As mentioned, the prevalence of smoking is considerably higher in men than in women, thus, it can be predicted that NGALR is involved in tobacco-associated lung carcinogenesis.

2.3.2.6. Expression Analysis of NGALR in Different Stages of Lung Cancer

Stages of cancer chiefly indicate the size of the tumor and the range of the disease spread. Aforesaid, precise staging of lung cancer is highly imperative for efficacious treatment of lung cancer patients (Carter *et al*., 2018). The TNM system is the internationally approved standard for the staging of cancer that includes tumor size and local growth (T), the extent of lymph node metastases (N), and the occurrence of distant metastases (Telloni, 2017). Based on this staging, a patient's cancer is divided into four different stages from stage I- IV. Our TMA slide is comprised of stage I, II and IIIa lung cancer tissues, and a stage-wise difference in the expression of NGALR protein was calculated in the lung cancer tissues compared to the normal lung tissues (Figure 2.5 A). Our analysis showed that NGALR was significantly upregulated in different stages of lung cancer i.e., stage I, II, and IIIa compared to normal lung tissues. Most importantly, NGALR expression was the highest at stage IIIa. Our result is in concordance with a previous finding wherein the expression level of NGALR was

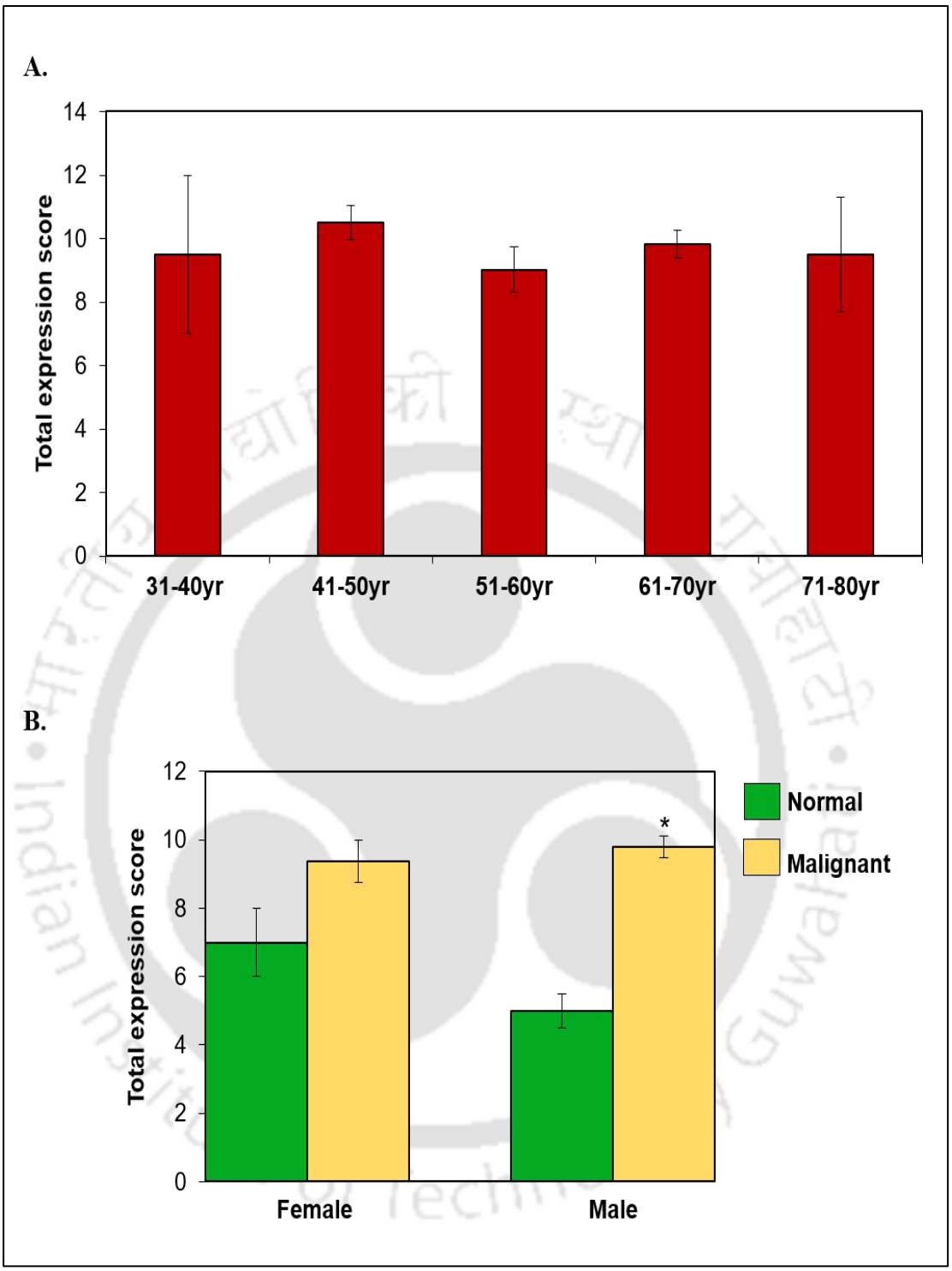

Figure 2.4. Expression of NGALR protein in lung cancer tissues of patients of different age groups and sex, A. Expression of NGALR protein in lung cancer tissues of patients of different age groups i.e., 31yr-40yr, 41yr-50yr, 51yr-60yr, 61yr-70yr and 71yr-80yr, B. Differential expression of NGALR protein in different sexes i.e., males and females. Data are represented as Mean ± SE, * denotes $p < 0.05$ compared to normal tissues.

significantly higher in advanced stages (stage II-III) than that of low stage (stage I) of hepatocellular cancer (HCC) tissues (Zhang *et al*., 2012b). Hence, our data suggest the involvement of the NGALR protein in the advanced stage of lung cancer. However, its role in the progression of lung cancer still requires further validation with more advanced stage or metastatic tissues.

2.3.2.7. Expression Analysis of NGALR in Different Grades of Lung Cancer

The grade of a tumor describes the level of differentiation and how abnormal the tumor cells/tissues appear under a microscope. Based on the degree of differentiation, the tumors are categorized into grade 1, 2, 3, and 4. Grade 1 denotes a well-differentiated (appear normal and does not grow rapidly/low grade), grade 2 signifies moderately-differentiated (cells appear slightly different than normal/ intermediate grade), grade 3 represents poorly differentiated (cells appear abnormal and grow and aggressively spread /high grade) and grade 4 denotes undifferentiated (grow rapidly/ high grade) tumors (https://www.cancer.gov/about-cancer/diagnosis-staging/prognosis/tumor-grade-fact-sheet). Further, cancer grading and staging enable clinicians to predict treatment outcomes, cure rates, and disease-free survival times (Telloni, 2017). The TMA slide contained lung cancer tissues from grade 1, 2, and 3 tumors. Consequently, we analyzed the expression of NGALR protein in different tumor grades or degree of differentiation (Figure 2.5 B). Similar to the findings observed in different lung cancer stages, NGALR was found to be significantly upregulated in different grades of lung cancer including grade 1, 2, and 3 when compared to normal lung tissues. The previous report has revealed that NGALR is upregulated in high-grade gliomas, particularly in grade IV of glioblastoma multiforme (GBM) and was not detected in low-grade gliomas and non-neoplastic brain tissue samples (Liu *et al*., 2011). Herein, we found that

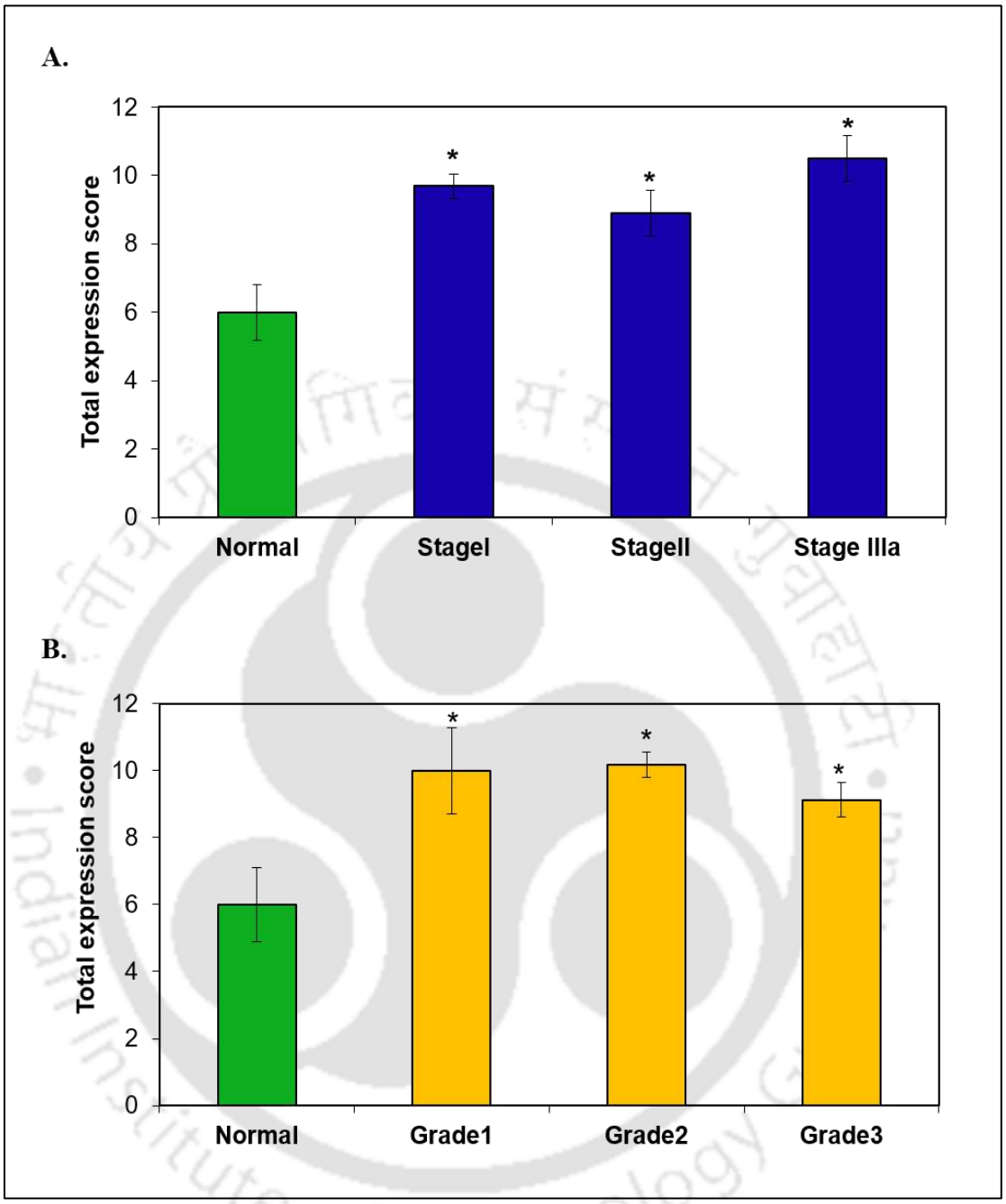

Figure 2.5. Differential expression of NGALR protein in different stages and grades of lung cancer. A. Differential expression of NGALR protein in different stages (Stage I, II, IIIa) of lung cancer, B. Differential expression of NGALR protein in different grades (Grade 1, 2, 3) of lung tumor. Data are represented as Mean ± SE, * denotes $p < 0.05$ compared to normal tissues.

NGALR expression was the highest in grade 2 with 4.1-folds increased in expression followed by grade 1 with 4-folds increased in expression and grade 3 with 3-folds increased in expression compared to the normal lung tissues. In concordance with our finding, Du *et al*. has shown a direct association between NGALR expression and histological differentiation grade of ESCC (Du *et al*., 2011). Moreover, in endometrial cancer, NGALR was overexpressed in patients with higher grade and advanced stage of the disease (Miyamoto *et al*., 2011). Hence, from both stage and grade-based analysis, it can be predicted that NGALR has a crucial role in the initiation and progression of lung cancer. Nonetheless, further studies with a larger number of tissue samples are warranted for the validation of our findings.

2.4. Conclusion

This chapter aimed to assess the NGALR expression in lung cancer tissues of different types, different age groups, pathologies, stages, grades, and sexes. Determining the significance of the expression of NGALR with these factors would be highly beneficial in ascertaining whether NGALR can serve as a predictive biomarker for lung cancer. Interestingly, our results revealed that NGALR is strongly involved in the positive regulation of lung carcinogenesis. It is noteworthy that NGALR was upregulated in different types of lung cancer as well as different pathological conditions. This chapter also indicates that NGALR may possess a direct correlation with tobacco-mediated lung carcinogenesis based on its expression profile in tissues of different age groups and sexes. Moreover, this study also showed that the expression of NGALR is directly correlated with the degree of differentiation as well as the stage of tumors, thus implying its potential as a prognostic factor and novel therapeutic target for lung cancer. Overall, it can be inferred that overexpression of NGALR may be closely associated

with poor clinical outcome in lung cancer patients. However, further investigation is imperative with a larger number of tissue samples to validate our findings. Also, more in-depth studies on the molecular mechanisms involving NGALR protein in the pathogenesis of lung cancer, especially in tobacco-induced lung carcinogenesis are highly warranted for a better understanding of the disease as well as to provide new avenues for effective treatment of this malignancy.

Chapter 3

Effect of various Tobacco Components and Tumor Necrosis Factors on the Expression of NGALR in Human Lung Epithelial Cells and Lung Cancer Cells and Analysis of the Upstream Regulators of NGALR

3.1. Introduction

In the preceding chapter, we have shown a marked upregulation of NGALR in lung cancer tissues (both NSCLC and SCLC) compared to normal lung tissues and its strong association with the positive regulation of lung carcinogenesis. It is a well-known fact that lung cancer development has a strong etiological association with tobacco in both males and females. More than 85% of lung cancers are attributed to smoking, with an additional portion triggered by exposure to secondhand smoke in nonsmokers (Warren and Cummings, 2013). This suggests that the main risk factor of lung cancer i.e., tobacco may partake in altering the expression of NGALR in lung cancer tissues. Moreover, it is well established that the prevalence of tobacco smoking is more common in men compared to women as men generally smoke five times more than women globally (Hitchman and Fong, 2011). It has been shown in the previous chapter that NGALR is significantly upregulated in the tissues of malignant males compared to that of malignant females. Thus, these results provide a strong base for the existence of a correlation between NGALR with carcinogens found in tobacco products. Tobacco smoke comprises approximately 5000 compounds (Pfeifer *et al*., 2002; Wang *et al*., 2015b). Out of these, more than 20 compounds are carcinogenic to the lung which includes polycyclic aromatic hydrocarbons (PAH), tobacco-specific nitrosamines, e.g. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), arsenic, aromatic amines, etc. (Pfeifer *et al*., 2002; Ge *et al*., 2015; Wang *et al*., 2015b). NNK and N-nitrosonornicotine (NNN) are two tobacco-specific nitrosamines that are strong carcinogenic agents (Hecht and Hoffmann, 1988). PAHs compounds are composed of two or more fused benzenoid rings and possess potent carcinogenic and mutagenic properties. Benzo[a]pyrene (BaP) is considered the most potent carcinogen among PAH (Vu *et al.*, 2015). Apart from them, nicotine is the addictive component of tobacco

smoke which promotes proliferation, migration, and invasion of cells *in vitro* and facilitates tumor growth and metastasis *in vivo*. It exerts its tumor-promoting effect through the binding and activation of nicotinic acetylcholine receptors (nAChRs), specifically the α7 subunit (Schaal *et al*., 2018). Further, reports suggest that the binding of nicotine to nAChRs results in the upregulation of epidermal growth factor (EGF) that in turn activated EGFR (Sanner *et al*., 2015). In addition, nAChRs have been found to play a crucial role in lung carcinogenesis through regulation of stimulatory or inhibitory signaling cascades, especially the subtype, α7nAChR is an important mediator in the proliferative, pro-angiogenic and pro-metastatic action of nicotine (Hajiasgharzadeh *et al*., 2019; Wang and Hu, 2018). As the role of the above-mentioned tobacco components in the regulation of the expression of NGALR has not yet been elucidated thus far, this chapter focuses on examining the effect of tobacco extract and various tobacco components (carcinogens) on the expression of NGALR in human lung epithelial cells and NSCLC cells.

Additionally, a growing body of evidence has supported the notion that chronic inflammation elicits tumorigenesis (Wang and Lin, 2008). Chronic inflammation is also known to contribute to lung cancer and alteration in inflammatory cytokine serum levels has been implicated both in smokers and lung cancer patients (Seifart *et al*., 2005). Accumulating studies revealed that tumor necrosis factor (TNF)-α and TNF-β although once believed as anti-tumor factors, are pro-inflammatory cytokines that play a crucial role in chronic diseases including cancer. TNF-β is also known as lymphotoxin α, the closest structural homolog to TNF-α has been found to activate NF-κB in cancer cells with a similar potency as TNF-α, thereby contributing to the progression of cancer (Buhrmann *et al*., 2020). Further, TNFs have been reported to participate in various cellular processes such as cell survival, proliferation, differentiation, etc., and may be

involved in inflammation-related carcinogenesis via activation of NF-κB (Balkwill *et al*., 2006; Wang and Lin, 2008; Drutskaya *et al*., 2010). The TNF superfamily is primarily composed of 19 ligands and 29 receptors. Among the ligands, TNF-α and TNF-β (lymphotoxin-α) produced by macrophages and lymphocytes respectively are important modulators of airway inflammation and have also been strongly implicated in tumorigenesis (Seifart *et al*., 2005; Aggarwal *et al*., 2012). There are two receptors for TNF i.e., TNF receptor-1 (TNFR-1, p55 receptor) and TNFR-2 (p75 receptor). The pathways mediated via TNFR-1 have been comprehensively studied. When TNF binds to TNFR-1, the TNFR-1 forms a homotrimer to recruit TNFR-associated death domain (TRADD). TRADD then recruits downstream adaptor proteins including receptor-interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and Fas-associated death domain (FADD) that further recruits vital molecules that promote activation of various signaling pathways such as NF-κB, MAPKs, and cell death, respectively (Wang and Lin, 2008). Notably, studies revealed that TNF may induce resistance of lung cancer cells to reactive oxygen species (ROS)-based therapies as well as doxorubicin treatment (Pogrebniak *et al*., 1991; Prewitt *et al*., 1994). As there is a lack of information on the effect of TNF-α and TNF-β on NGALR expression, in this chapter we attempt to decipher whether these two pro-inflammatory cytokines have any effect on the expression of NGALR in lung cancer cells, which would provide better knowledge on the function of NGALR in lung carcinogenesis. Additionally, to elucidate the molecular mechanisms of NGALR in lung cancer, determining the upstream regulators of NGALR is inevitable and promoter analysis is an indispensable approach. Therefore, in this chapter, we aimed to investigate the transcription factors that have potential binding sites in the promoter region of the *NGALR* gene.

3.2. Materials and Methods

3.2.1. Materials

NNK (Cat No. 78013), NNN (Cat No. 75285), BaP (Cat No. B1760) and Nicotine (Cat No. N3876) were bought from Sigma- Aldrich, Missouri, USA. Tobacco extract 'Tuibur', was purchased from a local store in Aizawl, Mizoram and was filtered, lyophilized, and then dissolved in sterile distilled water for use. The TNF-α and TNF-β were gifted by Dr. BB Aggarwal, USA and a stock solution of 200ng/ml was prepared using DMEM media.

3.2.2. Cell Culture

The human lung adenocarcinoma cell line A549, large cell carcinoma cell line NCIH460, and embryonic lung epithelial cell line L132 were procured from National Centre for Cell Science, 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 or proliferative concentrations of tuibur and other tobacco components, an MTT assay was performed. L132, A549 and NCIH460 cells were harvested with 0.05% trypsin-0.02% EDTA solution (HiMedia) and seeded in 96-well plates at a concentration of 2000 cells/100µl in sextuplicates. After 24h of incubation, the cells were treated with various 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), BaP (0, 0.05, 0.1, 0.25 and 0.05 µg/ml) and nicotine (0, 0.25,

0.5, 0.75 and 1 µM) for 0 and 24h. After each time point, 10 µL of 5 mg/mL MTT (Cat. No. M2128, Sigma-Aldrich, Missouri, USA) was added to each well followed by incubation for 2 h at 37 °C. After incubation, the culture media containing MTT was removed and 100µl 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 for dissolving the MTT-formazan product. Then, the absorbance of the colored solution obtained was taken at 570 nm using a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland). Then, the percentage of proliferation was determined by normalizing the absorbance value of 24 h with 0 h absorbance while considering the absorbance of untreated control as 100%.

3.2.4. Reverse Transcription-Polymerase Chain Reaction

To elucidate the effect of tuibur and different tobacco components on the expression of NGALR in normal and lung cancer cells, reverse transcription-polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted using Trizol reagent (Cat No. T9424-200 ML, Sigma-Aldrich, Missouri, USA) and subsequently cDNA synthesis was carried out using 1µg of RNA using High-Capacity cDNA Reverse Transcription Kit (Cat No. 4368814, Applied Biosystems™, USA) as per the manufacturer's protocol. Primers for amplifying NGALR were obtained from Integrated 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 NGALR was then amplified by 35 cycles of PCR using a specific primer and 1µL of cDNA as a template. The amplicons obtained were then resolved in 1% agarose gel electrophoresis and the intensity of the band was determined using Image lab software. 100 bp DNA Ladder (Cat No. N3231S, NEB, USA) was used as a standard. The

housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal gene control.

Table 3.1. Forward primers (FP) and reverse primers (RP) used for the amplification of the *NGALR* gene

Gene	**Primer Sequence**		**Melting temperature Tm (°C)**	**Amplicon size**
NGALR	FP	5'GCCTGGCCCTTGTCTCTAAG 3'	65	564 bp
	RP	5'CAGCCTCGTTCAGATAATCCCA 3'	67.2	

3.2.5. Promoter Analysis

To analyze the putative transcription factors that have potential binding sites in the NGALR promoter, an in-silico approach using a software tool Genomatix: MatInspector. MatInspector works by using a large collection of matrix descriptions for transcription factor binding sites that help in locating matches in DNA sequences.

3.2.6. siRNA Transfection

To knockdown α7nAChR and transcription factors that have potential binding sites on the NGALR promoter, NCIH460 cells were seeded in a 6 well plate. Upon reaching 70% confluency, the cells were transiently transfected with α7nAChR siRNA and transcription factors siRNAs (10pmol/µl) (Eurofins Genomics) mixed with 5x universal siMAX siRNA buffer (Eurofins genomics) using transfection reagent lipofectamine RNAiMax (Invitrogen; cat no: 13778-075) according to manufacturer's protocol. The cDNA was prepared from isolated RNA 48h post-transfection. Gene silencing was analyzed by real-time PCR.

3.2.7. Quantitative Real-time PCR (q-PCR)

To confirm the siRNA-mediated knockdown of α7nAChR and transcription factors, q-PCR was performed. The total RNA was isolated from the cultured NCIH460 cells using TRI Reagent®. 1µg of total RNA was further processed to synthesize cDNA by RT-PCR using High-Capacity cDNA Reverse Transcription Kit (Catalog no: 4368814; Applied Biosystems™). After reverse transcription, quantitative real-time PCR was performed to quantify the expression levels using PowerUp SYBR Green Master Mix (Catalog no: A25742; Applied Biosystems™). The relative fold change in gene expression was calculated using the $\Delta\Delta_{CT}$ method. β-actin was used as an internal control.

3.2.8. Western Blot

To determine the effect of TNF-α and TNF-β on the expression of NGALR in lung cancer cell line NCIH460, Western blot was performed. In brief, after treatment of NCIH460 cells with different concentrations of TNF-α and TNF-β (0, 0.1 and 1nM) for 24h, the total protein lysates were prepared using whole-cell lysis buffer containing 20 mM HEPES buffer, 2 mM EDTA, 250 mM NaCl, 0.1% (v/v) Triton-X100 and protease inhibitors such as 1 mM DTT, 1 mM PMSF, 2 µg/mL Aprotinin and 2 µg/mL Leupeptin hemisulfate. Protein concentrations were determined by Bradford protein assay (Cat. No. 500-0205; Bio-rad, California, USA) using bovine serum albumin (BSA) as standard, and equal amounts of protein were resolved on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel with 5X Laemmli Buffer (250 mM Tris-HCl, 5% β-mercaptoethanol, 10% SDS, 30% Glycerol and 0.02% Bromophenol blue) at a voltage of 70-90 V. After resolving, the proteins were transferred to nitrocellulose membranes and the protein transfer was checked by Ponceau-S stain (Cat. No. ML045; HiMedia).

Following the successful protein transfer, the membranes were blocked in 5% BSA for phospho-proteins and 5% non-fat dry milk (Amulya) for other proteins. Following 2-3 hours of blocking, the membranes were incubated with respective primary antibodies in appropriate dilution overnight at 4 °C. The next day, membranes were removed from primary antibodies, washed with 1X TBST, and incubated with horseradish peroxidase (HRP) – conjugated anti-rabbit or anti-mouse secondary antibodies for 2 h. After 2 h incubation, the membranes were again washed with 1X TBST and the blots were developed using Clarity Western ECL Substrate (Cat. No. 1705061; Bio-Rad, California, USA) in a ChemiDoc™ XRS System (Bio-Rad, California, USA). The housekeeping gene β-actin was used as a loading control.

3.2.9. Statistical Analysis

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

3.3. Results and Discussion

3.3.1. Effect of Tobacco Smoke-Infused Water "Tuibur" on the Expression of NGALR in Human Lung Epithelial Cells and Lung Cancer Cells

Among the different states of India, lung cancer is the most prevalent in Mizoram. Tobacco consumption is the highest in Mizoram, wherein the Aizawl district also leads the incidence rates of all tobacco-associated cancers in India (Mahanta *et al*., 1998; Madathil *et al.*, 2018). It has been reported that many people of North-Eastern states of India, especially Mizoram consume a unique form of tobacco smoke-saturated aqueous concentrate locally known as "tuibur". It is extremely addictive and deeply rooted in

the culture of the region (Madathil *et al.,* 2018). This tobacco smoke-infused water is made locally by releasing smoke generated from the burning of tobacco into the water until the preparation turns cognac in color and has a pungent smell. It is sipped and retained in the mouth for 5 to10 minutes and spat out and the users consume it few times a day (Phukan *et al.,* 2005). It has been reported that about 7% of persons surveyed in Aizawl (872 of 12185) and Churchandpur (139 of 2137) used tobacco water (Sinha DN *et al*., 2004). Heavy metals such as arsenic (1.02–1.08 mg/g), cadmium (0.011–0.012 mg/g), lead (0.015–0.018 mg/g), nickel (0.01–0.03 mg/g), and triethylene glycol (2321.42–2399.72 mg/g) were found in tobacco water. Also, a high concentration of N-nitrosonornicotine (NNN) (19.65–20.12 mg/g) was also reported in tuibur and NNN is the most abundant and the strongest carcinogen found in tuibur (Sinha and Gupta, 2006). Thus, analyzing whether tuibur has any effect on the expression of NGALR would help us in a better understanding of the mechanism involved in the initiation of lung cancer by this carcinogenic agent. Firstly, in order to determine the non-toxic concentrations of tuibur, L132, A549 and NCIH460 cells were treated with different concentrations of tuibur (0.25µg/ml, 0.50µg/ml, 0.75µg/ml, 1.0µg/ml and 5µg/ml) for 0h and 24h and the proliferative effect of tuibur was measured by MTT assay. It was found that 0.25, 0.5 and 0.75µg/ml of tuibur did not impart any cytotoxicity to the lung epithelial cells and the lung cancer cells. Next, we determined the expression of *NGALR* in L132, A549, and NCIH460 cells treated with different concentrations of tuibur (0.25 µg/ml, 0.50µg/ml, and 0.75µg/ml). Our results showed that in L132 cells, the mRNA expression of *NGALR* was upregulated dose-dependently up to 0.5 µg/ml of tuibur treatment whereas in A549 and NCIH460 the mRNA expression of *NGALR* was upregulated in a dose-dependent manner up to 0.75µg/ml compared to untreated control (Figure 3.1). These results showed that tuibur

significantly enhanced the expression of NGALR. As there is no report on tuibur mediated cancer signaling pathway and its role in lung carcinogenesis, further investigation is mandatory to reveal the downstream targets of NGALR after treating lung cancer cells with tuibur. Nonetheless, our result suggests that NGALR may have a substantial role in the pathogenesis of tobacco-associated lung cancer which is in concordance with IHC-based predictions as discussed in the previous chapter. Moreover, it is well established that tobacco smoke contains more than 20 compounds which are considered lung carcinogens. Thus, determining the effect of these carcinogens on the expression of NGALR in human lung epithelial cells and lung cancer cells will further reinforce our understanding concerning the tobacco-associated lung carcinogenesis. For this purpose, three potent carcinogens found in tobacco such as NNK, NNN and BaP, and the prime addictive compound nicotine were used to analyze their effect on the expression of NGALR.

3.3.2. Effect of NNK on the Expression of NGALR in Human Lung Epithelial Cells and Lung Cancer Cells

Of all the lung carcinogens, tobacco-specific nitrosamines 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is considered the most potent one. The oncogenic mechanisms associated with NNK are not wholly understood, thus deterring the development of an effective treatment approach for smoking-associated lung cancer (Ge *et al*., 2015). NNK, generated through nitrosation of nicotine is registered as group 1 human carcinogens by the IARC (Gupta *et al*., 2019; Shen *et al*., 2012). Naturally-occurring NNK is a procarcinogen, which is metabolically activated to elicit its carcinogenic activity by numerous CYPs which promote methylation,

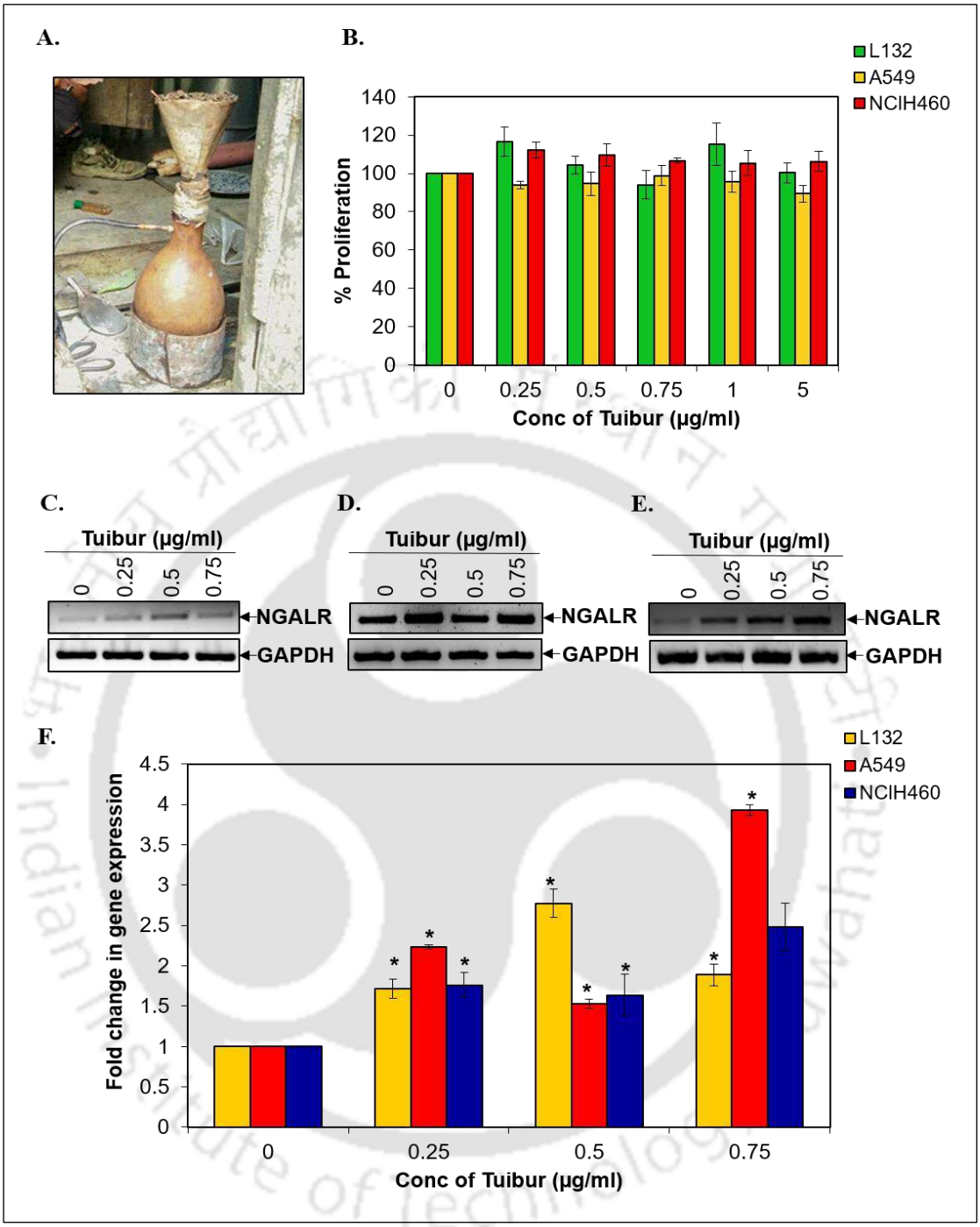

Figure 3.1. Effect of Tuibur on the expression of *NGALR* in human lung epithelial cells and lung cancer cells A. Preparation of Tuibur; made locally by passing smoke generated by burning tobacco through water till the preparation turns cognac in colour (Mahanta *et al*., 1998), B. MTT assay showing non-cytotoxic concentrations of Tuibur in L132, A549, NCIH460 cells. RT-PCR analysis of the expression of *NGALR* after treating C. L132, D. A549, E. NCIH460 with Tuibur for 24 h, F. Graphical representation of the expression of *NGALR* in Tuibur treated L132, A549, NCIH460 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.

pyridyloxobutylation and pyridylhydroxybutylation of nucleobases of DNA that contribute to the formation of DNA adducts which thereby promote tumor initiation (Xue *et al*., 2014). Further, it has been reported that NNK activates phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB/Akt), protein kinase C (PKC), NF-κB, and other key signals which all induce cell proliferation, survival, and angiogenesis, thereby facilitating the development of smoking-associated lung cancers (Ge *et al*., 2015). Additionally, studies have also shown that NNK induces tumor metastasis via alteration of cell motility and enhances migration and invasion of human lung cancer cells via upregulation of a protein kinase cascade such as c-Src, PKCι and focal adhesion kinase (FAK) (Shen *et al*., 2012). Furthermore, this potent carcinogen promoted reactive oxygen species (ROS) production which activates the Wnt signaling pathway in A549 cells (Hirata *et al*., 2017). NNK has been used *in vivo* to successfully induce lung tumors in various animal models including A/J mice, rats, hamsters, and ferret (Zheng and Takano, 2011; Ge *et al*., 2015). Further, NNK has a high affinity for α7nAChR and binds to it to activate voltage-gated Ca2+ channels and caused influx of Ca2+ into lung cells, which led to the activation of PKC, the serine/threonine kinase RAF1, ERK1/2, and transcription factors Fos, Jun, and Myc, which facilitate the proliferation of lung cancer cells (Xue *et al*., 2014). NNK was found to alleviate lung cancer progression risk and poorer outcomes in patients having p53 mutations via disrupting proper mitotic progression and chromosome integrity (Park *et al*., 2017). Therefore, examining the effect of NNK on the expression of *NGALR* would reveal novel molecular mechanisms in NNK-induced lung carcinogenesis. For that, we first determined the non-toxic concentrations of NNK on L132, A549, and NCIH460 cells using MTT assay and found that 0.05, 0.1, and 0.25μM did not have any cytotoxic effects on the cells. Next, these concentrations of NNK were used to treat the three cell

lines for 24 hours, and then RT-PCR was performed to determine the expression NGALR. Our findings revealed that the mRNA expression level of *NGALR* was upregulated in a dose-dependent manner in all three cell lines (Figure 3.2). Aforementioned, NNK has been found to promote lung carcinogenesis via activating PI3K/Akt, PKC, NF-κB, and other important molecular pathways involved in the proliferation, survival, and angiogenesis of lung cancer cells (Ge *et al.,* 2015). This suggests the regulation of NGALR expression by NNK and NGALR may be one of the mediators involved in the initiation and progression of NNK-associated lung carcinogenesis. Nevertheless, additional studies are required to elucidate the exact mechanism involved.

3.3.3. Effect of NNN on the Expression of NGALR in Human Lung Epithelial Cells and Lung Cancer Cells

N-Nitrosonornicotine (NNN), a tobacco-specific nitrosamine is classified as a Group 1 carcinogen. NNN is found in wide arrays of tobacco products including smokeless tobacco, cigarettes, and cigars (Zarth *et al*., 2016). The structure of NNN resembles that of nicotine (Schuller, 2007). NNN can be metabolized by cytochrome P450s via two activation pathways: 2′-hydroxylation and 5′-hydroxylation (Siminszky *et al*., 2005). Once activated, it binds with a nucleotide, forming DNA adducts. This induces deleterious mutations to both oncogenes and tumor suppressor genes, hence initiating tumor formation. Moreover, like NNK, NNN also binds to nAChR and promotes tumor growth via enhancing proliferation, survival, migration, and invasion (Xue *et al*., 2014). NNN has been used extensively to produce tumors in the lungs of mice (Konstantinou *et al.,* 2018). Thus, examining the effect of NNN on the expression of *NGALR* would

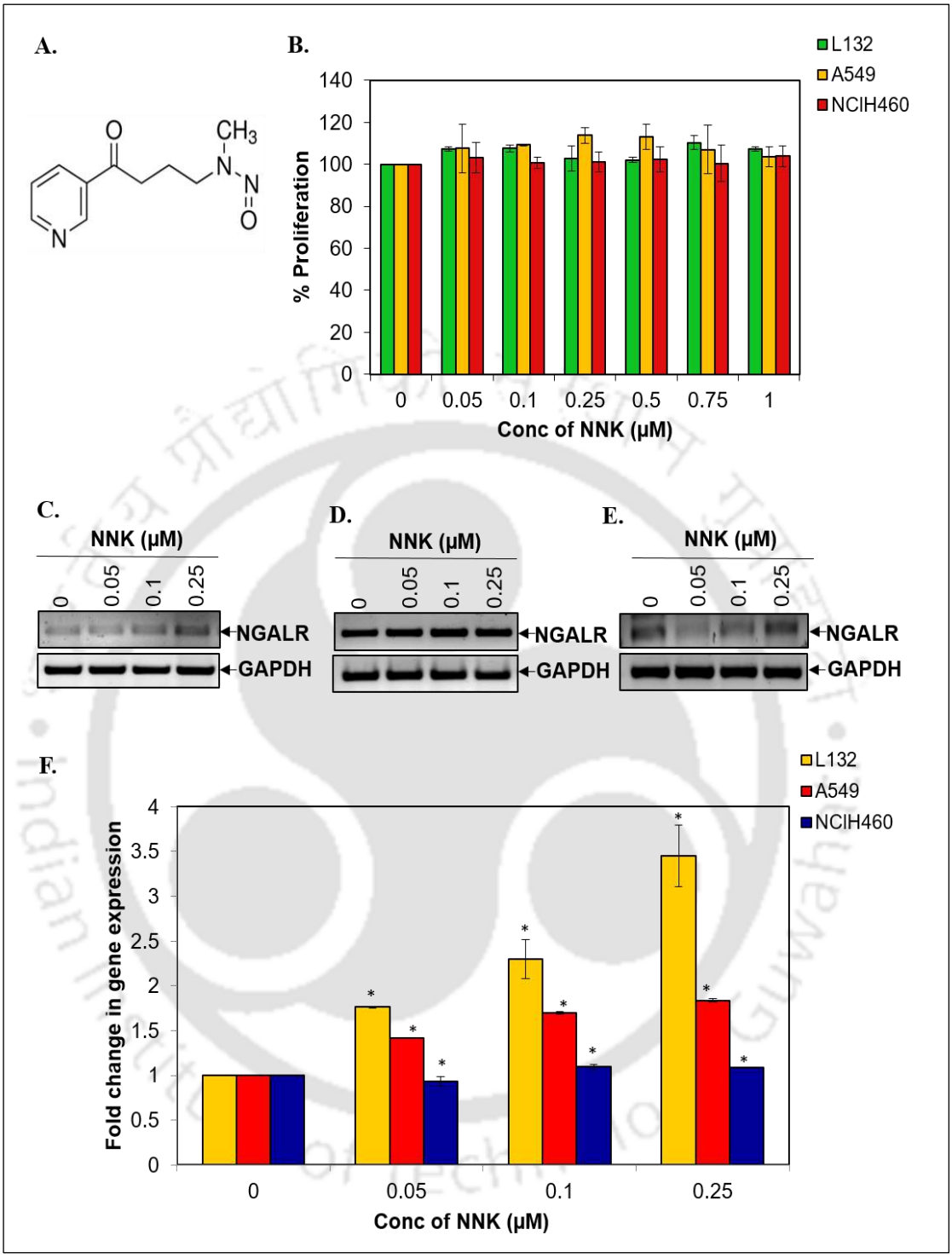

Figure 3.2. Effect of NNK on the expression of *NGALR* in human lung epithelial cells and lung cancer cells A. Structure of NNK, B. MTT assay showing non-cytotoxic concentrations of NNK in L132, A549, NCIH460 cells. RT-PCR analysis of the expression of *NGALR* after treating C. L132, D. A549, E. NCIH460 with NNK for 24 h, F. Graphical representation of the expression of *NGALR* in NNK treated L132, A549, NCIH460 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.

reveal whether NGALR is involved in NNN-mediated lung carcinogenesis. Initially, we determined the non-toxic concentrations of NNN on L132, A549, and NCIH460 cells using MTT assay and found that 0.05, 0.1, and 0.25μM did not induce any cytotoxicity to the cells. Secondly, these concentrations of NNN were used to treat the cells for 24 hours, and then RT-PCR was carried out to analyze the expression *NGALR*. Our result showed that the mRNA expression of *NGALR* was found to be significantly upregulated dose-dependently in all the cell lines (Figure 3.3). NNN is well evinced to activate nAChRs, which can instigate the constitutive activation of several transcription factors like GATA3, NF-κB, and STAT-1 which in turn upregulate the expression of proliferative and anti-apoptotic genes leading to augmented cell proliferation (Arredondo *et al.,* 2006). Therefore, it can be inferred that NGALR may be involved in NNN-mediated lung cancer development and progression. Nevertheless, in-depth studies are compulsory to fully unravel the exact molecular mechanism involved in the enhanced expression of NGALR induced by NNN.

3.3.4. Effect of Nicotine on the Expression of NGALR in Human Lung Epithelial Cells and Lung Cancer Cells

Nicotine is the main addictive constituent of tobacco that contributes directly to lung carcinogenesis via the activation of nAChRs (Improgo *et al.*, 2011). Nicotine is involved in inducing proliferation, migration, and invasion of cells *in vitro* and tumor growth and metastasis *in vivo* by activating nAChRs (Schaal *et al.*,2018). nAChRs have been found to play a crucial role in lung carcinogenesis through regulation of stimulatory or inhibitory signaling cascades, especially the subtype, α7nAChR is an important mediator in the proliferative, pro-angiogenic, and pro-metastatic properties of nicotine in lung cancer (Hajiasgharzadeh *et al.*, 2019; Wang and Hu, 2018).

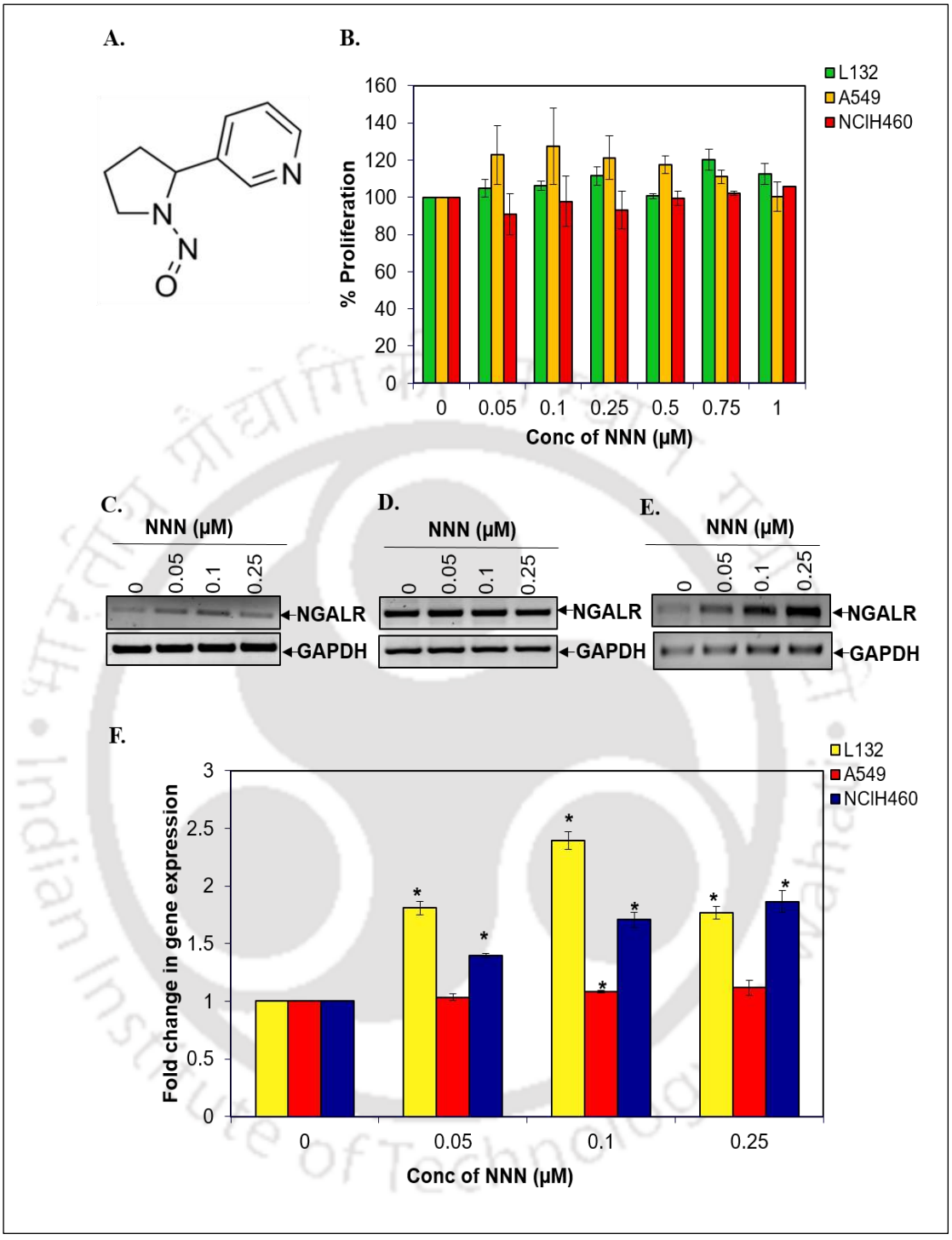

Figure 3.3. Effect of NNN on the expression of *NGALR* in human lung epithelial cells and lung cancer cells A. Structure of NNN, B. MTT assay showing non-cytotoxic concentrations of NNN in L132, A549, NCIH460 cells. RT-PCR analysis of the expression of *NGALR* after treating C. L132, D. A549, E. NCIH460 with NNN for 24 h, F. Graphical representation of the expression of *NGALR* in NNN treated L132, A549, NCIH460 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.

Stimulation of nAChR by nicotine leads to constitutive activation of key intracellular pathways including JAK/STAT, PI3K/Akt/mTOR, RAS/RAF/MEK/ERK, and enhanced NF-κB activity, increased transcription of mitogenic promoters, suppressed mitochondrial death pathway, and activated pro-angiogenic factors (Czyżykowski *et al*., 2016). A recent report also suggested that nicotine facilitated the development of NSCLC via activation of long intergenic non-protein coding RNA 460 (LINC00460) and PI3K/Akt signaling (Zhao *et al*., 2019). This addictive component has also been reported to enhance epithelial-mesenchymal transition (EMT), and stimulate invasion and metastasis of lung cancer *in vitro* (Hou *et al*., 2016). Further, nicotine has been reported to confer chemoresistance in lung cancer *in vitro* via promoting Mcl-1 phosphorylation and enhancing its binding to Bak (Liu *et al*., 2019). Notably, nicotine has also been shown to induce resistance of lung cancer cells to cisplatin via enhancing Bcl-2 stability (Nishioka *et al*., 2014). As nicotine is involved in the regulation of various molecules involved in lung cancer pathogenesis, we attempt to see whether it has any effect on the regulation of *NGALR* expression. For this purpose, we initially determined the non-toxic concentrations of nicotine on L132, A549, and NCIH460 cells using MTT assay and found that the concentrations 0.25, 0.5, and 0.75µM did not impart any cytotoxicity. Next, using the above concentrations of nicotine for treating the cells for 24 hours, the expression of *NGALR* was analyzed by RT-PCR. Our results showed that nicotine markedly enhanced the expression of *NGALR* in NCIH460 cells up to 0.5µM, although not much difference in the fold change compared to control was detected. In the case of L132 and A549 cells, the *NGALR* expression was significantly upregulated up to 0.75µM compared to control (Figure 3.4). Overall, these findings indicate the involvement of NGALR in nicotine-induced lung carcinogenesis. Moreover, it has been demonstrated that nicotine could instigate invasion and EMT in

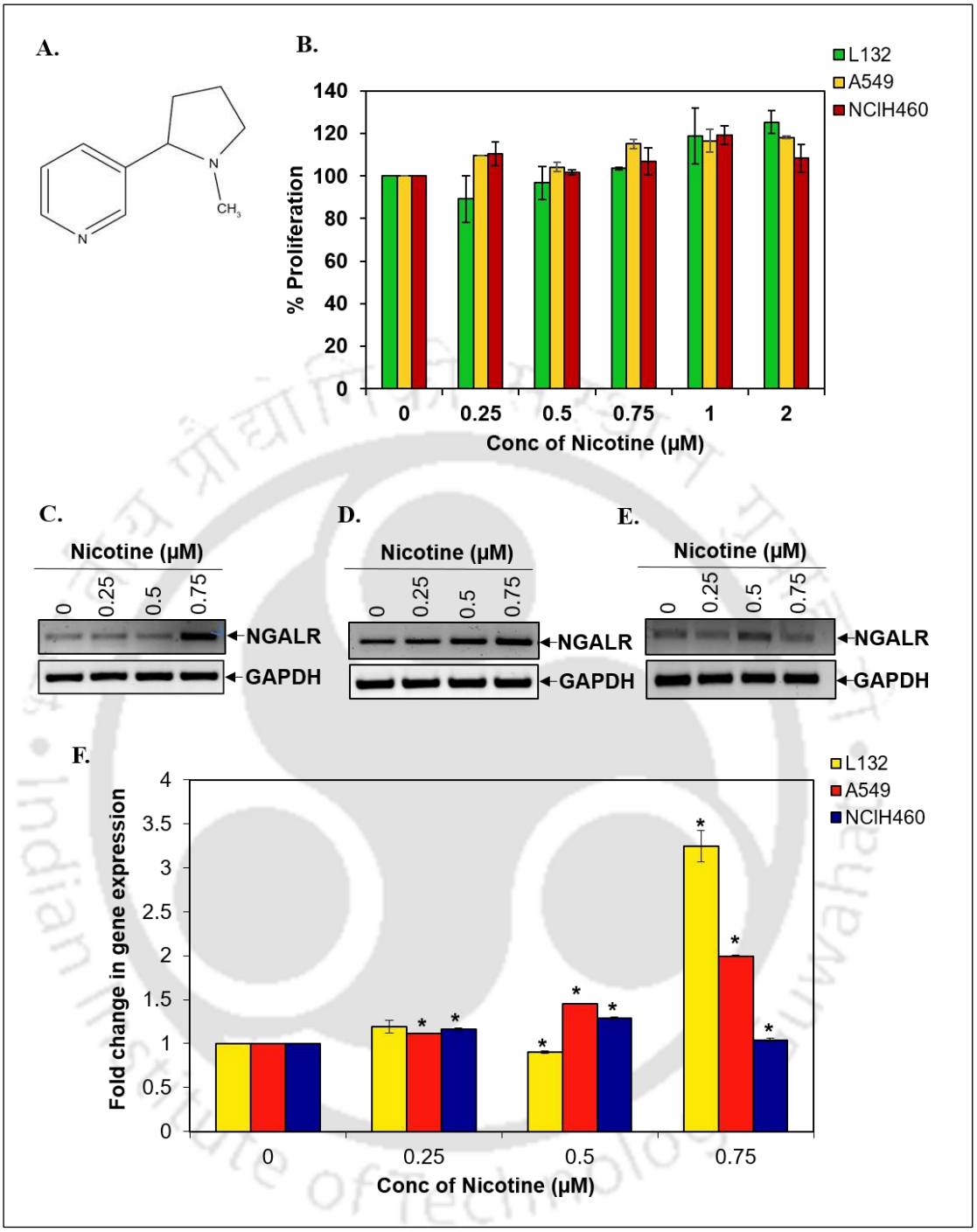

Figure 3.4. Effect of Nicotine on the expression of *NGALR* in human lung epithelial cells and lung cancer cells. A. Structure of Nicotine, B. MTT assay showing non-cytotoxic concentrations of nicotine in L132, A549, NCIH460 cells. RT-PCR analysis of the expression of *NGALR* after treating C. L132, D. A549, E. NCIH460 with nicotine for 24 h, F. Graphical representation of the expression of *NGALR* in nicotine treated L132, A549, NCIH460 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.

lung cancer *in vitro* (Davis *et al*., 2009). Additionally, multiple signaling cascades including PI3K/Akt, MAPK/ERK and JAK/STAT signaling pathways are activated by nicotine via nAChRs, thereby enhancing tumor progression (Schaal and Chellappan, 2014). Hence, it can be concluded that NGALR may be one of the downstream targets of nicotine that facilitates the development and progression of lung cancer. Nevertheless, additional investigation is imperative to determine the associated mechanism of action.

3.3.5. Effect of BaP on the Expression of NGALR in Human Lung Epithelial Cells and Lung Cancer Cells

Benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon, is one of the principal constituents of tobacco smoke that plays a major role in lung carcinogenesis. When consumed, it is converted into its reactive metabolites benzo[a]pyrene-diol-epoxide (BPDE) in the liver and lung by the CYP enzyme superfamily including CYP1A1/1A2 and CYP1B1. BPDE then binds to DNA and forms deoxyguanoside-DNA (BPDE-dG) adduct (Alexandrov *et al.*, 2010; Kasala *et al*., 2015; Wang *et al*., 2015a). Additionally, BaP also promotes tumor development through signal transduction mediated by aryl hydrocarbon receptor (AhR) (Kasala *et al*., 2015). It has been shown that BaP also mediated the activation of AhR/Src/ERK signaling cascade which promotes induction of CYP1A1 and formation of DNA adducts in lung cells (Vázquez-Gómez *et al*., 2018). BaP has been extensively used to induce lung cancer in mice and has been used by researchers with substantial success in ameliorating the pathophysiological alterations of lung cancer (Kasala *et al*., 2015). This tobacco component has also been found to induce cell cycle progression in lung cancer cells by ERK-associated activation of Chk1 pathway (Wang *et al.*, 2015a). It has been reported that BaP may be involved in lung

cancer migration and invasion via upregulating IL-8, CCL-2, and CCL-3 *in vitro* (Zhang *et al*., 2016). Additionally, it has been demonstrated that BaP promoted cell migration, invasion, and EMT in A549 lung cancer cells via enhancing the expression of a long non-coding RNA, linc00673 in an AhR-mediated manner (Wu *et al*., 2020). Therefore, analyzing the effect of BaP on the expression of NGALR protein would help us elucidate its involvement in BaP-mediated lung carcinogenesis. Primarily, to determine the non-toxic concentrations of BaP, L132, A549, and NCIH460 cells were treated with various concentrations of BaP for 24 hours and an MTT assay was performed. BaP concentrations 0.05, 0.1 and 0.25µg/ml were found to be non-toxic. Hence, these concentrations were used to treat L132, A549, and NCIH460 cells for 24h, and then the mRNA level of *NGALR* was analyzed by RT-PCR. Our results showed that BaP enhanced the expression of *NGALR* in L132 and A549 cells dose-dependently, although, in A549 cells, the difference in fold change was not prominent compared to untreated control. In the case of NCIH460, BaP also significantly increased the mRNA level of *NGALR* compared to untreated control but was not dose-dependent (Figure 3.5). Overall, this result suggested that NGALR may possess an invaluable role in BaP-mediated development and progression of lung cancer. Nonetheless, further studies are highly warranted to decipher the molecular mechanism associated with the enhanced expression of NGALR by BaP.

3.3.6. Effect of α7nAChR on the Expression of NGALR in NCIH460 Lung Cancer Cells

Nicotinic acetylcholine receptors (nAChRs) are ion channels found in the cell membrane of all mammalian cells, including cancer cells. Emerging evidence suggested that nAChRs not only facilitate addiction of nicotine in the brain but also

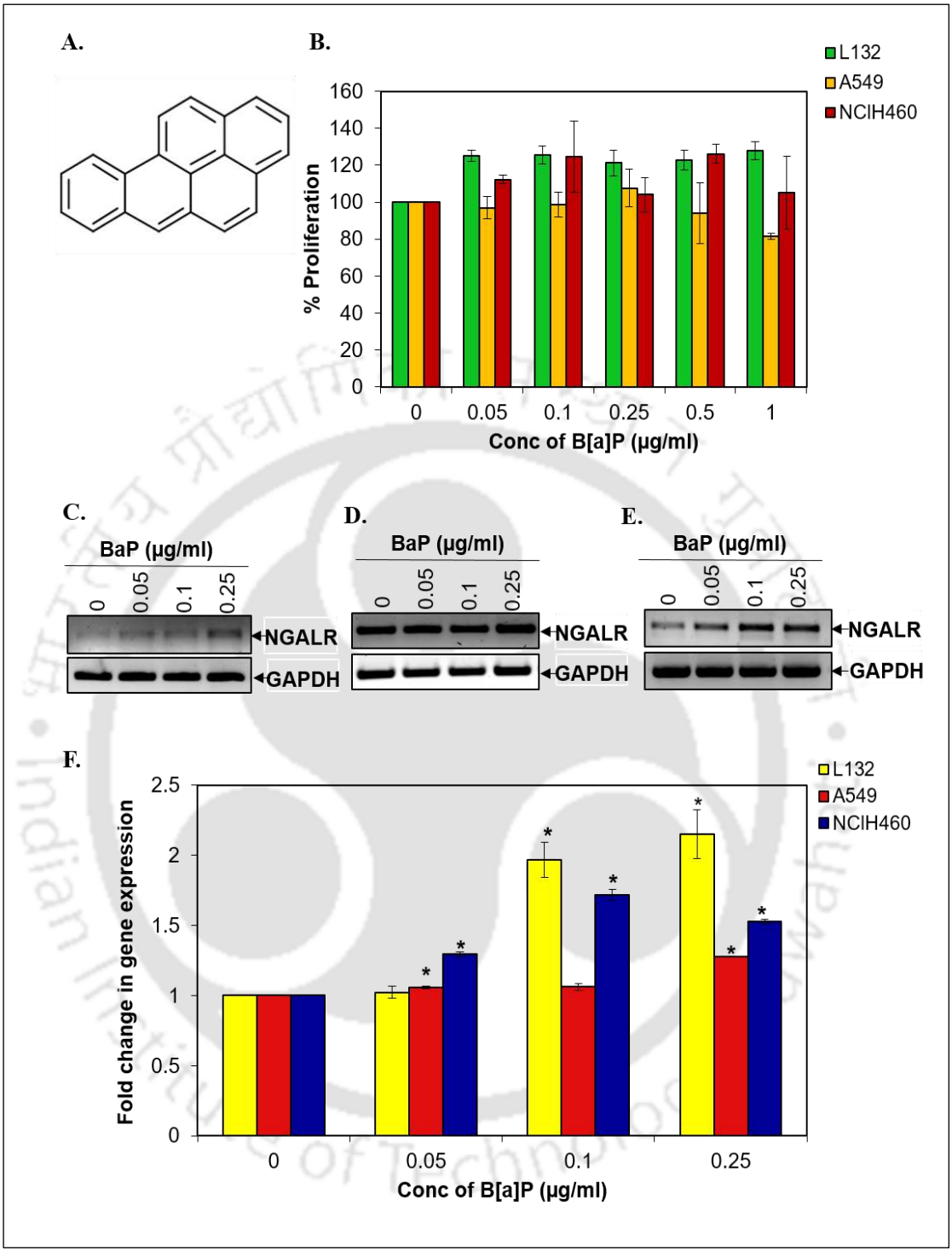

Figure 3.5. Effect of BaP on the expression of *NGALR* in human lung epithelial cells and lung cancer cells A. Structure of BaP, B. MTT assay showing non-cytotoxic concentrations of BaP in L132, A549, NCIH460 cells. RT-PCR analysis of the expression of *NGALR* after treating C. L132, D. A549, E. NCIH460 with BaP for 24 h, F. Graphical representation of the expression of *NGALR* in BaP treated L132, A549, NCIH460 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.

directly partake in the development and progression of cancers induced by nicotine and its derivative nitrosamines including NNK and NNN (Zhao *et al*., 2016). These carcinogens have been found to bind to nAChRs, subsequently activating the serine/threonine kinase Akt, protein kinase A (PKA), and various other important molecules involved in cancer progression (Zhao *et al*., 2016). The nAChRs comprises five subunits with homo- or hetero-pentamers that form ligand-gated ion channels found in the cell membranes of various neurons and on the presynaptic and postsynaptic region of the neuromuscular junction. Nicotine mimics acetylcholine and binds to the α subunit of nAChRs as an agonist (Xue *et al*., 2014). The homomeric α7nAChR subtype is the most powerful regulator of responses that activate cancer cells and is accountable for the proliferative, pro-angiogenic, and pro-metastatic properties of nicotine in lung cancer (Xue *et al*., 2014; Wang *et al*., 2018b). Aforementioned, nicotine promoted NSCLC cell progression, which is mediated via α7nAChRs (Zhang C *et al*., 2016). Besides nicotine, NNK binds to α7nAchR, with 5000 times more affinity compared to nicotine and activates voltage-gated Ca2+ channels and causes an influx of Ca2+ into lung cells, which leads to the activation of PKC, the serine/threonine kinase RAF1, ERK1/2, and transcription factors Fos, Jun, and Myc, thereby augmenting the proliferation of lung cancer cells (Xue *et al*., 2014). In addition, studies have shown that in A549 lung cancer cells, α7nAChR activates the Akt and MEK–ERK pathways and modulates nicotine-associated cell proliferation. Further, it has been indicated that obstruction of α7nAChRs impedes nicotine-induced tumor growth via the MEK–ERK signaling pathway in NSCLC. Thus, the interaction of α7nAChR with nicotine elevates epidermal growth factor (EGF) and binds to EGFR, thereby activating the RAS–RAF–ERK cascade, leading to enhanced cell proliferation (Hajiasgharzadeh *et al*., 2019). Hence, to determine whether α7nAChRs has any effect on the expression

of NGALR in lung cancer cells, knockdown of α7nAChRs in NCIH460 cells was carried out using siRNA. The gene silencing was analyzed by quantitative real-time PCR. Our results showed that upon knockdown of α7nAChR, the expression of *NGALR* was also downregulated (Figure 3.6 A & B). This finding gave an indication that α7nAChR may be a positive regulator of NGALR and activation of α7nAChR by nicotine and its derivatives may plausibly have led to upregulation of NGALR, thereby contributing to tobacco-mediated lung cancer pathogenesis. However, extensive studies are still necessary to fully establish the exact mechanism of action.

3.3.7. Effect of TNF-α and TNF-β on the Expression of NGALR in NCIH460 Lung Cancer Cells

Aforementioned, pro-inflammatory cytokines TNF-α and TNF-β (lymphotoxin-α) produced by macrophages and lymphocytes respectively play significant roles in airway inflammation as well as tumorigenesis (Seifart *et al*., 2005; Aggarwal *et al*. 2012). TNF is considered a key mediator of inflammation-induced cancer (Gong *et al*., 2018). Although TNF-α has been reported to exert its anticancer effect by inducing cancer cell death, TNF-α has been evinced to induce cell survival, proliferation, angiogenesis, and metastasis in most cancer cells that are resistant to TNF-α-induced cell death (Ooppachai *et al*., 2019). In such case, TNF-α binds to its receptor and activates various molecular pathways including NF-κB, and MAPKs which in turn induce cancer cell survival and progression via upregulation of antiapoptotic (e.g., survivin, XIAP, Bcl-2), proliferative (e.g., cyclin D and cyclin B1), invasive (e.g., MMP-9), and angiogenic (e.g., VEGF, and COX-2) proteins (Wang and Lin, 2008; Subkamkaew *et al*., 2019). Endogenous cytokine TNF-α has also been reported as a potent mutagen due to its ability to induce DNA damage via the stimulation of reactive

oxygen species (ROS). The production of TNF-α by inflammatory cells or tumor cells in the tumor microenvironment supports tumor cell survival by activating NF-κB-related anti-apoptotic proteins (Gomes *et al*., 2014). Notably, studies revealed that TNF may induce resistance of lung cancer cells to ROS-based therapies as well as doxorubicin treatment (Pogrebniak *et al*., 1991; Prewitt *et al*., 1994). It has been revealed that in lung cancer cells, TNF-driven adaptive response facilitates resistance to EGFR inhibition (Gong *et al*., 2018). A study also showed that TNF-β genetic variant may interact with environmental factors and contribute to the susceptibility to NSCLC (Liu *et al*., 2015). It has also been demonstrated that silencing of TNF-α augmented radiosensitivity in radioresistant lung cancer cells (Zhu *et al*., 2019). Therefore, analyzing the effect of TNF-α and TNF-β on the expression of NGALR along with other proteins involved in lung cancer progression would help us determine whether NGALR has any role in TNF-associated lung carcinogenesis. For this purpose, NCIH460 cells were treated with 0.1 and 1nM of TNF-α and TNF-β for 24h, and then the expressions of NGALR, NGAL, NF-κB and cyclin D1 were analyzed using Western blot. Our results showed that the protein level of NGALR was upregulated from its basal level upon treatment with the TNF-α and TNF-β. Moreover, its ligand NGAL was also upregulated at 1nM and 0.1nM of TNF-α and TNF-β respectively (Figure 3.6 C). Numerous studies demonstrated that TNF members mediate the activation of NF-κB and its regulated genes which induce cancer cell survival and metastasis (Gaur and Aggarwal, 2003; Ooppachai *et al*., 2019). In support to this, our result also showed that TNF-α and TNF-β upregulated the expression of NF-κB and p-NF-κB^{S536}. Moreover, TNF-α and TNF-β treatment also enhanced the expression of cyclin D1 which is involved in lung cancer cell proliferation and growth (Figure 3.6 C). Hence, results evidenced the involvement of NGALR in TNF-mediated lung carcinogenesis.

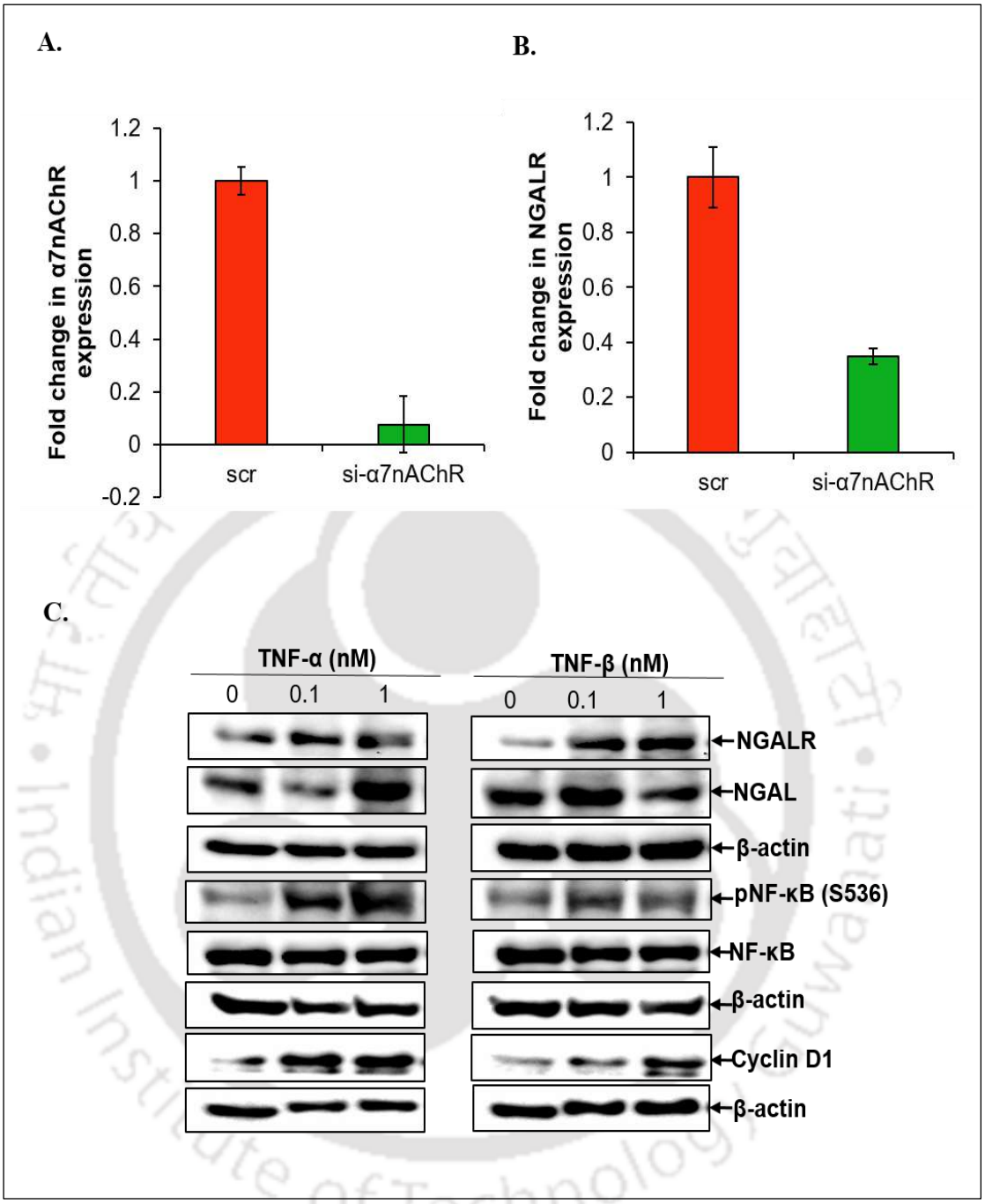

Figure 3.6. A. siRNA mediated knockdown of α7 nicotinic acetylcholine receptor (α7nAChR) on NCIH460 cells analyzed by qPCR, B. mRNA expression of NGALR post knockdown of α7nAChR on NCIH460 cells analyzed by qPCR. Data are represented as Mean± SE, C. Effect of cytokines TNF-α and TNF-β on NGALR expression and its related genes on NCIH460 cells analyzed by Western blot. β-actin was used as loading control.

3.3.8. Promoter Analysis to Determine the Putative Transcription Factor Binding Sites in *NGALR* Promoter Region via In Silico Approach

Promoter analysis is a crucial step for identifying regulatory networks. Accurate prediction of functional transcription factor binding sites (TFBS) is pivotal for successful promoter analysis. Control of transcription initiation is an essential mechanism to determine if a gene is expressed and how much mRNA is translated into proteins (Cartharius *et al*., 2005). Since the actual mechanism involved in the regulation of the *NGALR* gene has not been clearly elucidated, we have used in silico approach to determine the putative TFBS in the promoter region of the *NGALR*. Analyzing the potential transcription factors that can bind to the promoter region of NGALR enables us to determine the *NGALR* gene regulation at the transcriptional level. For this purpose, promoter analysis was done using Genomatix: MatInspector. Our results showed that the NGALR promoter has the potential to bind to numerous transcription factors (676 matches identified). Among these transcription factors, several of them have been reported to play significant roles in lung cancer development, progression, and metastasis and are listed below with their matrix similarity score and their binding site sequence:

i) Nuclear factor-kappaB (NF-κB)

ii) Signal transducer and activator of transcription (STAT)

iii) Activating enhancer-binding protein (AP)4

iv) Kruppel-like factor (KLF) family of transcription factor

v) Homeobox transcription factor NANOG

vi) Sox (sex-determining region Y (Sry) box) family transcriptional factors

vii) cAMP-responsive element binding protein (CREB)

viii) Smad

ix) Nuclear factor of activated T-cells (NFAT)

3.3.8.1. NF-κB: It is well established that NF-κB regulates the expression of more than 200 genes intricate in diverse processes including cell survival, inflammation, adhesion, differentiation, as well as growth. Overexpression of NF-κB in both SCLC and NSCLC has been reported and is significantly correlated with disease advancement in TNM stages and poor prognosis of the patients (Chen *et al*., 2011). MatInspector promoter analysis of NGALR showed that NF-κB has two potential binding sites and may modulate the expression of NGALR expression in lung cancer (Table 3.2; figure 3.7).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$NFKAPPAB.01	NF-kappaB	0.903	acGGGAatggcccta
V$NFKAPPAB50.01	NF-kappaB (p50)	0.84	accGGGAccccccgag

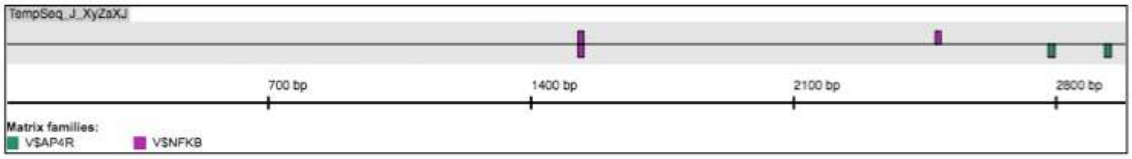

Table 3.2. NF-κB and their TFBS in NGALR promoter. Figure 3.7. Putative binding sites of NF-κB in NGALR promoter sequence.

3.3.8.2. STAT: STAT is constitutively activated in lung cancer and has also been projected to contribute to the development of tumor resistance to conventional and targeted therapies (Dutta *et al*., 2014). Our MatInspector promoter analysis of NGALR showed that STAT family has six potential binding sites in the NGALR promoter and may modulate the expression of NGALR in lung cancer (Table 3.3; figure 3.8).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$STAT3.02	Signal transducer and activator of transcription 3	0.957	agcaTTCCtgggagcttgt
V$STAT1.02	Signal transducer and activator of transcription 1	0.886	aagctcccaGGAAtgctgc
V$STAT.01	Signal transducers and activators of transcription	0.915	agagtttggGGAAagaagt
V$STAT.01	Signal transducers and activators of transcription	0.924	gtgtttagaGGAAgagggg
V$STAT3.02	Signal transducer and activator of transcription 3	0.971	tacgTTCCaggacttgcac
V$STAT1.01	Signal transducer and activator of transcription 1	0.775	gcaagtcctGGAAcgtagt

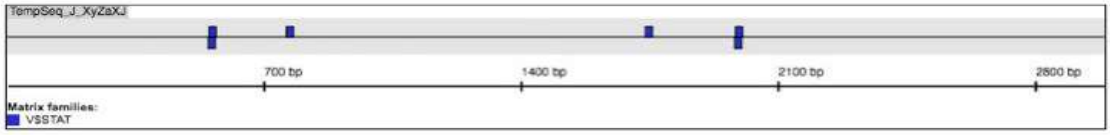

Table 3.3. STAT and their TFBS in NGALR promoter. Figure 3.8. Putative binding sites of STAT in NGALR promoter sequence.

3.3.8.3. AP4: The basic helix-loop-helix transcription factor activating AP4 has been implicated in tumor biology. Expression of AP4 was found to correlate with poor prognosis in NSCLC (Gong *et al*., 2014). Hu *et al*. have found that silencing of AP4 suppressed proliferation, induced cell cycle arrest, and facilitates apoptosis in human lung cancer cells (Hu *et al*., 2016). Our TFBS analysis showed that AP4 has two potential binding sites in the promoter region of NGALR and may regulate the expression of NGALR in lung cancer (Table 3.4; figure 3.9).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$AP4.02	Activator protein 4	0.946	ccctgcAGCTgctccgt
V$AP4.02	Activator protein 4	0.965	agctgcAGCTgctggcc

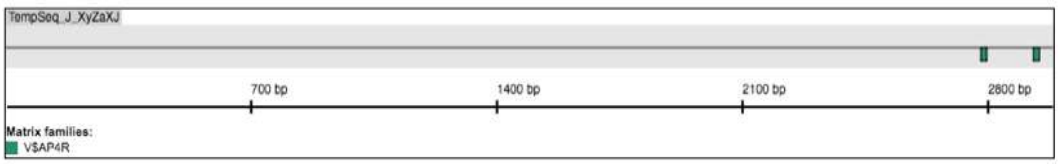

Table 3.4. Activating enhancer-binding protein 4 (AP4) and their TFBS in NGALR promoter. Figure 3.9. Putative binding sites of AP4 in NGALR promoter sequence.

3.3.8.4. Krueppel like factors (KLF): KLF family of transcription factors has several functions in regulating the cell cycle, proliferation and tumorigenesis and have been found to be overexpressed in various types of malignancy. For instance, KLF8 was found to aid in the progression of lung adenocarcinoma (Liu *et al*., 2017). Another report revealed that KLF5 instigated hypoxia-induced survival and hindered apoptosis in NSCLC cells (Li *et al*., 2014). Our MatInspector analysis has shown that KLF has twenty-eight possible binding sites on NGALR promoter sequence, hence may have a role in regulating the expression of NGALR in lung cancer (Table 3.5; Figure 3.10).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$KKLF.01	Kidney-enriched kruppel-like factor, KLF15	0.932	gcttgagctGGGGaggcgg
V$EKLF.01	Erythroid krueppel like factor (EKLF)	0.926	ttcttcagacaGGGTctca
V$GKLF.02	Gut-enriched Krueppel-like factor	0.97	tatacattAAAGgtagaga
V$EKLF.02	Erythroid krueppel like factor (EKLF, KLF1)	0.937	ggtctgGGTGaggcttgga
V$GKLF.02	Gut-enriched Krueppel-like factor	0.98	gcttgttcAAAGgcctcgt
V$KLF12.01	Krueppel-like factor 12 (AP-2rep)	0.916	gccttcaGTGGtgtctcct
V$KLF7.02	Kruppel-like factor 7 (ubiquitous, UKLF) (secondary DNA binding preference)	0.897	agtctgGGCGtcaggtttg
V$KLF2.01	Krueppel-like factor 2 (lung) (LKLF)	0.986	tgtatGGGTggtgattgaa
V$GKLF.01	Gut-enriched Krueppel-like factor	0.903	gatgagaggAGGGcctagg
V$GKLF.01	Gut-enriched Krueppel-like factor	0.874	agggagggaAGGGgggctc
V$BKLF.02	Kruppel-like factor 3 (basic)	0.935	ggggagGGAGggaagggggg
V$BKLF.02	Kruppel-like factor 3 (basic)	0.942	cccaggGGAGggagggaag
V$GKLF.01	Gut-enriched Krueppel-like factor	0.905	gagagaggaAGGGgggcca
V$KKLF.01	Kidney-enriched kruppel-like factor, KLF15	0.941	acgtgggaaGGGGaggaga
V$KKLF.01	Kidney-enriched kruppel-like factor, KLF15	0.926	ctagaggaaGGGGaggggga
V$BKLF.02	Kruppel-like factor 3 (basic)	0.986	ggaaggGGAGgggaaagca
V$KLF12.01	Krueppel-like factor 12 (AP-2rep)	0.94	ggtcccgGTGGccacccgg
V$KLF6.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers (KLF6, ZF9)	0.993	atggcgGGGGcggcggcaa
V$KLF7.01	Kruppel-like factor 7 (ubiquitous, UKLF)	0.953	cgcgggGGCGggggtgctg
V$KLF6.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers (KLF6, ZF9)	0.987	tccgcgGGGGcgggggtgc
V$BKLF.02	Kruppel-like factor 3 (basic)	0.933	tgggagGGAGggctaggaa
V$BKLF.02	Kruppel-like factor 3 (basic)	0.94	cagggtgGGAGggagggcta
V$KLF6.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers (KLF6, ZF9)	0.967	gggcagGGGGtggcaggag
V$EKLF.01	Erythroid krueppel like factor (EKLF)	0.896	cgcatctccgaGGGTcccg
V$KLF12.01	Krueppel-like factor 12 (AP-2rep)	0.927	ctgctccGTGGgcaccgca
V$BKLF.02	Kruppel-like factor 3 (basic)	0.989	cgagcgGGAGggggaggga
V$KLF6.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers (KLF6, ZF9)	0.949	cgggagGGGGagggagacg
V$BKLF.02	Kruppel-like factor 3 (basic)	0.942	ggagggGGAGggagacgcg

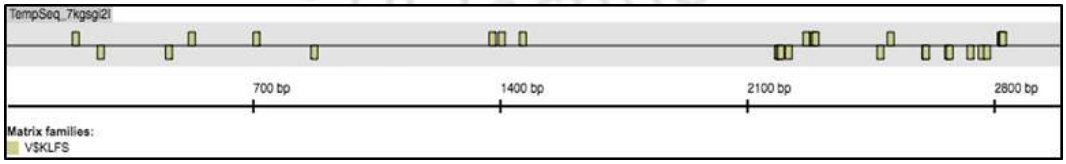

Table 3.5. KLF and their TFBS in NGALR promoter. Figure 3.10. Putative binding sites of krueppel like transcription factors in NGALR promoter sequence.

3.3.8.5. Homeobox transcription factor NANOG: NANOG has been reported to be overexpressed in lung cancer tissues compared to the normal lung tissues, and its expression was directly correlated with tumor differentiation and clinical stages of lung cancer patients. Hence, NANOG may serve as a promising prognostic marker in lung cancer (Du *et al*., 2013; Cheng *et al*., 2018). Our promoter analysis showed that NANOG has eight potential binding sites in the promoter region of NGALR suggesting that it may regulate the expression of NGALR at the transcript level in lung cancer (Table 3.6; figure 3.11).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$NANOG.01	Homeobox transcription factor Nanog	0.945	agacgggAATGgccctaga
V$NANOG.01	Homeobox transcription factor Nanog	1	attagtgAATGggtgaatg
V$NANOG.01	Homeobox transcription factor Nanog	0.942	atgggtgAATGaatgaatg
V$NANOG.01	Homeobox transcription factor Nanog	0.947	gtgaatgAATGaatgaatg
V$NANOG.01	Homeobox transcription factor Nanog	0.947	atgaatgAATGaatgaatg
V$NANOG.01	Homeobox transcription factor Nanog	0.947	atgaatgAATGaatgaatg
V$NANOG.01	Homeobox transcription factor Nanog	0.947	atgaatgAATGaatgatgg
V$NANOG.01	Homeobox transcription factor Nanog	0.942	atgaatgAATGatggcaaa

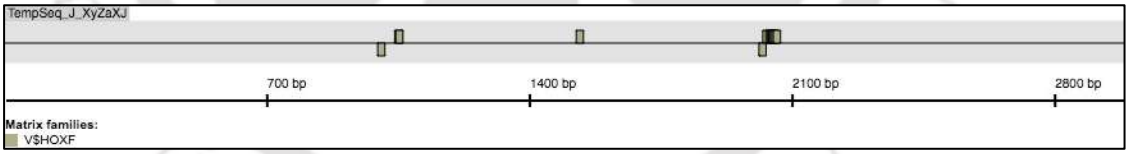

Table 3.6. NANOG and their TFBS in NGALR promoter. Figure 3.11. Putative binding sites of NANOG in NGALR promoter sequence.

3.3.8.6. Sox (sex-determining region Y (Sry) box) family transcriptional factors: Sox family of transcriptional factors are strong modulators of multiple processes involved in stem cell maintenance, tissue homeostasis, and carcinogenesis. Studies have shown that deregulation of *Sox* genes is implicated in various cancer including lung cancer. Recent studies revealed the crucial role of the members of the *Sox* gene

family in lung carcinogenesis and progression (Zhu *et al*., 2012). From promoter analysis of NGALR, we have found that Sox has nine potential binding sites, and may determine the expression of NGALR in lung cancer (Table 3.7; Figure 3.12).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$SOX5.01	Sox-5	0.872	ggctgaCAATgatagaacactgc
V$SOX21.03	SRY (sex determining region Y)-box 21, dimeric binding sites	0.817	cttGAATcataatctgtatttta
V$SOX1.04	SRY (sex determining region Y)-box 1, dimeric binding sites	0.802	ctaGAATtgctactcaaagtgtg
V$SOX6.01	SRY (sex determining region Y)-box 6	0.99	tataaACAAagcggacgctgaac
V$SOX21.03	SRY (sex determining region Y)-box 21, dimeric binding sites	0.767	gttGAATtctttacaaaattctg
V$SOX9.02	SRY (sex-determining region Y) box 9	0.947	tggccACAAtgcgatgttgaggg
V$SOX1.01	SRY (sex determining region Y)-box 1, dimeric binding sites	0.768	ccaCAATgcgatgttgagggtgc
V$SOX5.01	Sox-5	0.997	tacaaaCAATacaaacctgacgc
V$SOX1.04	SRY (sex determining region Y)-box 1, dimeric binding sites	0.795	tttGAATctccatttctcaacag

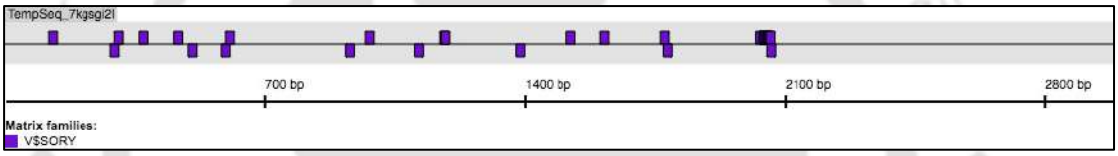

Table 3.7. Sox (sex-determining region Y (Sry) box-containing) family transcriptional factors and their TFBS in NGALR promoter. Figure 3.12. Putative binding sites of Sox in NGALR promoter sequence.

3.3.8.7. cAMP-responsive element binding protein (CREB): Studies have shown that genes regulated by CREB partake in hindering apoptosis, enhancing cell proliferation, inflammation, and metastasis. Additionally, it has been reported that constitutive activation of CREB is chiefly involved in supporting the growth and survival of NSCLC cells (Aggarwal *et al*., 2008). Our promoter analysis by MatInspector showed that NGALR could be one of the genes regulated by CREB as it has four potential binding sites on the NGALR promoter sequence (Table 3.8; figure 3.13).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$CREB1.01	cAMP-responsive element binding protein 1	0.98	ccagacTGACgtctccagctt
V$CREB.02	cAMP-responsive element binding protein	0.917	cgcccagacTGACgtctccag
V$CREB.02	cAMP-responsive element binding protein	0.973	ccagcggggTGACgcaactgc
V$CREB.02	cAMP-responsive element binding protein	0.971	aggttctgcTGACgtgggaag

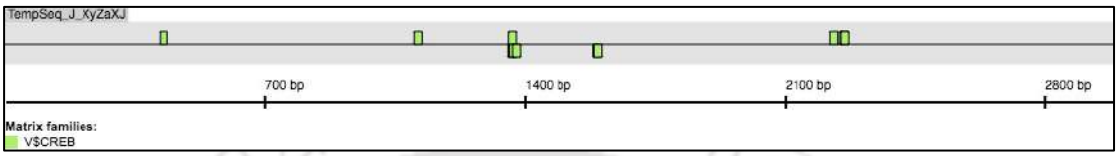

Table 3.8. CREB and their TFBS in NGALR promoter. Figure 3.13. Putative binding sites of CREB in NGALR promoter sequence.

3.3.8.8. Smad: Studies have shown that transforming growth factor (TGF)-β/Smad signaling promotes metastasis of lung adenocarcinoma (Yu *et al*., 2015). Further, TGF-β activates Smad2 and Smad3, which partner with Smad4 and then translocate into the nucleus and regulates various genes involved in carcinogenesis. Particularly, in lung cancer, Smad6 is found to be overexpressed in a portion of the tumors and is correlated with poor survival in NSCLC patients (Jeon *et al*., 2010). In addition, the TGF-β/Smad3 pathway has also been recently found to contribute to NSCLC progression regulated by miR-133a (Shen *et al*., 2020). The TFBS analysis showed that Smad has eight potential binding sites in the promoter region of NGALR, hence may in part regulate the expression of NGALR at the transcript level (Table 3.9; figure 3.14).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$SMAD3.01	Smad3 transcription factor involved in TGF-beta signaling	0.997	aggGTCTgggt
V$SMAD3.01	Smad3 transcription factor involved in TGF-beta signaling	0.994	tcaGTCTgggc
V$SMAD3.02	Smad3 transcription factor involved in TGF-beta signaling factor PU.1	0.988	agtGTCTggaa
V$SMAD4.01	Smad4 transcription factor involved in TGF-beta signaling	0.943	actGTCTtggc
V$SMAD.01	Sma- and Mad-related proteins	0.989	catGTCTgtct
V$SMAD3.01	Smad3 transcription factor involved in TGF-beta signaling	0.996	agaGTCTggac
V$SMAD.01	Sma- and Mad-related proteins	0.966	gctGTCTgtgt
V$SMAD3.02	Smad3 transcription factor involved in TGF-beta signaling factor PU.1	0.945	agcGTCTggca

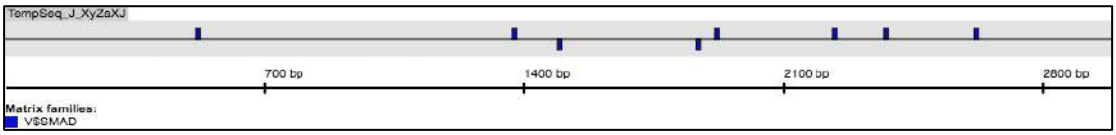

Table 3.9. Smad transcription factors and their TFBS in NGALR promoter. Figure 3.14. Putative binding sites of Smad in NGALR promoter sequence.

3.3.8.9. NFAT: NFAT has been found to contribute to the development of various types of cancer. NFAT is upregulated in lung cancer cells and plays important roles in the proliferation and migration of human NSCLCs (Guo *et al*., 2015; Zhang *et al*., 2007). NGALR promoter analysis showed that NFAT has five potential binding sites that may be involved in regulating the expression of NGALR in lung cancer (Table 3.10; figure 3.15).

Matrix	Detailed Matrix Information	Matrix sim.	Sequence
V$NFAT5.02	Nuclear factor of activated T-cells 5	0.994	acgtGGAAaatttctgaac
V$NFAT5.02	Nuclear factor of activated T-cells 5	0.879	gctgGGAAaactagaagaa
V$NFAT5.02	Nuclear factor of activated T-cells 5	0.895	agcaGGAAagatacatgat
V$NFAT5.02	Nuclear factor of activated T-cells 5	0.876	aggaGGAAaatgccagacg
V$NFAT.01	Nuclear factor of activated T-cells	0.971	gcaggaGGAAaatgccaga

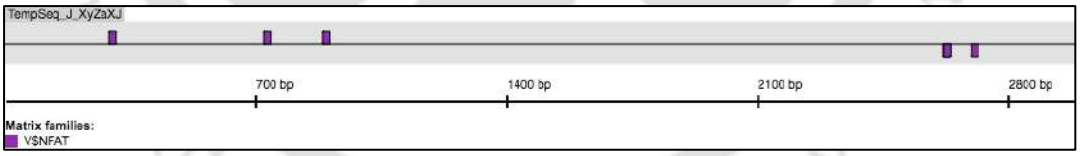

Table 3.10. NFAT and their TFBS in NGALR promoter. Figure 3.15. Putative binding sites of NFAT in NGALR promoter sequence.

3.3.9. Silencing of the Transcription Factors that have Potential Binding Sites in NGALR Promoter

In order to further evaluate whether the above transcription factors have any role in regulating the expression of NGALR, they were silenced using siRNA mediated knockdown in NCIH460 cells and the expression of NGALR was determined. Knockdown of these transcription factors and expression of NGALR post-knockdown

was analyzed by western blot analysis and real-time PCR on cDNA prepared from isolated RNA 48h post-transfection. Our results showed that upon knockdown of the transcription factors STAT-3, AP4, KLF12, NANOG, SOX6, CREB, SMAD3, and NFAT, the expression of NGALR was also found to be significantly downregulated. However, the expression of NGALR was not found to be downregulated upon knockdown of NF-κB (Figure 11 A to I). Moreover, these transcription factors have been reported to play important roles in lung carcinogenesis. Collectively, our data suggest that NGALR expression at the transcript level maybe in part regulated by STAT-3, AP4, KLF12, NANOG, SOX6, CREB, SMAD3 and NFAT transcription factors. Nevertheless, further *in vitro* studies are warranted to validate our findings.

3.4. Conclusion

It has been well evinced that tobacco is the primary risk factor for lung carcinogenesis. This is the first report that showed a correlation between tobacco carcinogens and the regulation of *NGALR* in human lung cancer. Precisely, we have shown that exposure of human lung epithelial cells and lung cancer cells to tobacco smoke-infused water (tuibur) and other tobacco constituents such as NNK, NNN, BaP and nicotine resulted in significant upregulation of *NGALR*. This further strengthens our IHC data wherein NGALR expression was markedly higher in lung cancer tissues compared to normal lung tissues. Moreover, silencing of α7nAChR resulted in downregulation of *NGALR* which implies that activation of α7nAChR by tobacco-specific nitrosamine consequently upregulated NGALR expression in lung cancer cells. Therefore, our results indicate that NGALR may function as an important molecule involved in the development and progression of tobacco-induced lung carcinogenesis. Furthermore, increasing lines of evidence have evinced the essential role of pro-inflammatory

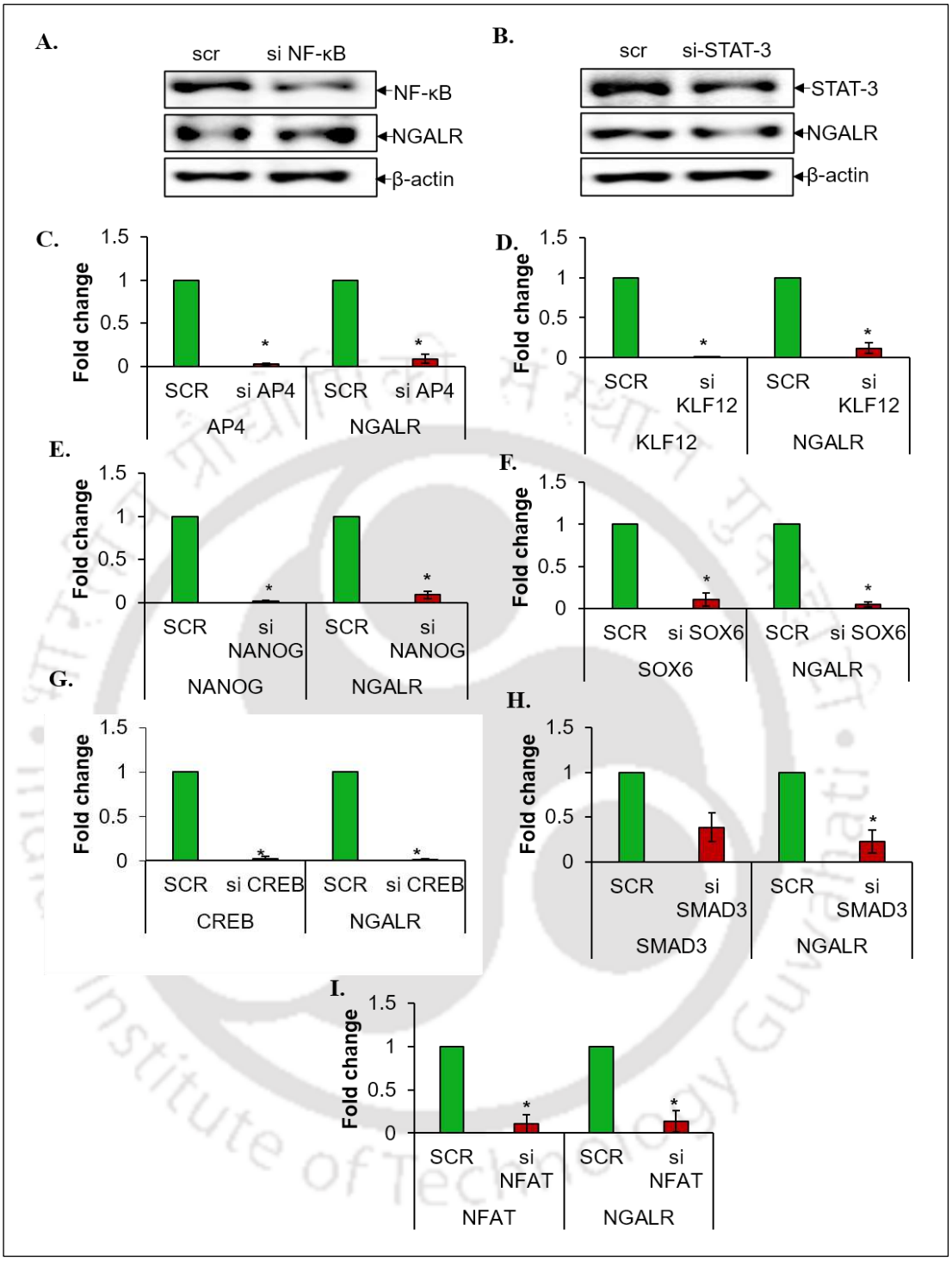

Figure 3.16. Silencing of the transcription factors that have potential binding sites in NGALR promoter via siRNA-mediated knockdown. Silencing of A. NF-κB and B. STAT-3 on NCIH460 cells and NGALR expression analyzed on the knockdown cells by western blot. β-actin was used as loading control; Silencing of C. AP4, D. KLF12, E. NANOG, F. SOX6, G. CREB, H. SMAD3, I. NFAT by siRNA on NCIH460 cells and NGALR expression analyzed on the knockdown cells by qPCR. Data are represented as Mean± SE, * =p <0.05 compared to scramble control.

cytokines such as TNF in the development of lung cancer. Our study has shown that treatment of lung cancer cells with TNF-α and TNF-β significantly upregulated NGALR and its ligand NGAL, along with NF-κB and cyclin D1 which are essential proteins involved in diverse lung cancer hallmarks. This suggests that NGALR may partake in TNF-induced lung carcinogenesis, which warrants further studies. Moreover, to further elucidate the upstream regulators of NGALR, transcription factors that have potential binding sites in the promoter region of NGALR was determined. Upon silencing of these transcription factors with potential TFBS in NGALR promoter such as STAT-3, AP4, KLF12, NANOG, SOX6, CREB, SMAD3, and NFAT, the expression of NGALR was found to be significantly downregulated in lung cancer cells. Accumulating studies have shown that these transcription factors are involved in the development and progression of lung cancer via multiple molecular pathways. Hence, this indicates that these transcription factors may function as positive regulators of NGALR expression in lung cancer.

Chapter 4

Role of NGALR in Different Processes Involved in the Development and Progression of Lung Cancer

4.1. Introduction

In the previous chapters, we have shown a significant upregulation of NGALR in lung cancer tissues compared to the normal lung tissues. Additionally, tobacco infused water and tobacco components that are potent lung carcinogens were found to remarkably upregulate the expression of NGALR in human lung epithelial cells as well as in lung cancer cells. Importantly, exposure to NNK, which is a strong carcinogen used regularly to induce lung carcinogenesis *in vivo* significantly increased the level of NGALR in human lung epithelial cells and lung cancer cells. From these results, it is evident that NGALR is one key molecule involved in the development and progression of tobacco-related lung cancer. Further, the upregulation of NGALR upon treatment of lung cancer cells with pro-inflammatory cytokines TNF-α and TNF-β indicated the involvement of NGALR in TNF-induced lung carcinogenesis. Also, we have determined the transcription factors that have putative binding sites on the promoter region of NGALR in lung cancer which gave us an insight into the upstream regulation of NGALR in lung cancer cells. Notably, previous studies have shown that NGALR is overexpressed in various cancers and is associated with the pathogenesis of various cancers including CRC, endometrial cancer, ESCC, glioma, and HCC. However, its expression and role in lung cancer have not been unraveled thus far. Moreover, to assess the exact function of NGALR and its downstream targets, it is imperative to disrupt NGALR completely and examine its effect on the expression of proteins involved in lung carcinogenesis. Hence, in this chapter, we have performed CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas9- mediated knockout of NGALR in NCIH460 human lung cancer cells and analyzed the effect of *NGALR* gene knockout on the different hallmarks of cancer. Moreover, we determined the effect of *NGALR* gene knockout on

the expression of different proteins involved in the various processes of lung cancer. Besides these, we also attempt to decipher the role of NGALR in the mechanism involved in tobacco-mediated as well as TNF-mediated lung carcinogenesis.

4.2 Materials and Methods

4.2.1 Cell Culture

The human NSCLC cell line NCIH460 was procured from National Centre for Cell Science (NCCS), Pune, India. The 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% CO2 and 95% humidity.

4.2.2. CRISPR/Cas9 Mediated Gene Knockout

To completely inhibit the NGALR gene, CRISPR/Cas9 mediated gene-editing technology was used (Figure 4.1). For this purpose, CRISPR/Cas9 All-in-one Lentivector Set (Human) expressing human Cas9 and sgRNA i.e. scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (Cat no: K010) and SLC22A17 sgRNA CRISPR/Cas9 All-in-One Lentivector (Cat no: K2173805) were obtained commercially from Applied Biological Materials (ABM), Richmond, BC, Canada. The sequences of the sgRNA target are given in Table 4.1. Initially, NCIH460 cells were seeded at a density of 25000 cells/well in a 24 well plate and allowed to achieve 70-80% confluency. Following this, the cells were transfected with 1 µg of respective plasmids with the help of Lentifectin™ transfection reagent (Cat. No. G074, Applied Biological Materials, Richmond, BC, Canada) in incomplete opti-MEM media. After

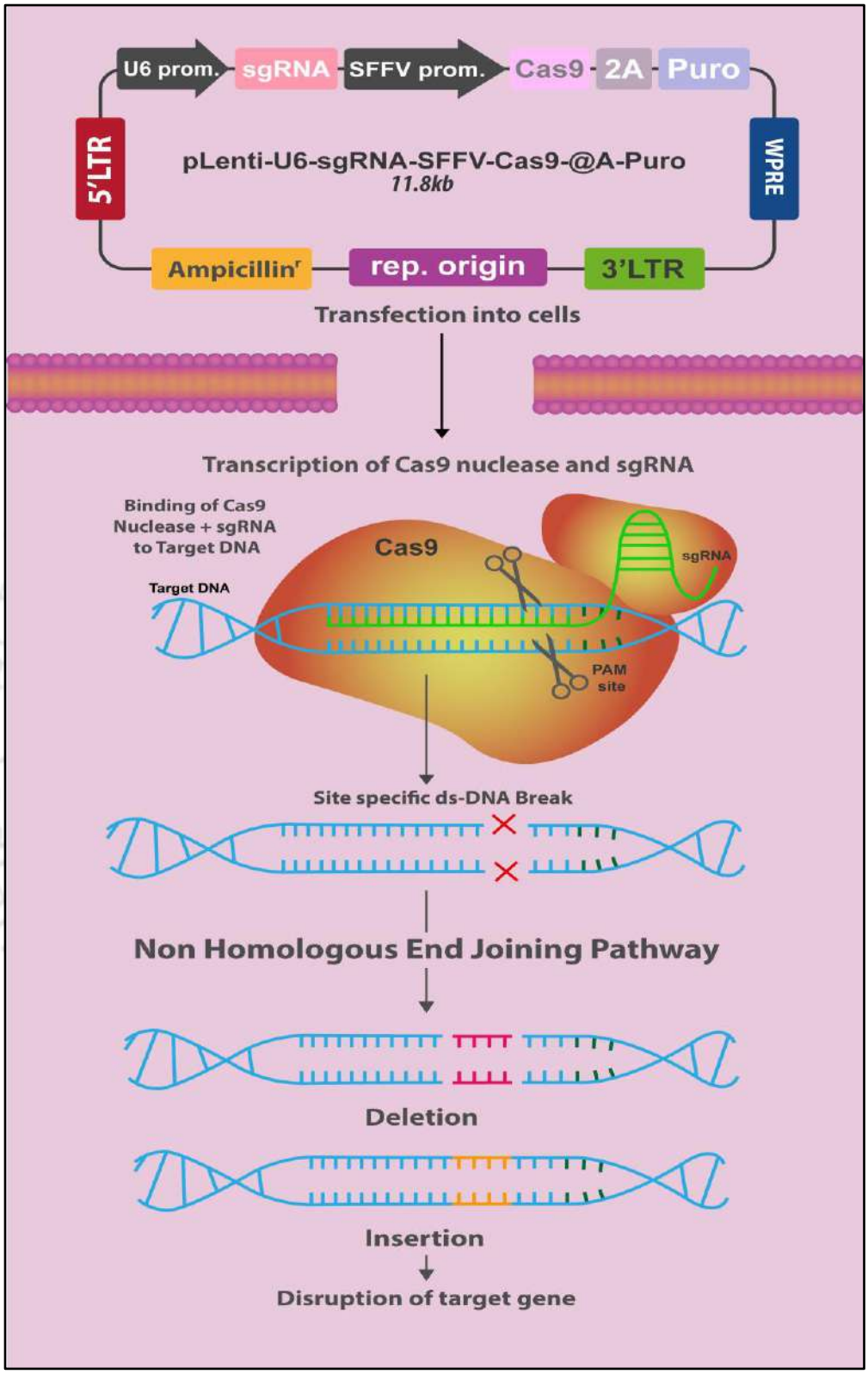

Figure 4.1. Mechanism of CRISPR/Cas9 mediated gene knockout

transfection, the cells were then incubated at 37°C in a CO2-regulated incubator in a humidified 95% air/5% CO2 atmosphere for 5-8h, followed by addition of 50µl 10% FBS (Gibco®, NY, USA) in each well and was incubated for 24h for recovery. After that, the media containing plasmid was removed and a fresh DMEM medium (with 10% FBS and 1X Penstrep) was added to the wells and kept for recovery. After that, a positive selection of transfected cells was carried out by adding 2.5 µg/ml of puromycin (Cat no: P8833, Sigma Aldrich, Missouri, USA). As the CRISPR/Cas9 plasmids contained puromycin resistant gene, only the transfected cells will be resistant to puromycin treatment. The puromycin resistant cells were then allowed to form colonies and single-cell selection was subsequently performed. Confirmation of CRISPR/Cas9 mediated knockout out of the selected single-cell clones was done using Western blot analysis. Clones showing complete inhibition of the expression of the NGALR protein was considered as the successful knockout cells and were grown for further studies.

Table. 4.1. sgRNA target sequences

Gene	Target	sgRNA target sequence
Scramble	-	GCACTCACATCGCTACATCA
NGALR	Target 1	GCCCCGTAGTGGCAATGCAG
	Target 2	GACGCTGACGCCGCTGGCAT
	Target 3	AGTACCGACCCCTCGTGCAG

4.2.3. MTT Assay

To examine the effect of NGALR knockout on the proliferation of human lung cancer cells MTT assay was performed. For this, the scramble sgRNA transfected cells (CRISPR/Cas9 scramble), as well as NGALR knockout cells (CRISPR/Cas9 NGALR)

were seeded in 96-well plates at a density of $2X10^3$ cells/well and incubated for 24h in a CO_2 incubator at 37°C. The MTT assay was carried out at 0 and 48 h. At 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 incubation, the culture medium was discarded and 100µl of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added in all the wells followed by incubation for 1h at room temperature (RT) to dissolve the MTT-formazan product. Lastly, the absorbance of the colored solution was measured using a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland) at 570 nm. The inhibition of proliferation caused by the NGALR knockout was calculated by normalizing the 48 h absorbance value with 0 h while considering the absorbance of CRISPR/Cas9 scramble as 100%. Further, the effect of NGALR knockout on the chemosensitivity to chemotherapeutic drugs in NCIH460 cells was analyzed using this assay. For this, CRISPR/Cas9 scramble and CRISPR/Cas9 NGALR cells were seeded in 96 well plates at a density of $2X10^3$ cells/well. After 24 h incubation, different concentrations of various chemotherapeutic drugs such as 5-fluorouracil (5-FU) (0, 1, 5, 10 and 50 µM), docetaxel (0, 0.5, 1 and 5 µM), carboplatin (0, 5, 10, 50 and 100 µM), cisplatin (0, 5, 10 and 50 µM), crizotinib (0, 0.5, 2.5, 5 and 10 µM) and erlotinib (0, 1, 10 and 50 µM) were added to the CRISPR/Cas9 scramble and CRISPR/Cas9 NGALR cells. The MTT assay was performed at 0 and 72 h after the addition of the drugs and the same protocol was followed as mentioned. Then, the inhibition in the proliferation of the treated scrambled and knockout cells was calculated by normalizing the absorbance value of 72 h with 0 h while considering the percentage of the proliferation of untreated scrambled and knockout cells as 100%.

4.2.4. Colony Formation Assay

To determine the clonogenic potential of NGALR knockout NCIH460 cells, colony formation assay was performed. Briefly, CRISPR/Cas9 scramble and CRISPR/Cas9 NGALR knockout cells were seeded at a density of 500cells/2ml/well in a 6-well plate. It was then incubated and grown for 15 days with replenishing of media as required. After 15 days, the plates were washed with 1X PBS and the colonies were fixed with 70% ethanol. This was followed by washing with 1X PBS again and then staining using 0.01% (w/v) crystal violet (Cat No: 548-6209; SRL Pvt. Ltd., Mumbai, India). For removal of the excess stain, gentle washing with 1X PBS was done again. The images of each well were taken, the individual clone types were identified, and the survival fraction was calculated using the formula: PE (plating efficiency) = (Number of colonies counted/ Number of cells plated) x 100; SF (survival fraction) = (PE of treated sample/PE of control) (Franken *et al*., 2006). Additionally, the effect of tobacco component NNK and pro-inflammatory cytokines TNF-α and TNF-β on the clonogenic potential of NGALR knockout cells was also determined using this assay by seeding 500cells/2ml/well in a 6-well plate and 0.05 µM NNK, 1nM TNF-α, and 1nM TNF-β were added to the CRISPR/Cas9 scramble (Cr/Cas9 Scr) and CRISPR/Cas9 NGALR knockout cells (Cr/Cas9 NGALR) and incubate for 24h. After that, the media from all the wells were changed, cells were allowed to grow for 10 days and the same procedure was followed hereafter.

4.2.5. Cell Cycle Analysis

The effect of gene knockout of NGALR on the cell cycle progression of NCIH460 cells was determined by flow cytometer assisted cell cycle analysis. Briefly, the Cr/Cas9 Scr

and Cr/Cas9 NGALR cells were plated in 6-well plates at a density of 200,000 cells/2 mL/well and allowed to grow for 24h. After 24h, the cells were trypsinized and collected, washed with 1X PBS, and fixed using 75% ethanol without any formation of clumps overnight at -20 °C. Right before the analysis, the ethanol was removed by centrifugation, followed by washing with 1X PBS and stained with Propidium Iodide (PI)/RNase solution for 20 min in dark. Following the 20 min incubation, the cells were analyzed by flow cytometer (BD FACS Celesta™, Becton-Dickinson, New Jersey, USA) and the percentage of cells in each phase of the cell cycle was analyzed using FCS Express software.

4.2.6. Migration Assay

To evaluate the migratory potential of NCIH460 cells after knockout of NGALR compared to Scramble control, wound healing assay was performed. For this, Cr/Cas9 Scr and Cr/Cas9 NGALR cells were seeded at a density of $6x10^5$ cells/mL/well in 12 well plates and allowed to form a monolayer. The medium was then replaced with a serum-free DMEM medium to inhibit cell proliferation and allowed 6-8h for adaptation. After serum starvation, a wound was formed across the monolayer using a 100 µL micro tip. The detached cells were then washed using 1X PBS and then fresh serum-free DMEM was added to the cells. Finally, migration of the cells was assessed by analyzing the difference in the area of the scratched wounds using an inverted microscope (Nikon T1-SM, Japan). Images were captured at different time intervals and then examined using Image J software. Additionally, to analyze the migratory potential of NNK, TNF-α and TNF-β treated NGALR knockout cells, this assay was performed. For this, after serum starvation followed by scratching of wound, NNK

(0.05 µM), TNF-α (0.1nM), and TNF-β (0.1nM) were added to the Cr/Cas9 Scr and Cr/Cas9 NGALR cells, and the migration of the cells was assessed as mentioned above.

4.2.7. Western Blot

The successful *NGALR* gene knockout in NCIH460 cells was determined by Western blot analysis. Additionally, this analysis was also used to determine the effect of NGALR knockout on the expression of its different targets. Briefly, Cr/Cas9 Scr and Cr/Cas9 NGALR 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 was measured using Bradford reagent (Cat. No. 500-0205; Bio-Rad, California, USA) using Bovine serum albumin (BSA) as standard. 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 the 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 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 housekeeping gene GAPDH and β-actin were

used as the loading controls. Further, this process was also used to analyze the expression of different targets in NNK, TNF-α and TNF-β treated NGALR knockout cells. For this, lysis of the cells was done post 24 h of treatment with NNK (0.05 µM), TNF-α (0.1nM), and TNF-β (0.1nM) followed by the procedure mentioned above.

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

Name	Details	Dilutions used
Anti-24p3R antibody	ab124506; abcam®, Cambridge, USA	1:5000
Anti-β-actin antibody	4697S; Cell Signaling Technology, Massachusetts, USA	1:2000
Anti-β-catenin antibody	8480S; 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-Jak2 (Tyr1007/1008) antibody	3776; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti- Jak2 antibody	3230; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- mTOR (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-Phospho-p44/42 MAPK (Thr202/Tyr204) antibody	4370; Cell Signaling Technology, Massachusetts, USA	1: 2000

Anti-p44/42 MAPK antibody	4695; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Myt1 antibody	4282; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho-Histone H3 (Ser10) antibody	3377; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-p53 antibody	2524T; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-p27 Kip1 antibody	3686; Cell Signaling Technology, Massachusetts, USA	1:2000
Anti-p18 INK4C antibody	2896; Cell Signaling Technology, Massachusetts, USA	1:2000
Anti-PTEN antibody	11-7539; ABGENEX Pvt. Ltd., Odisha, India	1: 1000
Anti-LC-3B antibody	2775S; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-Lipocalin-2 / NGAL antibody	ab41105; abcam®, Cambridge, 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-cIAP-1/HIAP-2 antibody	20-1054; ABGENEX Pvt. Ltd., India	1: 1000
Anti-caspase-9 antibody	9508T; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-caspase-3 antibody	20-1039; ABGENEX Pvt. Ltd., Odisha, India	1: 2000
Anti-CDK2 antibody	2546; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-CDK6 antibody	3136; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-cyclin B1 antibody	12231; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-cyclin D3 antibody	2936; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-cyclin D1 antibody	2978; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-cyclin E2 antibody	4132; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-EGFR antibody	4267; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-MMP-2 antibody	4022S; Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-MMP-9 antibody	13667P; Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-N-Cadherin antibody	13116; Cell Signaling Technology, Massachusetts, USA	1: 2000

Anti-Twist-1 antibody	46702; Cell Signaling Technology, Massachusetts, USA	1: 2000
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 carried out using Student's *t*-test. All the data are represented as Mean±SE. p-value < 0.05 was represented as statistically significant.

4.3. Results and Discussion

In this present chapter, we aimed to determine the role of NGALR in the development and progression of lung cancer. For this, we first established a stable NGALR knockout in NCIH460 cells using CRISPR/Cas9 mediated gene knockout. Then, we accessed the effect of NGALR knockout on the cell proliferation, cell cycle, survival, and migration of lung cancer cells. Moreover, we also determined the effect of NGALR knockout on the expression of genes involved in these different processes. Also, the effect on the proliferation, survival, and migration of NNK, TNF-α and TNF-β treated NGALR knockout cells and the associated mechanism of action of NGALR in NNK and TNF-induced lung carcinogenesis was studied.

4.3.1. Confirmation of Knockout of NGALR

Firstly, to understand the role of NGALR in lung carcinogenesis, knockout of NGALR in NCIH460 human lung cancer cells was done using CRISPR/Cas9 gene-editing technology. From the three targets given for NGALR knockout, transfection with

target1 sgRNA/Cas9 plasmid generated successful NGALR knockout clones. Confirmation of the knockout was done using Western blot analysis (Figure 4.2 A). These clones were allowed to grow for further investigations.

4.3.2. Effect of NGALR Knockout on the Proliferation of Lung Cancer Cells

It is well established that the growth and proliferative signaling pathways are aberrated in cancer cells, thus making them proliferate uncontrollably and sustain their survival (Fouad and Aanei, 2017). Thus, we determined the effect of NGALR knockout on the proliferation of lung cancer cells using MTT assay. We have found that NGALR knockout significantly decreased the proliferation of NCIH460 cells (Figure 4.2 B). The % inhibition of proliferation of NGALR knockout NCIH460 cells was found to be 75% compared to scrambled control cells after 48h. This is the first report that showed the participation of NGALR in enhancing the proliferation of lung cancer cells. Further, to confirm this decrease in proliferation, we analyzed the effect of NGALR knockout on the different phases of the cell cycle (G1, S, G2, and M phases) by flow cytometer. Our results showed that disruption of NGALR expression led to an increased number of cells in the G1 phase and decreased in the subsequent S phase (Figure 4.2 C & D). During the G1 phase, growth-dependent cyclin-dependent kinase (CDK) facilitates DNA replication by promoting the G1-to-S phase transition. This transition from the G1-to-S phase of the cell cycle is vital for regulating eukaryotic cell proliferation, and its dysregulation elicits oncogenesis (Bertoli *et al*., 2013). Accumulation of NGALR knockout cells at the G1 phase indicates that the cells fail to enter the S-phase where DNA replication takes place. Hence, we have found that the reduction in the proliferation of lung cancer cells acquired by silencing of NGALR is mediated through inhibition of cell cycle progression due to cell cycle arrest at the G1 phase. Thus, this

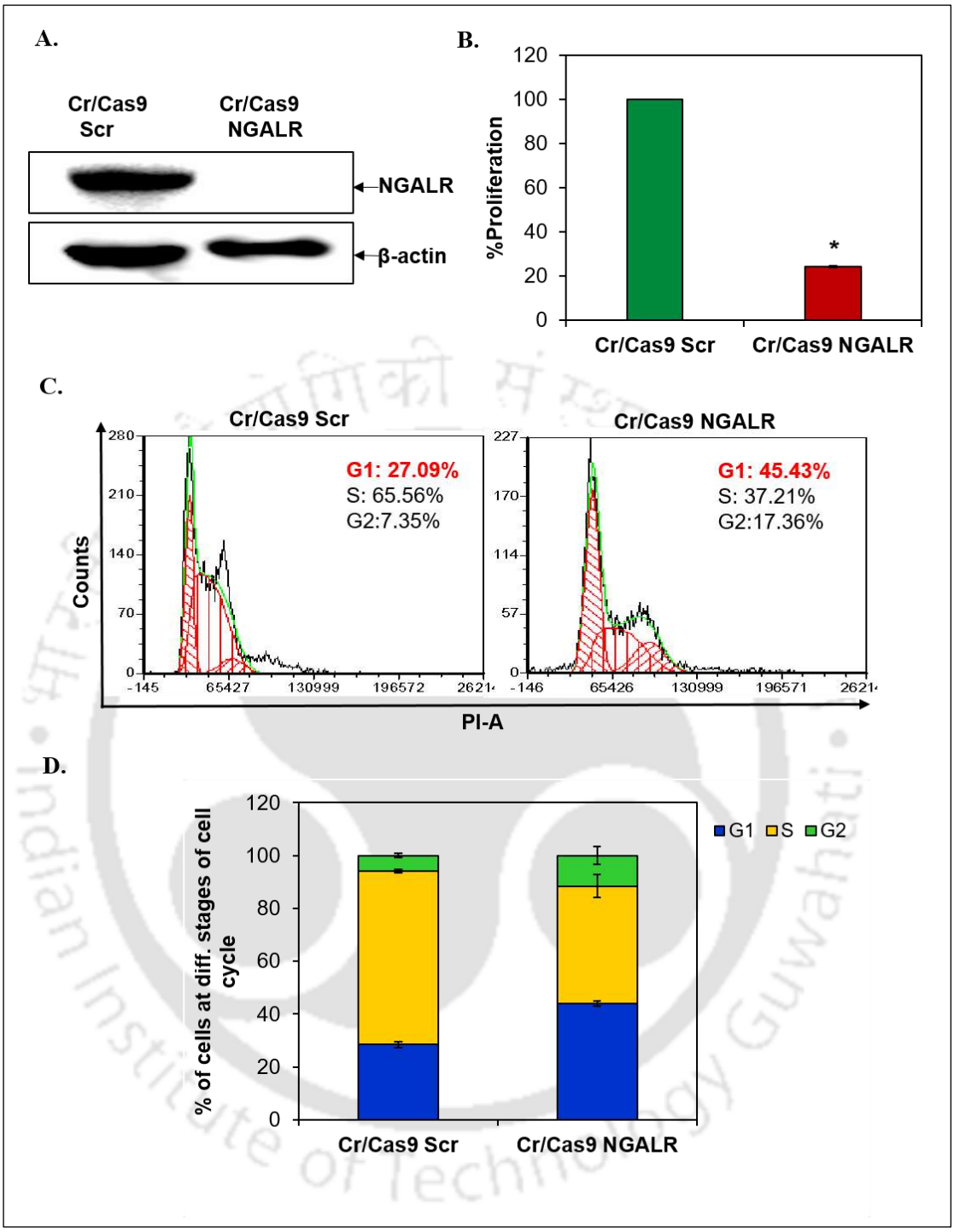

Figure 4.2. Knockout of NGALR in lung cancer cells and its effect on lung cancer cells' proliferation and cell cycle regulation. A. Representative Western blot image of NGALR knockout compared with scrambled control, B. Percentage change in the proliferation of NGALR knockout cells compared with scrambled control cells evaluated using MTT assay, C. Cell cycle distribution determined by flow cytometric analysis in NGALR knockout and scrambled control cells, D. Graphical representation of percentage of NGALR knockout cells and scrambled control cells at different stages of cell cycle. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

finding is in concordance with our previous results and further proved NGALR as one of the positive regulators of lung cancer development.

4.3.3. Effect of NGALR Knockout on the Clonogenic Potential of Lung Cancer Cells

Besides enhanced proliferation, increased survivability is another cardinal hallmark of cancer cells. Therefore, to access the effect of NGALR knockout on the survival potential of NCIH460 cells, colony formation assay was performed. Colony formation assay is a survival assay that determines the ability of a single cell to form a colony *in vitro*. It examines every cell in the population for its ability to undergo "unlimited" division (Franken *et al*., 2006). Our study showed that NGALR knockout considerably decreased the clonogenic potential of NCIH460 cells as demonstrated by the decrease in the number and size of colonies in NGALR knockout cells compared to scrambled control (Figure 4.3 A and B). This indicates that NGALR is involved in increasing the survival fraction of lung cancer cells. In line with our results, Du *et al*. reported that ESCC patients with positive expression of NGALR had a shorter survival time compared to those with negative expression (Du *et al*., 2010). Similarly, gliomas and HCC patients with overexpression of NGALR reportedly had low survival rates (Zhang *et al*., 2012b; Liu *et al*., 2011). Hence, this is the first report that showed that NGALR may have a significant role in sustaining the survival potential of lung cancer cells.

4.3.4. Effect of NGALR Knockout on the Migratory Potential of Lung Cancer Cells

Aforementioned, the mortality rate of lung cancer is exceedingly high which is the result of late-stage or metastatic stage detection. Cell migration has been an inevitable

process in initial lung cancer ontogeny, with preinvasive lung cancer cells migrating across the normal epithelium, leading to invasive disease (Millar *et al*., 2017). Thus, lung cancer metastasis involves enhanced motility, survival in circulation, and ability to form new tumors by exhibiting epithelial to mesenchymal transition (EMT) and enhanced survival and migratory signals (Chanvorachote *et al*., 2016). Growing evidence revealed that various signaling molecules residing in the tumor microenvironment have essential roles in facilitating the migration of the cancer cells (Luanpitpong *et al*., 2010). Hence, to determine whether NGALR is involved in the migration of lung cancer cells, wound healing assay was performed. The result of this assay revealed that disruption of NGALR significantly inhibited the migratory potential of NCIH460 cells. In the case of scrambled control cells, there was tremendous healing of the scratched wound whereas NGALR knockout NCIH460 cells showed significant inhibition of healing of the wound even after 24 h. It was observed that the percentage of wound area remaining in NGALR knockout cells was more than 80% after 24h (Figure 4.3 C and D). This finding clearly showed that NGALR has an integral role in promoting the migration of lung cancer cells.

4.3.5. Effect of NGALR Knockout in the Modulation of Multiple Signaling Pathways involved in Lung Carcinogenesis

From our previous findings, it is evident that NGALR has a crucial role in inducing lung cancer cell proliferation, survival, and migration as disruption of NGALR remarkably downregulated these processes. It is well established that cancer is characterized by aberrations of various signaling molecules or pathways that maintain cell homeostasis, thus consequently results in fueling the cells' ability to proliferate, survive, and invade neighboring tissues (Sever and Brugge, 2015). Hence, in order to

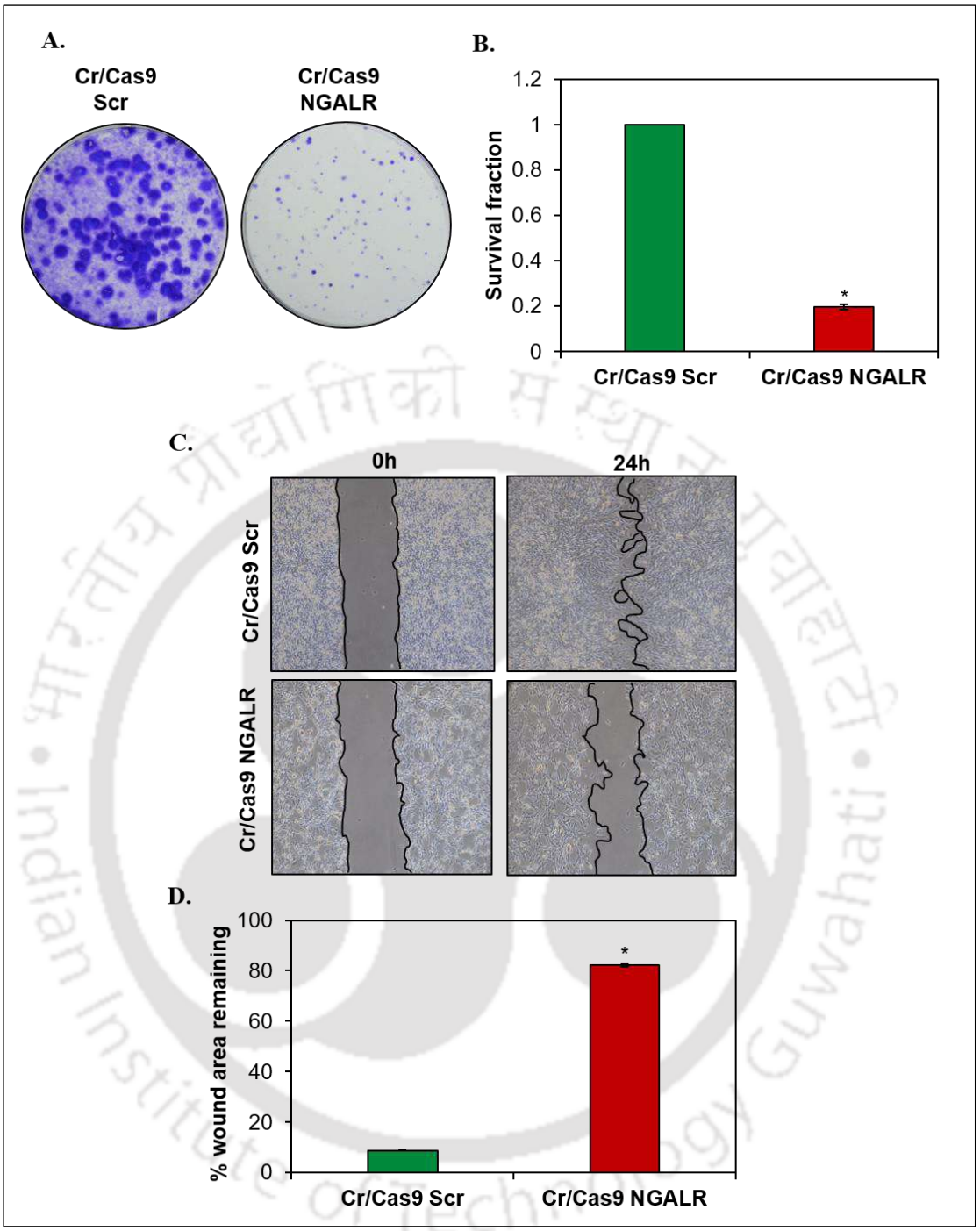

Figure 4.3. Effect of CRISPR/Cas9 mediated knockout of NGALR on the survival and migration of lung cancer cells. A. Representative images of the colonies formed in NGALR knockout cells along with scrambled control, B. Graphical representation of clonogenic potential of NGALR knockout cells in terms of survival fraction compared to scrambled control, C. Representative images of the effect of NGALR knockout on the migration of lung cancer cells, D. Graphical representation of percentage of wound area remaining in NGALR knockout cells compared to scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

unravel the mechanism of action of NGALR in lung cancer, it is imperative to analyze the involvement of NGALR in the modulation of these signaling molecules/pathways. For this purpose, expression analysis of various cancer-related proteins was carried out using Western blot (Figure 4.4). Our results demonstrated that knockout of NGALR resulted in downregulation of the expression of apoptosis regulatory proteins including survivin, cIAP-1, procaspase-9, and upregulated the expression of caspase-3. Survivin and cIAP-1 are the members of inhibitors of apoptosis protein (IAP) family and mediate apoptosis inhibition (Erkanli *et al*., 2007; Li *et al*., 2018). Procaspase-9 initiates the intrinsic apoptosis pathway and has a caspase activation domain (CARD) motif, by which caspase-9 is activated (Li *et al*., 2017). Caspase 3 is a prototypical apoptotic executioner which upon activation by either caspase 8 or caspase 9, facilitates apoptosis (Zhou *et al*., 2018). Further, in NGALR knockout lung cancer cells, downregulation of LC3B was observed. LC3B is a structural protein found in autophagosomal membranes and is considered a marker for autophagy (Chen *et al*., 2018). Autophagy is an important catabolic process that helps in cell survival in adverse conditions such as hypoxia. Precisely, after tumor development, autophagy aids in the persistence of the tumor. In lung cancer cells, autophagy has been also found to contribute to chemoresistance (Lee *et al*., 2015). Additionally, COX-2, which plays an important role in promoting apoptotic resistance, proliferation, angiogenesis, inflammation, invasion, and metastasis of cancer cells, was found to be downregulated upon loss of NGALR function (Hashemi Goradel *et al*., 2019). Also, NGALR knockout in lung cancer cells was found to modulate various cell cycle regulatory proteins such as downregulate phosphorylated histone H3 (p-HH3^{S10}), Myelin transcription factor 1 (Myt1), and cyclin D1, and upregulate G1 specific cyclin-dependent kinase inhibitors (CDKI), p27 (Kip1) and p18. Cyclin D1 has been reported to form active complexes with either

CDK-4/6, which then phosphorylate the retinoblastoma protein (Rb) and facilitate G1 to S phase transition of the cell cycle (Qie and Diehl, 2016). Studies also showed that p27 directly inhibits the enzymatic activity of CDK-cyclin complexes and arrests cells in G1 and p18 specifically inhibit the CDK-4/6 activity. Besides their role as CDKI, p27, and p18 function as tumor suppressor genes (Vlach *et al*., 1997; Lloyd *et al*., 1999; Noh *et al*., 1999). Importantly, the downregulation of cyclin D1 and upregulation of p27 (Kip1) and p18 obtained further evinced our previous findings wherein we showed that NGALR knockout resulted in lung cancer cell cycle arrest at the G1 phase. Notably, our study showed that loss of NGALR function led to the upregulation of p53. It is well evinced that p53 is a tumor suppressor protein, which regulates various cellular phenomena such as the promotion of cycle arrest, apoptosis, senescence, and metabolic adaptation (Hong *et al*., 2014). It has been reported that p53 instigates G1 phase arrest by the transcriptional activation of p21 which binds to cyclin E/CDK-2 and cyclin D/CDK-4 complexes (Chen, 2016). HistoneH3 has been found to facilitate chromosome condensation during mitosis (Dai *et al*., 2005). Myt1 is a protein kinase that regulates G2 checkpoint, by impeding mitosis promoting complex Cdc2-cyclin complexes (Booher *et al*., 1997; Liu *et al*., 1999; Wang *et al*., 2004). Depletion of Myt1 has been reported to potentiate DNA damage to suppress cell growth in clonogenic and tumor xenograft models (Chow and Poon, 2013). Further, proteins that are involved in cancer cell invasion, migration, metastasis, and angiogenesis such as MMP-2, MMP-9 and VEGF-A were found to be downregulated upon disruption of NGALR expression in lung cancer cells (Hao *et al*., 2007; Wu *et al*., 2019). Additionally, studies have shown that metastasis is promoted by a process called epithelial-to-mesenchymal transition (EMT) wherein the cancer cells transitioned from a non-motile, epithelial into a migratory, mesenchymal-like phenotype (Mrozik *et al*., 2018). Studies have shown

that Twist-1 and N-cadherin contribute to promoting EMT and metastasis (Yochum *et al*., 2018; Mrozik *et al*., 2018; Cao *et al*., 2019). Interestingly, we have found that expressions of Twist-1 and N-cadherin were considerably decreased in NGALR knockout lung cancer cells compared to scrambled control. Additionally, β-catenin was also found to be downregulated upon loss of NGALR function in lung cancer cells. β-catenin is the vital component of the Wnt pathway whose nuclear translocation promotes the transcription of genes such as c-Myc and cyclin D-1 and contributes to cancer cell proliferation and survival (Zhang *et al*., 2017). Also, studies have found that nuclear β-catenin induces EMT, cell migration, and invasion in NSCLC (Shang *et al*., 2017). Aforementioned, NGAL, the ligand of NGALR has been found to be highly expressed in lung cancer tissues and downregulation of NGAL suppressed the growth, migration, and invasion of lung cancer cells (Tang *et al*., 2015; Song *et al*., 2015). Moreover, overexpression of NGAL was linked with poor prognosis of lung cancer patients (Ruiz-Morales *et al*., 2015). Notably, NGAL was found to be markedly downregulated in NGALR knockout cells compared to the scrambled control. Further, a growing body of evidence has evinced the overexpression of EGFR in about 60% of NSCLC and its direct correlation with poor prognosis, thus making EGFR one of the oncogenic drivers for lung cancer. An increase in the kinase activity of EGFR results in the hyperactivation of downstream pro-survival pathways including MAPK /ERK, PI3K/Akt/mTOR and STAT-3 signaling pathways. Hence, activation of these pathways is pivotal for lung tumorigenesis (Hsu *et al*., 2019). So, we determined if these pathways are associated with NGALR mediated lung carcinogenesis. Our results showed that knockout of NGALR modulated the expression levels of key molecules of the Akt/mTOR pathway. We have found that loss of NGALR downregulated the expression of p-Akt^{S473}, Akt-1, p-mTOR^{S2448}, mTOR, p-S6$^{S235/236,}$ and S6. Also,

enhanced expression of phosphatase and tensin homolog (PTEN) was observed upon NGALR knockout. PTEN is an oncosuppressor protein that inhibits the activation of Akt (Phadngam *et al*., 2016). Furthermore, previous studies have found that co-activation of STAT-3 with NF-κB plays a crucial role in tumor maintenance and PI3K/Akt pathway is partially responsible for the constitutive activation of STAT-3 and NF-κB (Han *et al*., 2010). Interestingly, we have found that loss of NGALR function tremendously downregulated the expression of p-NF-κB^{S536} and NF-κB. Moreover, NGALR knockout also resulted in the downregulation of proteins involved in the STAT3 pathway including p-JAK-2$^{Y1007/1008}$, JAK-2, p-STAT-3^{S727} and STAT-3. Besides, the expression of EGFR was considerably decreased in NGALR knockout cells compared to scrambled control cells. Moreover, the expressions of p-MAPK$^{T202/Y204}$ and MAPK were also found to be downregulated upon NGALR knockout in lung cancer cells (Figure 4.5). Overall, NGALR knockout is found to decrease the proliferation, survival, and migration of lung cancer cells via downregulation of a wide range of intracellular signals including Akt/mTOR, NF-κB, JAK/STAT-3 and EGFR/MAPK signaling pathways (Figure 4.6). Thus, NGALR elicits its oncogenic effect via modulation of Akt/mTOR/NF-κB/STAT-3 and EGFR/MAPK signaling pathways in human lung cancer cells.

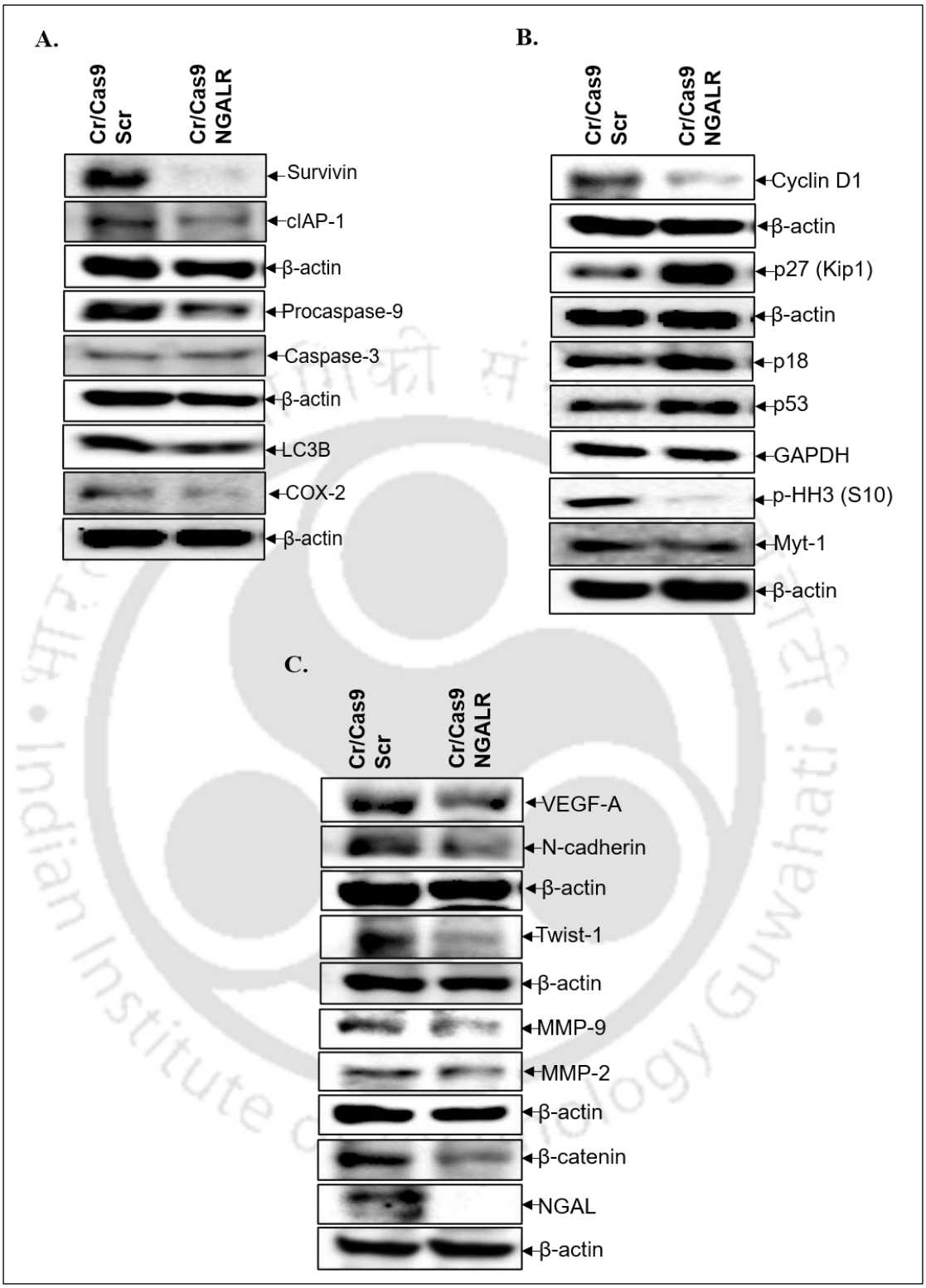

Figure 4.4. Effect of CRISPR/Cas9 mediated knockout of NGALR on different proteins involved in lung cancer. A. Effect of NGALR knockout on the expression of proteins involved in cell growth, proliferation, survival and apoptosis regulation, B. Effect of NGALR knockout on the expression of proteins involved in cell cycle regulation, C. Effect of NGALR knockout on the expression of proteins involved in EMT, migration, angiogenesis and metastasis. β-actin and GAPDH were used as loading controls.

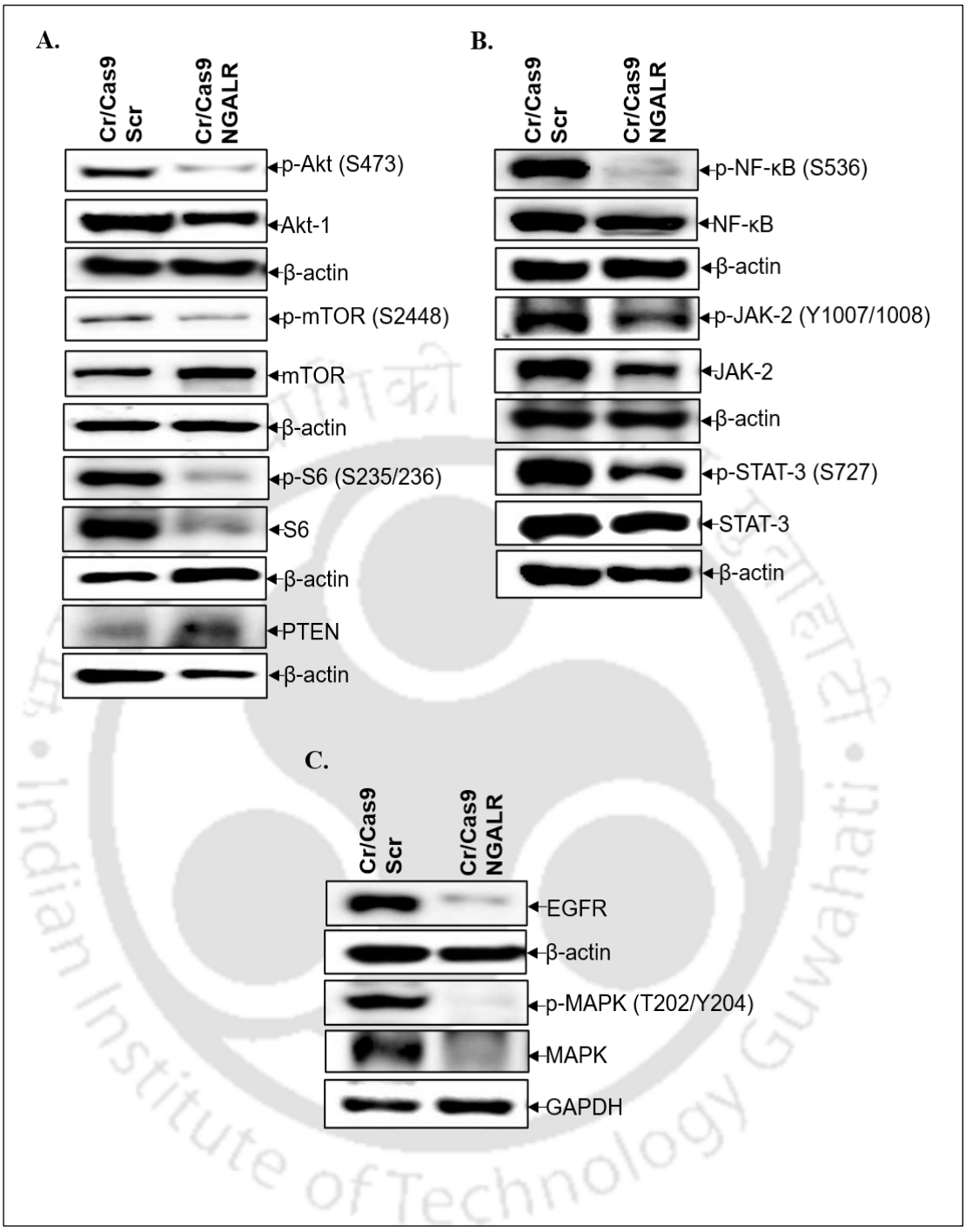

Figure 4.5. Effect of CRISPR/Cas9 mediated knockout of NGALR on different signaling molecules/pathways. A. Effect of NGALR knockout on Akt/mTOR signaling, B. Effect of NGALR knockout on NF-κB/ STAT-3 signaling, C. Effect of NGALR knockout on EGFR/MAPK signaling. β-actin and GAPDH were used as loading controls.

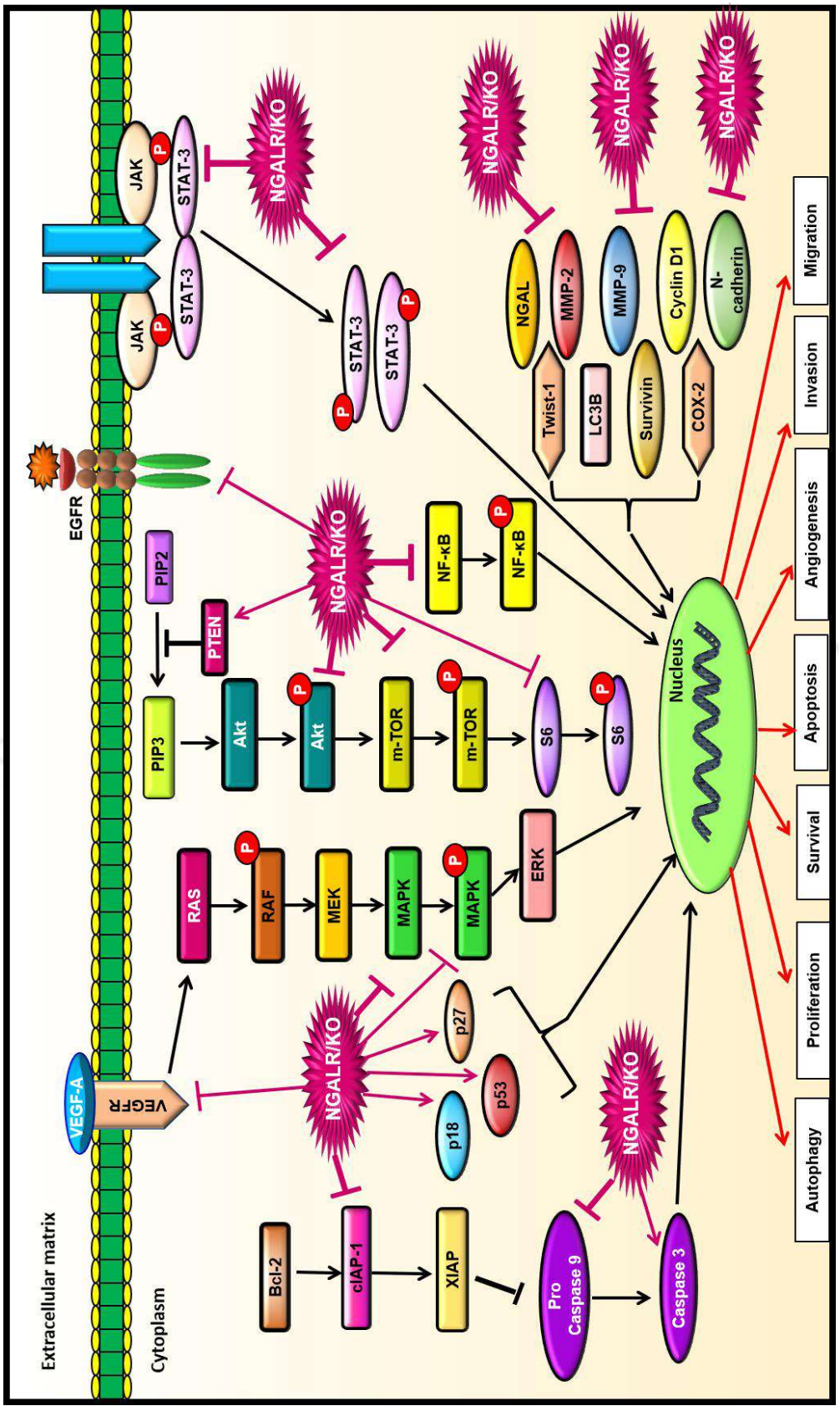

Figure 4.6. Knockout of NGALR modulates Akt/mTOR/NF-κB/STAT-3 and EGFR/MAPK signaling. NGALR/KO denotes Cr/Cas9 NGALR.

4.3.6. Role of NGALR in Tobacco-mediated Lung Carcinogenesis

As discussed in the preceding two chapters, NGALR may be involved in tobacco-induced lung carcinogenesis. First, our IHC data showed a significant NGALR upregulation in lung cancer tissue compared to the normal tissue with malignant male sample showing higher expression than that of malignant female sample. Plus, men reportedly smoke five times as women worldwide, thus we predicted that NGALR may be involved in tobacco-related lung carcinogenesis. Second, we have also revealed a marked overexpression of NGALR upon treatment of human lung epithelial cells and lung cancer cells with different tobacco components including NNK. Therefore, this implied that the upregulation of NGALR might serve as an important molecular event involved in the development and progression of tobacco-induced lung cancer. Aforementioned, NNK is a tobacco-specific nitrosamine considered the most potent carcinogen found in tobacco and has been extensively used for inducing lung cancer in different animal models. Thus, to further strengthen our hypothesis, we treated the NGALR knockout NCIH460 cells with NNK and analyzed its effect on the survival and migration of the cells. Further, the effect on the expression of its associated downstream targets was also studied.

4.3.6.1. Effect of NNK on the Clonogenic Potential of NGALR Knockout Lung Cancer Cells

Aforesaid, among the carcinogens that induce lung cancer, NNK is the most potent one. NNK has been reported to activate PI3K/Akt, NF-κB, and other vital signaling pathways that induce cell proliferation, survival, and angiogenesis, thereby facilitating the development of smoking-associated lung cancer (Ge *et al*., 2015). Further, NNK enhanced the proliferation of NSCLC cells in an Akt-dependent manner in association

with the upregulation of cyclin D1 expression (Tsurutani *et al.*, 2005). Further, NNK binds α7nAChR with high affinity which leads to the activation of PKC, RAF1, ERK-1/2, and transcription factors Fos, Jun, and Myc, which induce the proliferation of lung cancer cells (Xue *et al*., 2014). Moreover, NNK has been found to activate Bcl2 and Mcl-1 which augments their antiapoptotic activity. NNK also phosphorylates Bax and Bad which inactivated their pro-apoptotic functions, thus, contributing to improving survival and chemoresistance in human lung cancer cells (Deng, 2014). Hence, to examine the effect of NNK on the survivability of NGALR knockout cells, colony formation assay was performed. Our study showed that Cr/Cas9 NGALR cells treated with NNK showed a decrease in the colony forming ability compared to Cr/Cas9 Scr cells, which was evinced by the considerable decrease in the survival fraction (Figure 4.7). Noteworthy, the scrambled control treated with NNK showed a comparatively higher survival fraction than untreated scrambled control possibly due to the enhancement of the cell's growth induced by NNK. However, after NGALR knockout, the survival fraction does not increase even after treatment with NNK. This clearly shows that NGALR knockout significantly reduced the survival of NNK treated lung cancer cells. Overall, our data imply that NGALR is a positive regulator of tobacco-mediated growth and survival of human lung cancer cells.

4.3.6.2. Effect of NNK on the Migratory Potential of NGALR Knockout Lung Cancer Cells

Mounting evidence suggests that tobacco and its component not only promote tumorigenesis but also remarkably instigate the spread of cancer in the body. NNK also promotes tumor metastasis via alteration of cell motility and enhances migration and invasion of human lung cancer cells by activating c-Src/ protein kinase C iota (PKCι)/

focal adhesion kinase (FAK) signals (Shen *et al*., 2012). Additionally, NNK-induced migration and invasion of lung cancer cells were found to be facilitated via enhancement of the mRNA levels of N-cadherin and decreased the mRNA levels of E-cadherin (Wang *et al*., 2020b). Further, studies also showed that NNK enhanced chemokine CCL20 production, which consequently induces lung cancer cell proliferation and migration (Wang *et al.*, 2015b). Thus, to study the effect of NNK on the migratory potential of NGALR knockout lung cancer cells, scratch wound healing assay was performed. Our results revealed that the NGALR knockout cells treated with NNK exerted a significant inhibition of wound healing compared to scrambled control. After 24 h of NNK treatment, the percentage of wound area remaining in Cr/Cas9 NGALR was found to be 81%, whereas in the case of Cr/Cas9 Scr there was complete healing of the wound. Moreover, while the Cr/Cas9 Scr treated with NNK exhibited complete healing of the wound, the untreated Cr/Cas9 Scr was found to have some wound remaining after 24 h (Figure 4.7). Also, Cr/Cas9 NGALR cells treated with NNK were found to have a larger area of wound area remaining compared to untreated Cr/Cas9 NGALR cells after 24 h which implies the ability of NNK in increasing the invasion and migratory ability of the tumor cells. Overall, this is the first report that revealed NGALR as one of the positive regulators of NNK-induced migration of lung cancer cells.

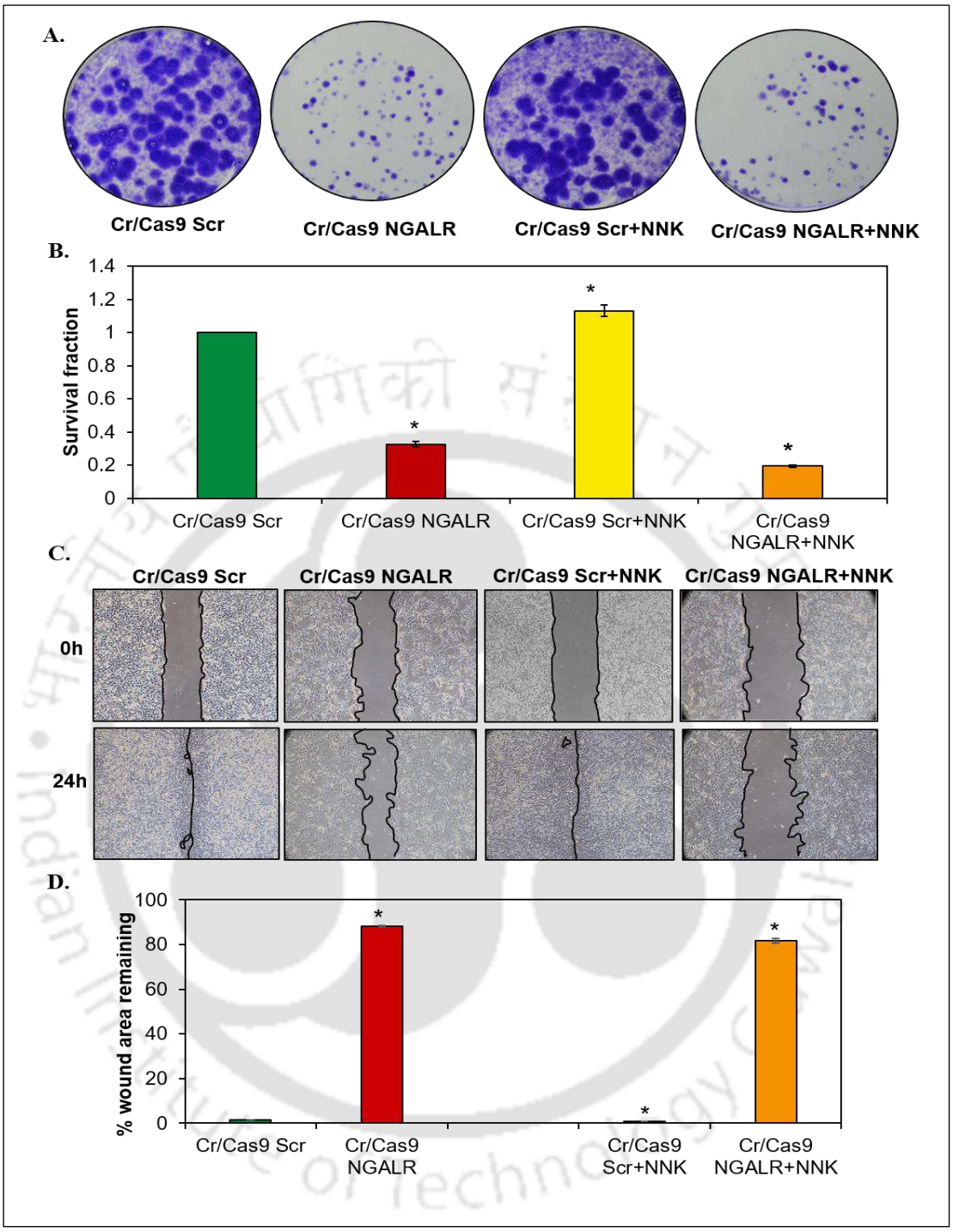

Figure 4.7. Effect of NGALR in NNK induced survival and migration of lung cancer cells. A. Representative images of the colonies formed in NNK treated NGALR knockout cells along with scrambled control, B. Graphical representation of clonogenic potential of NNK treated NGALR knockout cells in terms of survival fraction compared to the scrambled control, C. Representative image of the effect of NGALR on the migration of NNK treated NGALR knockout cells along with scrambled control, D. Graphical representation of percentage of wound area remaining in NNK treated NGALR knockout cells compared to scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

4.3.6.3. Effect of NNK in the Modulation of Different Molecular Pathways in NGALR Knockout Lung Cancer Cells

Aforesaid, tobacco consumption is the chief etiological factor in lung carcinogenesis which is driven by the aberration of signal pathways/ molecules involved in various aspects of lung cancer development and progression. From the above findings, it can be substantiated that NGALR positively regulates NNK-induced proliferation, survival, and migration of lung cancer cells. In order to determine the underlined mechanism by which NGALR mediates its oncogenic role in tobacco-induced lung carcinogenesis, we treated NGALR knockout and scramble control cells with NNK and performed Western blot to analyze the level of expression of different proteins involved in the development and progression of lung cancer (Figure 4.8). Herein, we reported that NNK treated NGALR knockout cells exhibit downregulation of apoptosis regulatory proteins including cIAP-1 and procaspase-9 compared to scramble control cells treated with NNK. Moreover, the expression of autophagosome marker LC3B was comparatively downregulated in NNK treated NGALR knockout cells than in scrambled control cells treated with the same. Besides, proteins facilitating proliferation, survival, and angiogenesis of cancer cells such as cyclin D1, COX-2, VEGF-A were found to be downregulated in NNK treated NGALR knockout cells compared to NNK treated scrambled control cells. Additionally, NNK treated NGALR resulted in downregulation of Twist-1, MMP-2, and MMP-9 which are involved in the EMT, migration, and metastasis of tumor cells compared to NNK treated scramble control cells. Studies have shown that enhanced activation of the PI3K/Akt/mTOR pathway leads to several hallmarks of cancer and has been profoundly implicated in the tumorigenesis and progression of NSCLC (Tan, 2020). Moreover, activation of Akt has been implicated

in lung tumors of NNK-treated A/J mice, and in human lung cancers obtained from smokers thus contributing to tobacco-related carcinogenesis (West *et al*., 2003) Notably, we have shown that NNK treated NGALR knockout cells exerted downregulation of vital molecules of the Akt/mTOR pathway including p-Akt ^{S473}, Akt-1, p-mTOR^{S2448} and S6. This shows that NGALR exerts its oncogenic effect in NNK mediated lung carcinogenesis by activating Akt/mTOR pathway. Additionally, it has been well established that NF-κB regulates numerous genes involved in lung carcinogenesis, and an increased level of nuclear NF-κB in lung cancer tissues and enhanced NF-κB activity is closely correlated with the advanced stage of the disease (Bordoloi *et al*., 2019). Further, it has been reported that exposure to NNK induced the proliferation of normal bronchial epithelial and small airway epithelial cells by activating NF-κB, which upregulated the expression of cyclin D1 (Ho *et al*., 2004). Herein, we found that NNK treated NGALR knockout cells showed decreased expression of p-NF-κB^{S536} compared to NNK treated scrambled control cells. Further, constitutive activation of STAT-3 plays a crucial role in promoting NSCLC progression by enhancing proliferation and survival (Njatcha *et al*., 2018). NNK has been reported to activate JAK/STAT in NSCLC cells (Guo *et al*., 2013). Our study showed downregulation of p-JAK-2$^{Y1007/1008}$, p-STAT-3^{S727} and STAT-3 in NNK treated NGALR knockout cells than the NNK treated scrambled control cells. Moreover, the MAPK pathway is another important molecular pathway that has been reported to be involved in the invasion of lung cancer (Zhang *et al*., 2018b). Previous studies also have revealed the involvement of MAPK pathways in tobacco smoke-induced EMT (Liang *et al*., 2018). Our data showed that NNK treated NGALR knockout cells showed decreased expression of p-MAPK$^{T202/Y204}$ and MAPK compared to NNK treated scrambled control cells. Collectively, this is the first report that shows the

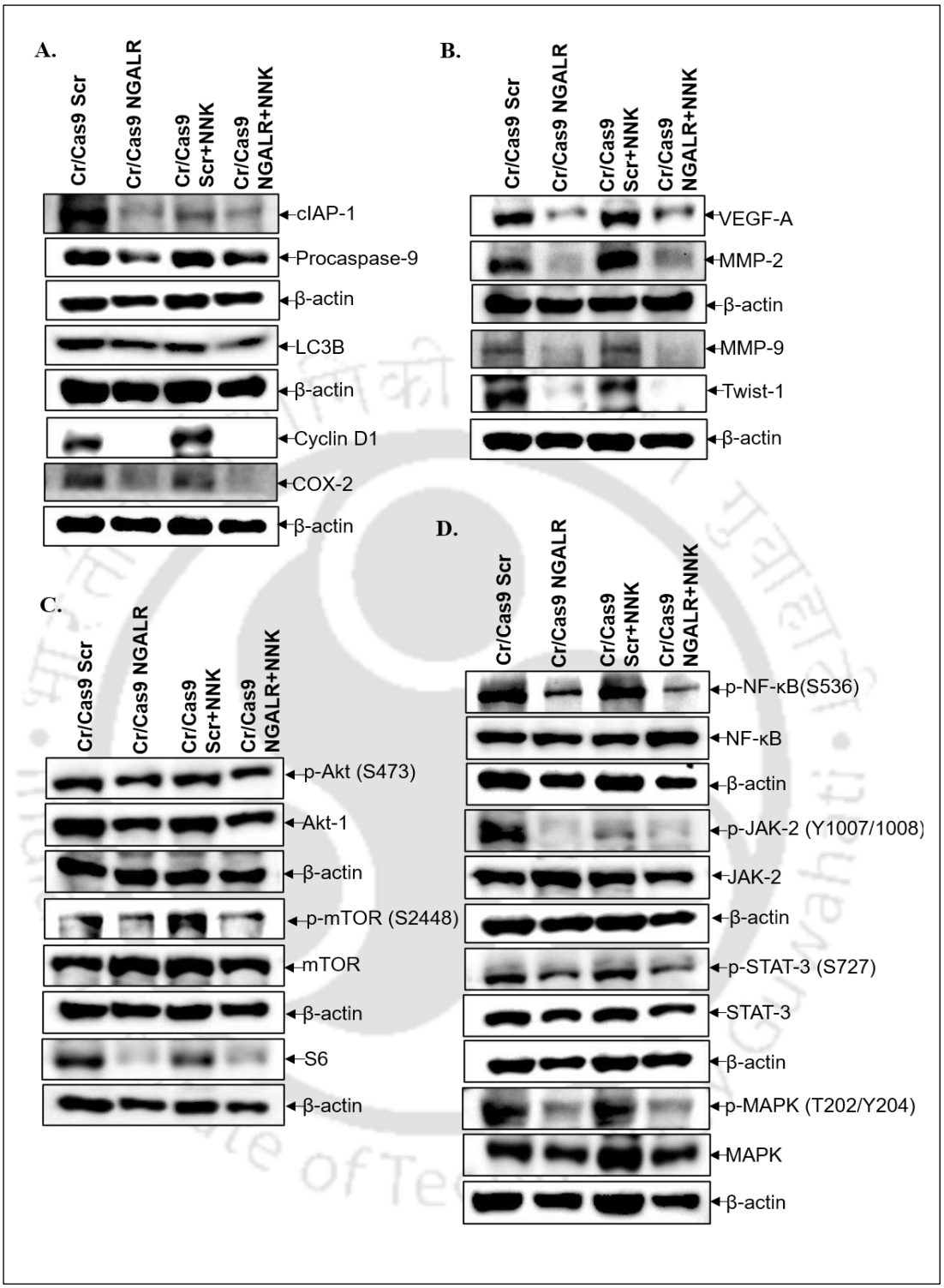

Figure 4.8. Effect of NNK in the modulation of different signaling molecules/pathways in NGALR knockout cells. A. Effect on the expression of proteins involved in cell growth, proliferation, survival and apoptosis regulation in NNK treated NGALR knockout cells. B. Effect on the expression of proteins involved in EMT, migration, angiogenesis and metastasis in NNK treated NGALR knockout cells, C. Effect on Akt/mTOR/S6 signaling in NNK treated NGALR knockout cells, D. Effect on NF-κB/STAT-3 and MAPK signaling in NNK treated NGALR knockout cells. β-actin was used as loading control.

involvement of NGALR in NNK-mediated increased proliferation, survival, and migration of lung cancer cells via the modulation of Akt/mTOR/NF-κB/STAT-3 and MAPK signaling pathways.

4.3.7. Role of NGALR in TNF-associated Lung Carcinogenesis

In the previous chapter, we have indicated that NGALR may be involved in TNF associated lung carcinogenesis. We have shown that exposure of lung cancer cells to TNF-α and TNF-β significantly upregulated the level of NGALR along with its ligand NGAL. Moreover, NF-κB and its downstream target cyclin D1 was also found to be upregulated in lung cancer cells when exposed to these cytokines. Hence, this has given us an indication that NGALR may play an important role in TNF-associated lung cancer progression. Accumulating epidemiological and clinical data evinced that chronic inflammation induces cancer development and progression. TNF-α is a multifunctional proinflammatory cytokine that endogenously promotes tumor by bridging inflammation and carcinogenesis. Also, studies have shown the involvement of TNF-α in various cancer hallmarks such as cell transformation, survival, proliferation, invasion, angiogenesis, and metastasis (Wang *et al*., 2008). TNF-α exerts its tumor-promoting effect through activating key signaling pathways such as NF-κB (Wang *et al*., 2008). TNF-β has also recently gained increased attention as it may activate NF-κB in cancer cells with a potency similar to that of TNF-α, consequently enhancing cancer cells' proliferation, invasion, and metastasis (Buhrmann *et al*., 2020). In the case of lung cancer, it has been reported that TNF-β genetic variants may interact with environmental factors and contribute to the susceptibility to NSCLC (Liu *et al*., 2015). It has also been reported that TNF-α along with its target NF-κB are considerably upregulated in cisplatin-resistant and radioresistant lung cancer cells, and silencing of

TNF-α tremendously augmented radiosensitivity in radioresistant lung cancer cells (Zhu *et al*., 2019). Like TNF-α, TNF-β also promotes proliferation, survival, invasion, migration, and colony formation in colorectal and ovarian cancer (Buhrmann *et al*., 2019b). Therefore, to further validate our previous findings, we exposed Cr/Cas9 NGALR cells with TNF-α and TNF-β and examine its effect on the survival and migration of the cells. Also, the effect on the expression of the associated proteins that are involved in the proliferation, survival, invasion, angiogenesis, and metastasis of lung cancer cells was studied.

4.3.7.1. Effect of TNF-α and TNF-β on the Clonogenic Potential of NGALR Knockout Lung Cancer Cells

Aforementioned, TNFs induce survival, proliferation, angiogenesis, and metastasis in most cancer cells by binding to its receptor and activating various molecular pathways including NF-κB, and MAPKs (Wang and Lin, 2008; Subkamkaew *et al*., 2019). TNF-induced NF-κB activation helps cells evade apoptosis by inhibition of JNK and upregulation of survival factors including XIAP and survivin. TNF functions by activating IκB kinase (IKK), which in turn, degrade IκB, thereby allowing NF-κB to enter the nucleus and activate the expression of its downstream anti-apoptotic targets (Wang *et al*., 2007). To elucidate the effect of these cytokines on the survival of NGALR knockout cells, Cr/Cas9 NGALR cells and Cr/Cas9 Scr cells were treated with TNF-α and TNF-β and colony formation assay was performed (Figure 4.9). Our results showed a significant decrease in the survival fraction of Cr/Cas9 NGALR cells treated with TNF-α compared to Cr/Cas9 Scr cells. Similarly, TNF-β treated Cr/Cas9 NGALR cells also displayed a significantly lesser survival fraction compared to Cr/Cas9 Scr cells. These results indicate that knockout of NGALR tremendously reduced the

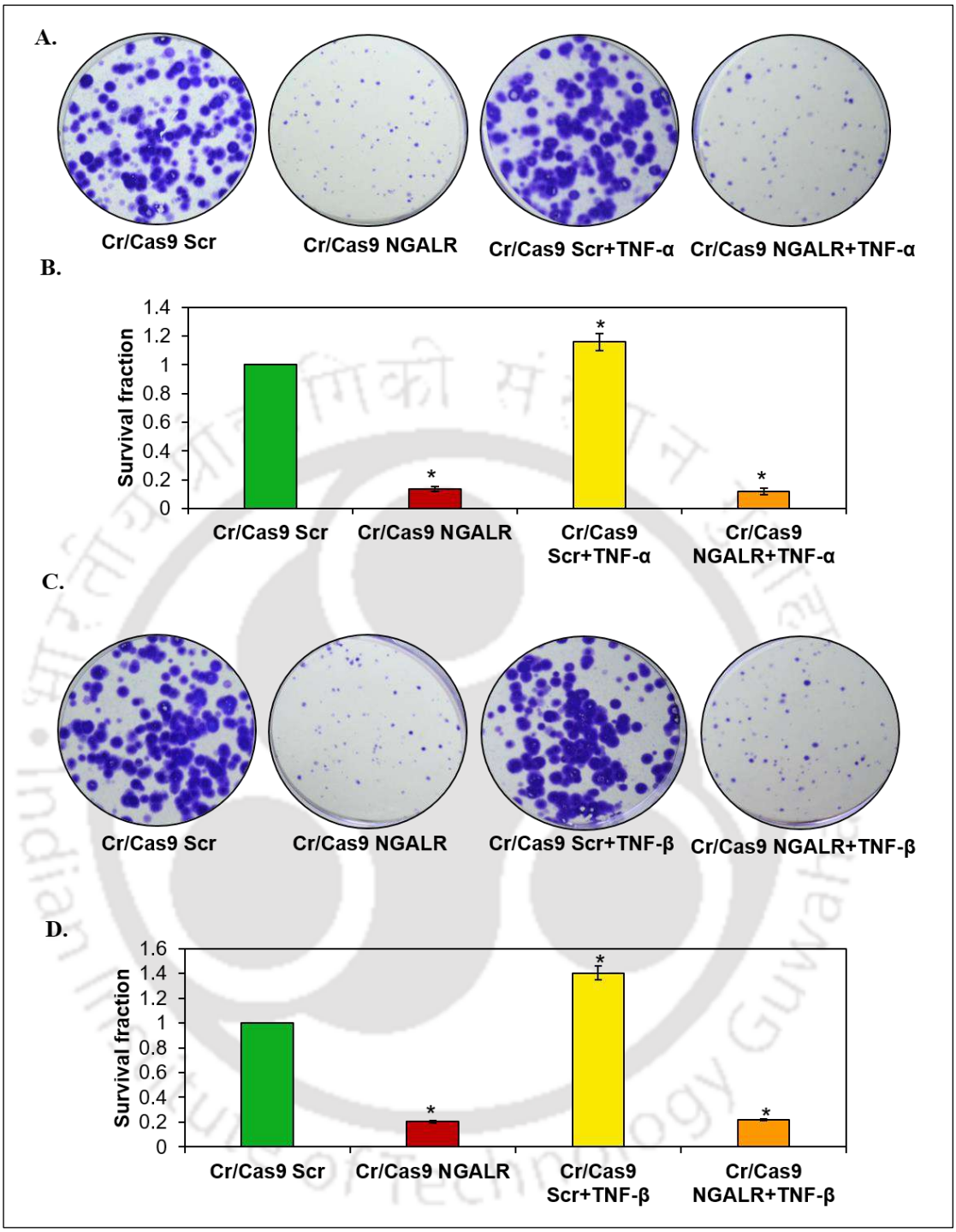

Figure 4.9. Effect of NGALR in TNF-α and TNF-β induced survival of lung cancer cells. A. Representative image of the colonies formed in TNF-α treated NGALR knockout cells along with scrambled control, B. Graphical representation of clonogenic potential of TNF-α treated NGALR knockout cells in terms of survival fraction compared to scrambled control, C. Representative image of the colonies formed in TNF-β treated NGALR knockout cells along with scrambled control, D. Graphical representation of clonogenic potential of TNF-β treated NGALR 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.

clonogenicity of the cytokines treated lung cancer cells. Also, the number of colonies formed on the TNF-α and TNF-β treated scrambled controls are higher than their respective untreated scrambled controls perhaps due to the enhancement of the cell's clonogenic potential induced by the TNFs. However, in TNF-α and TNF-β treated NGALR knockout cells, the number of colonies does not show a prominent increase as compared to the respective untreated NGALR knockout cells. Collectively, this is the first report that indicates that NGALR is involved in the positive regulation of TNF-associated growth and survival of lung cancer cells.

4.3.7.2. Effect of TNF-α and TNF-β on the Migratory Potential of NGALR Knockout Lung Cancer Cells

As mentioned previously, lung cancer has an extremely high mortality rate as the majority of the patients are diagnosed at the advanced or metastatic stage of the disease. The attainment of lung cancer cell metastasis involves migration of the cancer cells from their origin and invasion of surrounding tissue (Tungsukruthai *et al*., 2017). Interestingly, it has been reported that besides promoting survival and growth, TNFs also mediate the metastasis of cancer cells. Particularly in lung cancer, TNF-α has been found to participate in BaP induced metastasis of lung cancer cells and suppression of TNF-α repressed the induced metastasis (Wang *et al*., 2007). In a lung metastasis mouse model, TNFs receptor TNFR-1-mediated signals were found to maintain tumor neovascularization at least in part through enhancement of hepatocyte growth factor (HGF) expression, which was presumed to facilitate lung metastasis (Wang and Lin, 2008). Therefore, to study whether NGALR is involved in TNF-mediated lung cancer migration, scratch wound healing assay was carried out (Figure 4.10). Our findings revealed that after exposure of both NGALR knockout cells and scrambled control cells

to TNF-α for 24h, the NGALR knockout cells exhibited a significantly large area of wound remaining (approximately 65% wound area remaining) whereas in scrambled control complete healing of the wound was observed after 24h possibly due to TNF-α mediated enhancement of lung cancer cell migration. Similarly, NGALR knockout cells treated with TNF-β displayed a considerably large area of wound remaining (approximately 70% wound area remaining) whereas there was almost complete healing of the wound in TNF-β treated scrambled control after 24h which may be due to enhanced migration of the cells in wound area induced by TNF-β. However, post NGALR knockout, the migratory potential of the lung cancer cells significantly reduced even after treatment with these two cytokines. Hence, our finding ascertained that NGALR plays an invaluable role in TNF-mediated migration and metastasis of lung cancer cells.

4.3.7.3. Effect of TNFs on the Modulation of Different Molecular Pathways in NGALR Knockout Lung Cancer Cells

Extensive studies have affirmed the imperative role of chronic inflammation in tumor initiation and progression. This process requires the action of inflammatory mediators, consisting primarily of cytokines and chemokines such as TNFs to aid in facilitating tumor growth, angiogenesis, invasion and metastasis, and sustain a cancer-promoting inflammatory environment (Peng *et al*., 2020). Besides, aberrant TNF expression inside the tumor microenvironment has been found to promote invasion, migration, and metastasis of the cancer cells (Mocellin and Nitti, 2008). From the above findings, it is substantiated that NGALR is involved in the TNF-mediated proliferation, survival, and migration of lung cancer cells. Thus, to unravel the molecular mechanism of NGALR in facilitating TNF-mediated lung cancer pathogenesis, we performed Western blot

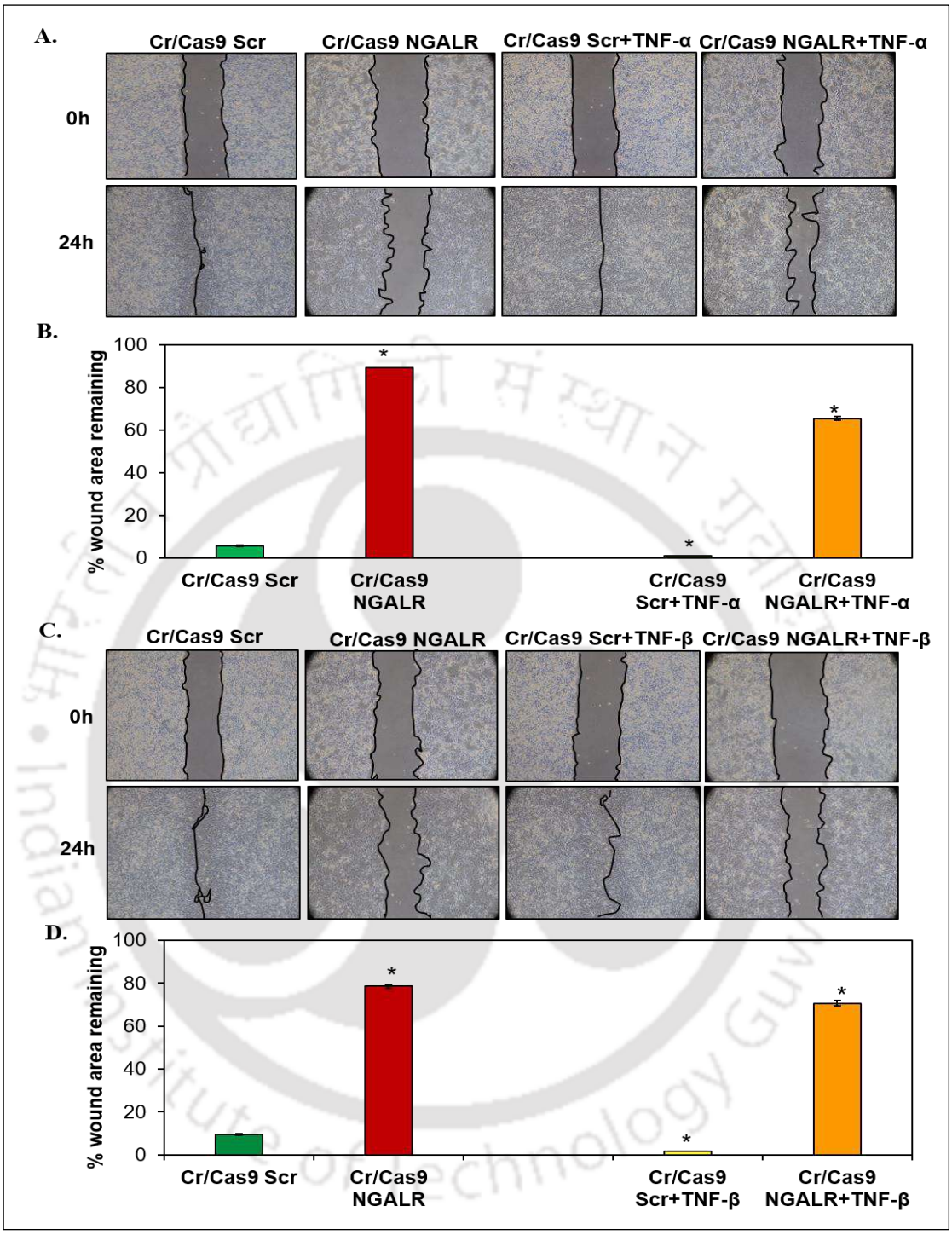

Figure 4.10. Effect of NGALR in TNF-α and TNF-β induced migration of lung cancer cells. A. Representative images of the effect of NGALR on the migration of TNF-α treated NGALR knockout cells compared to the scrambled control, B. Graphical representation of % of wound area remaining in TNF-α treated NGALR knockout cells compared to the scrambled control, C. Representative image of the effect of NGALR knockout on the migration of TNF-β treated NGALR knockout cells along with scrambled control, D. Graphical representation of % of wound area remaining in TNF-β treated NGALR knockout cells compared to scrambled control. Data are represented as Mean±SE, * denotes p <0.05 compared to scrambled control.

analysis to study the expression level of various proteins involved in diverse malignant processes. It is well established that cyclin-dependent kinases (CDKs) regulate the cell cycle progression and have also been reported to help in regulating gene transcription (Morgan *et al*., 1997). Transition through the G1 to S phases of the cell cycle requires the action of CDK4 and CDK6 associated with D-type cyclins (D1, D3), and CDK2 associated with cyclins E and A (Lauper *et al*., 1998). Cyclin D/CDK4/6 phosphorylates retinoblastoma tumor suppressor protein (pRb) and reduces the action of p21 Waf1/Cip1 and p27 Kip1, subsequently activating cyclin E/CDK2 which allow progression into S-phase (Lundberg *et al*.,1998; Ewen *et al*., 2000). The deregulation of the CDK4/6-cyclin D signal is a common aberration implicated in human malignancy (Deng *et al*., 2017). Also, aberrant cyclin E expression is frequently implicated in pulmonary dysplasia and lung cancer which results in an unfavorable clinical outcome in lung cancer. Hence, the deregulation of CDK and their associated cyclins makes them suitable targets for lung cancer therapy (Galimberti *et al*., 2010). Our results showed that TNF-α treated NGALR knockout cells displayed downregulation of cyclin E2, cyclin D1, cyclin D3, CDK-2, and CDK-6 than the TNF-α treated scrambled control cells. Also, downregulation of p-HH3^{S10} which is involved in chromosome condensation during mitosis was observed in TNF-α treated NGALR knockout cells (Goto *et al*.,1999). Additionally, a decreased in the expression of cyclin B1 was observed in TNF-α treated NGALR knockout cells compared to scrambled control cells treated with the same. Cyclin B1, the subunit of the M-phase promoting factor, controls the G2 to M phase transition (Yuan *et al*., 2004; Ou *et al*., 2013). Further, amplification of cyclin D1 has been reported in various human cancers, including lung cancer, and is found to be an adverse prognostic marker for stage I NSCLC patients (Soria *et al*., 2000). We have also found that TNF-α treated NGALR

knockout cells exhibited downregulation in the expression of proteins involved in proliferation, survival, angiogenesis, EMT, invasion, and metastasis including COX-2, survivin, VEGF-A, Twist-1, MMP-2, and MMP-9 compared to TNF-α treated scrambled control. Additionally, TNF-alpha induced protein 8 (TIPE) and TNF-alpha induced protein 8-like 2 (TIPE2) are oncoproteins that have been found to facilitate the survival, proliferation, invasion, and migration of lung cancer (Bordoloi *et al*., 2019; Bordoloi *et al*., 2020). Studies have shown that TIPE is induced by TNF-α via nuclear translocation of activated NF-κB (Padmavathi *et al*., 2018). Herein, we have shown that the expressions of both TIPE and TIPE2 were found to be downregulated in TNF-α treated NGALR knockout cells compared to TNF-α treated scrambled control cells. Aforementioned, studies have indicated that TNF activates important molecular pathways involved in inflammation-related carcinogenesis including NF-κB and MAPK pathways (Wang and Lin, 2008; Drutskaya *et al*., 2010). TNF-α has been found to induce cancer cell invasion, angiogenesis and metastasis by activation of the NF-κB pathway (Tang *et al*., 2017; Wang *et al*., 2017b; Mu *et al*., 2020). It is well known that aberrant or constitutive NF-κB and MAPK have been implicated in many human malignancies including lung cancer (Zhang *et al*., 2018b; Du *et al*., 2020). Besides, NF-κB and MAPK are key signaling pathways in promoting lung cancer progression and metastasis (Tsao *et al*., 2014). In this study, downregulation of p-NF-κB^{S536}, NF-κB, p-MAPK$^{T202/Y204}$ and MAPK was observed in TNF-α treated NGALR knockout cells compared to scrambled control cells treated with the same. Collectively, this is the first report that shows the involvement of NGALR in TNF-α mediated proliferation, survival, and migration of lung cancer via modulation of MAPK and NF-κB pathway and its regulated gene products (Figure 4.11).

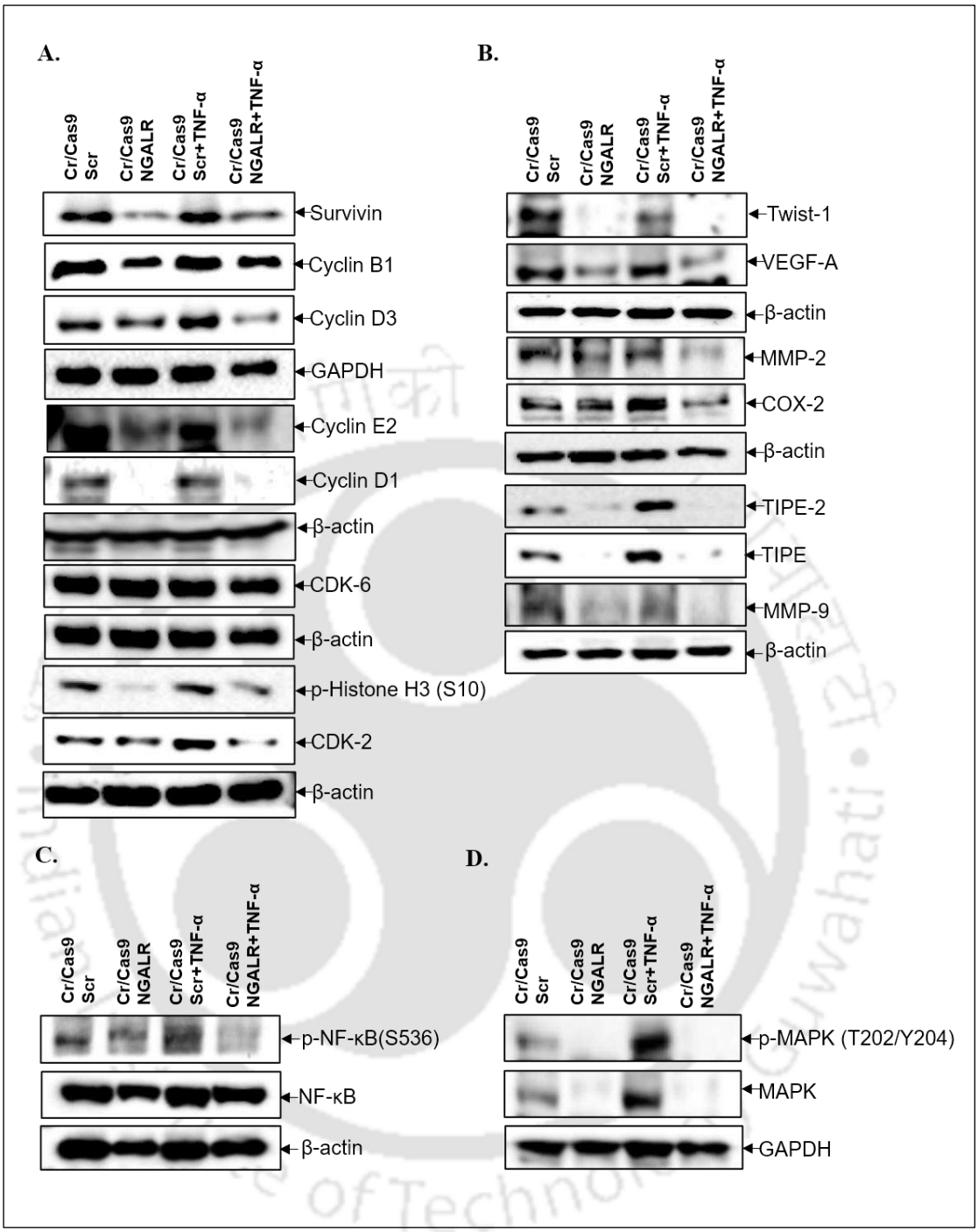

Figure 4.11. Effect of TNF-α in the modulation of different signaling molecules/ pathways in NGALR knockout cells. A. Effect on the expression of survivin and the different proteins involved in cell cycle regulation in TNF-α treated NGALR knockout cells, B. Effect on the expression of proteins involved in the EMT, migration, angiogenesis and metastasis in TNF-α treated NGALR knockout cells, C and D. Effect on NF-κB and MAPK signaling in TNF-α treated NGALR knockout cells. β-actin and GAPDH were used as loading controls.

In the case of TNF-β treated NGALR knockout cells, cell cycle regulatory proteins such as cyclin D1, cyclin D3, cyclin E, and cyclin B1 were found to be downregulated compared to TNF-β treated scrambled control cells. Similarly, downregulation of p-HH3^{S10} was also reported. Moreover, like the TNF-α treated cells, downregulation of various proteins involved in lung cancer cell proliferation, survival angiogenesis, EMT, invasion, and metastasis including COX-2, survivin, VEGF-A, Twist-1, MMP-2, and MMP-9 was observed in TNF-β treated NGALR knockout cells compared to scrambled control cells treated with the same. Furthermore, the expression of TIPE and TIPE2 which are involved in lung cancer tumorigenesis and progression were also found to be downregulated in TNF-β treated NGALR knockout cells compared to scrambled control cells treated with the same. Additionally, TNF-β treated NGALR knockout cells exhibited downregulation of important molecules involved in the Akt/mTOR pathway including p-Akt^{S473}, Akt-1, and p-mTOR^{S2448}. Thus, this showed that NGALR is involved in the TNF-β associated lung carcinogenesis by activating Akt/mTOR signaling pathway. Besides, it has been reported that activation of the PI3K/Akt consequently resulted in enhanced activation of STAT-3 and NF-κB (Han *et al*., 2010). Previous studies have revealed that TNF-β activates the NF-κB signaling pathway in colorectal cancer cells, thereby instigating cancer cell proliferation, EMT, invasion, and metastasis, and induces its own expression and further facilitates TNF-α expression (Buhrmann *et al*., 2019). Interestingly, our results showed that TNF-β treated NGALR knockout cells showed downregulation of p-NF-κB^{S536}, NF-κB, p-STAT-3^{S727} and STAT-3 compared to the scrambled control cells treated with the same. Moreover, downregulation of p-MAPK$^{T202/Y204}$ and MAPK was also observed in TNF-β treated NGALR knockout cells. Taken together, NGALR is involved in the induction of TNF-β-mediated lung carcinogenesis via modulation of Akt/mTOR/NF-κB/STAT-3 and

MAPK pathways which are involved in promoting proliferation, survival, invasion, migration, and metastasis of lung cancer cells (Figure 4.12).

4.3.8. NGALR Knockout Increases the Chemosensitivity of Lung Cancer Cells to Chemotherapeutic Drugs

Aforementioned, lung cancer patients are mostly diagnosed at the advanced or metastatic stage at which the 5-year survival rate remains only 15%. Platinum-based chemotherapy remains the backbone of treatment for advanced NSCLC patients (Sève *et al*., 2010). Combination of active agents with platinum (cisplatin or carboplatin)-based therapy is also extensively used for the management of NSCLC patients (Lemjabbar-Alaoui *et al*., 2015; Noonan *et al*., 2015; Zhang *et al*., 2019). Cisplatin or carboplatin with active agents such as taxanes (paclitaxel and docetaxel), vinorelbine, gemcitabine and irinotecan are used for the treatment of metastatic patients (Sève and Brugge, 2005). However, the therapeutic efficacy of these chemotherapeutic interventions is often rendered ineffective and limited due to the acquisition of chemoresistance by the cancer cells. For instance, most advanced lung patients relapse after a few cycles of treatment with platinum-based chemotherapy (Hamilton and Rath, 2014). Acquired resistance to various anticancer drugs used in clinics including 5-fluorouracil (5-FU), docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib has become a major hurdle for improving the outcome of patients with metastatic and advanced lung cancer (Noro *et al*., 2010; Friboulet *et al*., 2014; Xiao *et al*., 2017; Zhang *et al*., 2018a). Carboplatin has been reported to induce multi-drug resistance thereby causing disease progression (Wang *et al*., 2020c). It has been found that cytoplasmic RAP1, CXCR4, flap endonuclease 1 (FEN1), and overexpression of miRNA-328

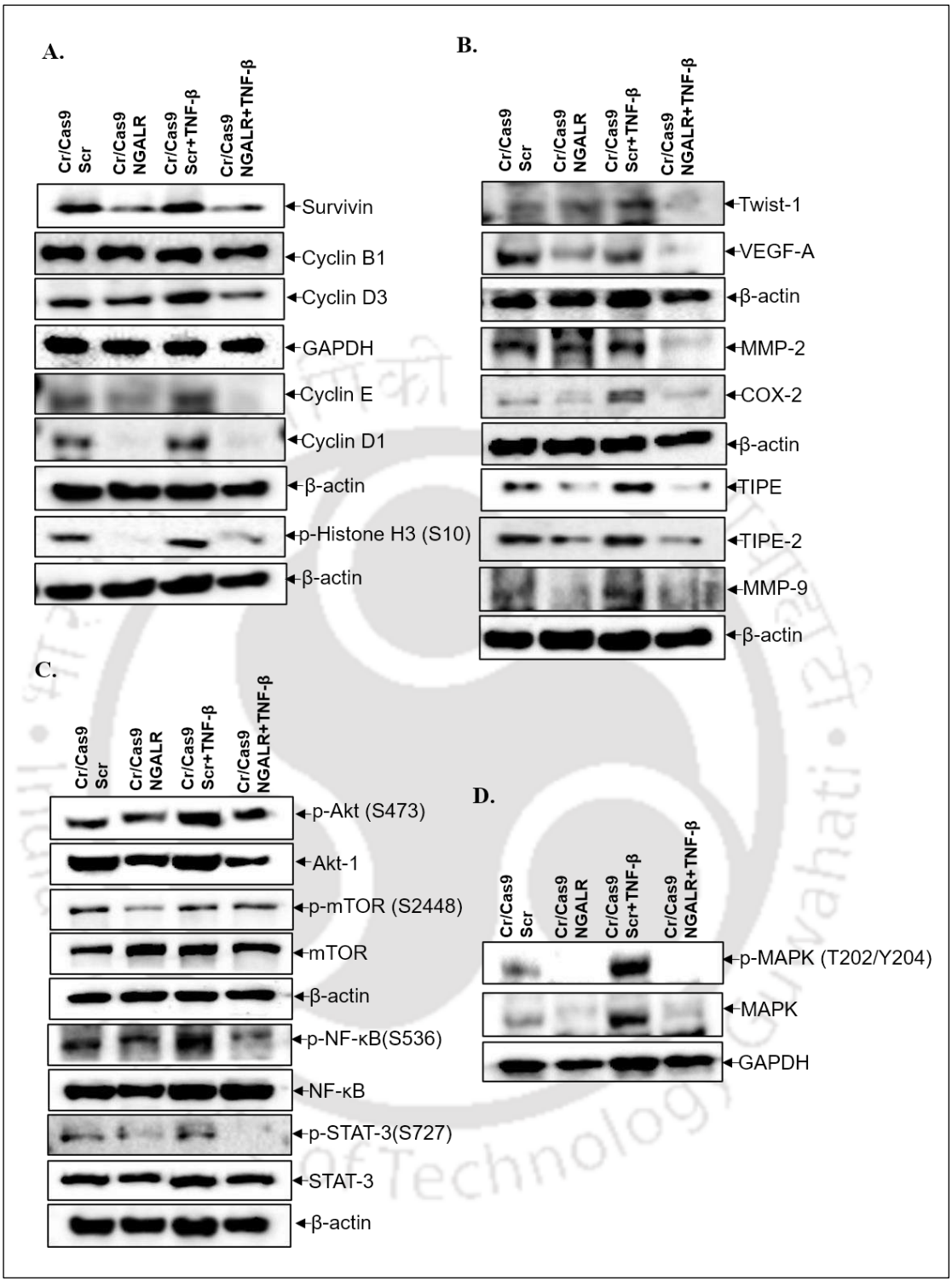

Figure 4.12. Effect of TNF-β in the modulation of different signaling molecules/ pathways in NGALR knockout cells. A. Effect on the expression of survivin and the different proteins involved in cell cycle regulation in TNF-β treated NGALR knockout cells, B. Effect on the expression of proteins involved in the EMT, migration, angiogenesis and metastasis in TNF-β treated NGALR knockout cells, C. Effect on Akt/mTOR/NF-κB/STAT-3 signaling in TNF-β treated NGALR knockout cells, D. Effect on MAPK signaling in TNF-β treated NGALR knockout cells. β-actin and GAPDH were used as loading controls.

conferred resistance to cisplatin treatment in NSCLC cells (Xiao *et al*., 2017; He *et al*., 2017; Xie *et al*., 2017; Wang *et al*., 2018a). Cisplatin resistance is also mediated by several molecular alterations, such as enhanced expression of metallothionein and the mRNA level of the excision repair cross-complementing (ERCC1) gene (Rosell and Felip, 2001). Additionally, studies have shown that Yes-associated protein (YAP) and upregulation of miR-641 mediated erlotinib resistance in NSCLC cells (Hsu *et al*., 2016; Chen *et al*., 2018). Moreover, induction of miR-141/ eukaryotic initiation factor-4E (EIF4E) and downregulation of miR-27b contributed to docetaxel resistance in NSCLC patients (Chen *et al*., 2016; Wang *et al*., 2017a). Hence, the effect of these chemotherapeutic drugs on improving patients' survival is presently still far-off from satisfactory (Noro *et al*., 2010). Consequently, sensitizing chemoresistant cancer cells is highly critical for better outcomes for the patients. Here, we attempt to see if NGALR knockout enhances the therapeutic actions of the standard chemotherapeutic drugs including 5-fluorouracil (5-FU), docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib in NSCLC cells using MTT assay (Figure 4.13). Our results revealed that the percentage of proliferation decreased in both the NGALR knockout and scrambled control cells treated with 5-FU, docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib. However, the percentage of proliferation was considerably lower in the NGALR knockout cells compared to the treated scrambled control cells. Noteworthy, the treated NGALR knockout cells showed considerably lower IC_{50} than that of treated scrambled cells. It was found that the IC_{50} of 5-FU treated NGALR knockout cells was found to be 1.5 µM whereas the IC_{50} of 5-FU treated scrambled cells was found to be 3.5 µM. Also, the IC_{50} in docetaxel treated NGALR knockout cells was found to be 2.75 µM whereas the IC_{50} in docetaxel treated scrambled cells was found to be 4.25 µM. Moreover, in NGALR knockout cells and scrambled cells treated both with

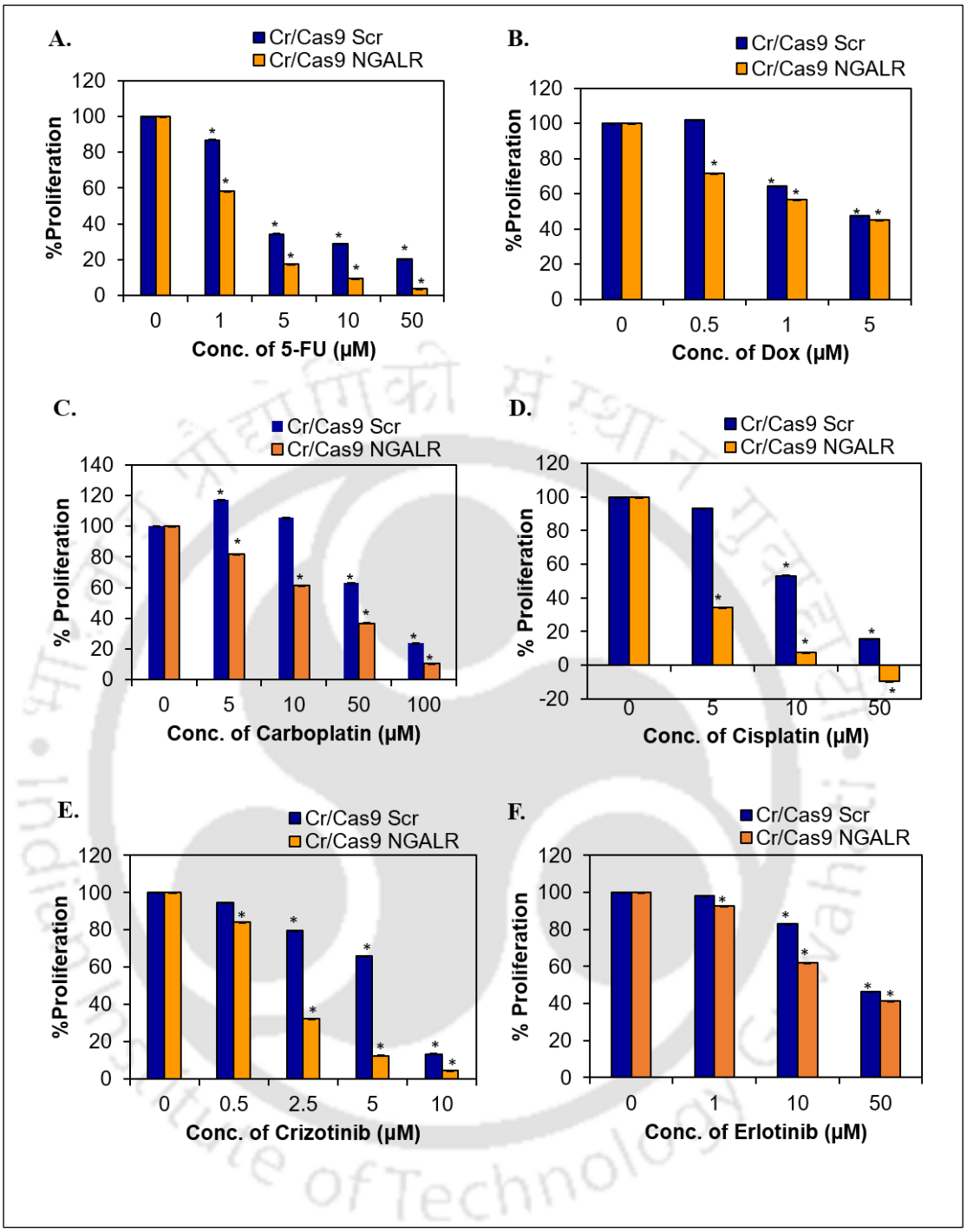

Figure 4.13. Disruption of NGALR enhances the efficacy of chemotherapeutic drugs in lung cancer cells. Percentage of change in cells' proliferation of NGALR knockout cells compared with scrambled control cells treated with A. 5-Fluorouracil, B. Docetaxel, C. Carboplatin, D. Cisplatin, E. Crizotinib, F. Erlotinib, analyzed by MTT Assay. Data are represented as Mean ±SE, * denotes $p < 0.05$ compared to untreated controls.

carboplatin, the IC_{50} was found to be 25 µM and 65 µM respectively. Additionally, upon treatment with cisplatin, NGALR knockout cells exhibited an IC_{50} of 4.5 µM whereas scrambled cells showed an IC_{50} of 12 µM. The IC_{50} of crizotinib in NGALR knockout and scrambled control cells were 1.5 µM and 6.5 µM respectively. In the case of erlotinib treatment, the IC_{50} in treated NGALR knockout cells was 29 µM and 45 µM in the case of treated scrambled cells. Hence, this study indicates the involvement of NGALR in imparting chemoresistance in lung cancer cells. Collectively, our results imply that NGALR knockout may sensitize the lung cancer cells to chemotherapeutic agents including 5-FU, docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib treatment. However, further studies are required to confirm the synergistic action of NGALR knockout and chemotherapy in reducing the growth of lung cancer cells.

4.4. Conclusion

In this chapter, we investigated the role of NGALR in the modulation of essential hallmarks of cancer and its underlined molecular mechanism. Our results have shown that knockout of NGALR significantly decreased the proliferation, survival, and migration of NCIH460 human lung cancer cells. Moreover, mechanistic studies revealed that NGALR imparted its tumorigenic effect on lung cancer cells via modulation of a wide range of signaling pathways including Akt/mTOR, NF-κB, JAK/STAT-3, and EGFR/MAPK pathways. Further, NGALR knockout was found to alter the expression of various proteins involved in the cell cycle regulation such as p-histone $H3^{S10}$, Myt-1, p27, p18, p53, and cyclin D1. NGALR knockout was also found to modulate proteins associated with apoptosis, cancer cell proliferation, survival, EMT, angiogenesis invasion, migration, and metastasis including pro-caspase 9, caspase-3, cIAP-1, survivin, COX-2, Twist-1, VEGF-A, MMP-2, and MMP-9.

NGALR also modulates LC3B expression, thus signifying its role in regulating autophagy. The ligand NGAL has been reported to induce chemoresistance in lung cancer cells and is associated with poor prognosis of lung cancer patients. Notably, the knockout of NGALR also resulted in the downregulation of its ligand NGAL in lung cancer cells. Collectively, our data suggest that NGALR is an oncoprotein that has a profound role in lung cancer pathogenesis. Next, we determined the role of NGALR in NNK-mediated lung carcinogenesis. Our results revealed for the first time that NGALR is involved in enhancing NNK-induced lung cancer proliferation, survival, and migration. Further, mechanistic studies displayed that NGALR mediates its oncogenic role in NNK-induced lung carcinogenesis via the modulation of Akt/mTOR/ NF-κB/STAT-3 and MAPK signaling pathways and their downstream targets which are involved in promoting lung cancer cell growth and progression such as cIAP-1, cyclin D1, COX-2, VEGF-A, Twist-1, MMP-2, MMP-9, etc. Subsequently, we decipher the role of NGALR in inflammation-mediated lung carcinogenesis induced by pro-inflammatory cytokines TNF-α and TNF-β. Our data showed for the first time the function of NGALR as one of the positive regulators of TNF-mediated lung cancer cell proliferation, survival, and migration. Moreover, we have found the involvement of the MAPK/NF-κB pathway and its regulated gene products in NGALR knockout lung cancer cells treated with TNF-α. Additionally, modulation of Akt/mTOR, NF-κB, STAT-3, and MAPK pathways in TNF-β treated NGALR knockout lung cancer cells was observed. Collectively, our data suggest the indispensable role of NGALR in the development and progression of lung cancer particularly in tobacco-induced as well as inflammation-mediated lung carcinogenesis. Moreover, we have found that disruption of NGALR tremendously enhanced the efficacy of multiple chemotherapeutic drugs in decreasing the proliferation of lung cancer cells. Therefore, NGALR is a promising

molecular target for the effective treatment of lung cancer. Nevertheless, further investigations in the *in vivo* and clinical settings are essential for validating the *in vitro* findings to fully establish NGALR as a prognostic marker and therapeutic target for lung cancer.

Chapter
5

Discussion, Conclusion and Future Prospects

5.1. Discussion and Conclusion

Despite the remarkable advances in cancer diagnosis and management in recent years, lung cancer continues to be the number one cause of cancer-related mortality. Lung cancer consists of two major histological types i.e., SCLC, which covers about 15% of all the cases and NSCLC that encompasses approximately 85% of all the cases (Lemjabbar-Alaoui *et al*., 2015). Lung cancer is a preventable disease as the predominant risk factor is tobacco smoking which is associated with 90% of lung cancer cases (Lemjabbar-Alaoui *et al*., 2015; Wang *et al*., 2015b). The smoke emerging from a cigarette contains lung carcinogens such as NNK, NNN, PAH, chromium, cadmium, arsenic, aromatic amines, etc. (Pfeifer *et al*., 2002; Ge *et al*., 2015; Wang *et al*., 2015b). Besides tobacco, other factors that contribute to lung cancer risk include unhealthy diet, exposure to air pollution and occupational carcinogens, aging, family history, genetic susceptibility, etc. (Molina *et al*., 2008; Alberg *et al*., 2005; Young *et al*., 2009). The basic treatment modalities for lung cancer consist of surgery, chemotherapy, radiation therapy and targeted therapy. Several tyrosine kinase inhibitors such as erlotinib and gefitinib, VEGFR inhibitors such as bevacizumab, ALK inhibitors such as ceritinib have been extensively used for the treatment of NSCLC patients (Duma *et al*., 2019). However, there exist several limitations associated with the current treatment of lung cancer due to the acquisition of chemoresistance, tumor relapse, and late-stage diagnosis consequently causing poor clinical outcome and elevated mortality rates. Resistance to multiple chemotherapeutic agents such as cisplatin, erlotinib, docetaxel, doxorubicin, gemcitabine, etoposide, vinorelbine, paclitaxel, and topotecan have been reported in NSCLC patients (Chang, 2011; Indovina *et al*., 2011). Moreover, tumor relapses arise post-surgery in 30-70% of cancer patients in stages I and II after adequate

treatment (Chudacek *et al*., 2014; Caulo *et al*., 2012). Also, approximately 70% of lung cancer patients are diagnosed at an incurable advanced or metastatic stage due to negligible symptoms at early stages (Lemjabbar-Alaoui *et al*., 2015; Horeweg and de Koning, 2014). Hence, to overcome these drawbacks, the identification of novel biomarkers for early diagnosis and promising molecular targets for developing highly efficacious therapeutic intervention is indispensable for the better management of this deadly malignancy. Interestingly, NGALR is one such protein that has a promising prospect in this respect.

NGALR, also known as SLC22A17 is a cell surface receptor for NGAL/Lipocalin-2 belonging to a major facilitator superfamily (MFS) (Miyamoto *et al*., 2011). In 2005 Devireddy *et al*. first isolated 24p3R, the mouse homolog of NGALR from mouse FL5.12 cell consisting of 12 transmembrane domains (Devireddy *et al*., 2005). NGALR with its ligand NGAL functions as a novel iron transporter that facilitates the survival, growth, and maturation of cells (Zhang *et al*., 2012a). Increasing lines of evidence demonstrated that alteration of NGALR is strongly implicated in the development and progression of various human malignancies including colon cancer, esophagus cancer, endometrial cancer, glioma, liver cancer, etc. However, the expression and role of NGALR lung cancer have not been elucidated. Yet, its ligand NGAL was found to be overexpressed and enhanced erlotinib resistance in NSCLC cells (Krysan *et al*., 2013). Also, NGAL depletion instigated apoptosis in lung adenocarcinoma cells by generating ROS (Song *et al*., 2015). For the ligand to exert its function, it requires its receptor NGALR. Therefore, in this study, we deciphered the exact function of NGALR in lung cancer by analysis of its expression and its underlined molecular mechanism of action in lung carcinogenesis with an emphasis on tobacco and TNF-mediated lung

carcinogenesis. Firstly, we analyzed NGALR expression using a lung cancer tissue microarray slide comprising of tissues from different lung cancer types, pathologies, age groups, sexes, stages and grades along with normal lung tissues. Our results revealed that NGALR is significantly overexpressed in lung cancer tissues compared to normal lung tissues. In support of our findings, mounting evidence has shown the upregulation of NGALR in various human cancers including colorectal carcinoma, ESCC, endometrial cancer, glioma, hepatocellular carcinoma, and pancreatic ductal adenocarcinoma (Du *et al*., 2011; Liu *et al*., 2011; Zhang *et al*., 2012b; Lv *et al*., 2010; Miyamoto *et al*., 2011; Gomez-Chou *et al*., 2017). This upregulation of NGALR was found to decrease the survival rate of ESCC and glioma patients (Du *et al*., 2011; Liu *et al*., 2011); cause unfavorable clinicopathologic features and poor prognosis in HCC cases (Zhang *et al*., 2012b); instigate deeper invasion and a high degree of TNM stages of CRC patients (Lv *et al*., 2010); enhance higher tumor grade, and lower survival rate in EC patients (Miyamoto *et al*., 2011); improve tumor growth and diminished survival rate in PDAC cases (Gomez-Chou *et al*., 2017). Hence, our results underpinned that NGALR has a potential role as a positive regulator of lung carcinogenesis. Further, our analysis showed upregulation of NGALR in both SCLC and NSCLC tissues compared to the normal lung tissues, with significant upregulation in NSCLC tissues. Next, the differential expression of NGALR in various lung cancer pathologies including adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma, small cell carcinoma and bronchioalveolar carcinoma was determined. Herein, we found that the upregulation of NGALR expression in all the mentioned pathologies with significant upregulation in adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma compared to the normal tissues. Besides, expression analysis of NGALR in different age groups showed that there was no

substantial difference of NGALR expression age-wise plausibly due to the different sample size among the different age groups and also may be due to the fact that the prevalence of tobacco smoking, the prime risk factor for lung cancer development, varies with ages. Further, our analysis revealed an upregulation of NGALR in both malignant females and males' tissues compared to normal females and males' tissues respectively with relatively more prominent upregulation of NGALR in malignant males' tissues than in malignant females' tissues. This may be because the habit of smoking is comparatively more prevalent among men than in women, with an estimation of men smoking almost five times more than women globally (Hitchman and Fong, 2011; Schwartz and Cote, 2016). Consequently, the risk of lung cancer is relatively greater in men compared to females (Bain *et al*., 2004). Thus, this indicated that NGALR may have a cardinal role in tobacco-induced lung carcinogenesis. Further, stage-wise analysis of NGALR expression showed a significant increase in NGALR expression in stages I, II, and IIIa of lung cancer tissues compared to the normal lung tissues. Notably, the NGALR expression was the highest at stage IIIa. This is the first report to indicate the direct correlation of NGALR expression with the advanced clinical stage of this deadly malignancy. Similar to our findings, a previous study has shown a higher expression level of NGALR in advanced TNM stage II–III compared to low TNM stage of HCC tissues (Zhang *et al*., 2012b). Moreover, upon analyzing the expression of NGALR protein in different tumor grades, NGALR was found to be significantly upregulated in different grades (grade 1, 2, and 3) of lung cancer tissue when compared to normal lung tissues with a more prominent upregulation in grade 1 and 2. In concordance with our findings, Du *et al*. also showed a positive correlation between NGALR expression and histological differentiation grade of ESCC, and ESCC patients with positive NGALR expression showed a shorter survival time than those

with negative expression (Du *et al*., 2011). Further, it has been demonstrated that NGALR was not expressed in normal endometrial glands, but was overexpressed in endometrial cancer (EC) patients with higher grade and advanced stages of the disease. Moreover, EC patients with NGALR overexpression have lower survival periods (Miyamoto *et al*., 2011). A previous report has also revealed an enhanced expression of NGALR in high-grade gliomas, particularly in grade IV of glioblastoma multiforme (GBM) (Liu *et al*., 2011). NGALR was also reported to be significantly associated with a deeper invasion, a high degree of TNM stages, and poor cellular differentiation in CRC patients (Lv *et al*., 2010). Moreover, previous studies have shown that overexpression of NGALR and its ligand NGAL is significantly associated with poor prognosis in glioma, ESCC, and HCC patients (Du *et al*., 2011; Liu *et al*., 2011; Zhang *et al*., 2012b). Hence, from both stage and grade-wise analysis, it can be certainly predicted that NGALR has a key role in the initiation and progression of lung cancer. Also, based on the enhanced NGALR expression on malignant males' tissues compared to malignant females' tissues, it can be predicted that NGALR has a key role in tobacco-mediated lung carcinogenesis. However, the effect of tobacco and its components on the regulation of NGALR expression has not been elucidated thus far. It is well evinced that more than 85% of lung cancers are due to tobacco smoking, with an additional portion triggered by exposure to secondhand smoke among nonsmokers (Warren and Cummings, 2013). Tobacco smoke comprises more than 5000 compounds (Pfeifer *et al*., 2002; Wang *et al*., 2015b). Out of these, more than 20 compounds are carcinogenic to the lung including arsenic, aromatic amines, PAH, tobacco-specific nitrosamines such as NNK and NNN (Pfeifer *et al*., 2002; Ge *et al*., 2015; Wang *et al*., 2015b). Therefore, we examined the effect of tobacco and its carcinogenic components on the expression of NGALR in human lung epithelial cells L132, and NSCLC cells A549 and

NCIH460. For this purpose, we have used a tobacco extract in the form of tuibur as well as tobacco components including NNK, NNN, BaP and nicotine. Tuibur is made locally by releasing the smoke produced from the burning of tobacco into the water until it becomes cognac in color and generates a pungent smell (Phukan *et al*., 2005). Reports have shown the frequent consumption of this highly addictive tobacco form in the North-Eastern states of India, especially Mizoram (Madathil *et al*., 2018). The presence of cadmium, lead, nickel, arsenic, and triethylene glycol has been reported in tobacco water. Also, a high concentration of NNN was reported in tuibur which is the strongest carcinogen found in it (Sinha and Gupta, 2006). Of the carcinogens associated with lung cancer, NNK and NNN are two tobacco-specific nitrosamines considered strong carcinogenic agents (Hecht and Hoffmann, 1988). NNK and NNN have been extensively used *in vivo* to successfully induce various types of lung tumors (Zheng and Takano, 2011; Ge *et al*., 2015; Zarth *et al*., 2016). Metabolic activation of NNK and NNN induces deleterious DNA adducts formation thereby inducing mutations in oncogenes and tumor suppressor genes consequently leading to tumor initiation (Sturla *et al*., 2005; Xue *et al*., 2014). Moreover, these tobacco-specific nitrosamines bind to nAChRs and instigate tumor growth via enhancing proliferation, survival, migration, and invasion. These two actions of NNK and NNN synergistically mediate tobacco-mediated carcinogenesis (Xue *et al*., 2014). Nicotine is the addictive component of tobacco smoke which is found to facilitate tumor growth and metastasis *in vitro* and *in vivo*. It also exerts its tumor-promoting effect through the binding and activation of nAChRs, specifically the α7 subunit (Schaal *et al*., 2018). Apart from this, BaP is considered the most potent carcinogen among PAH (Vu *et al*., 2015). When consumed, it is converted into its reactive metabolites BPDE that binds to DNA and forms deoxyguanosine-DNA (BPDE-dG) adduct, thereby exerting its tumor-promoting effect

(Alexandrov *et al.*, 2010; Kasala *et al*., 2015; Wang *et al*., 2015a). This PAH has also been extensively used to induce lung cancer *in vivo* (Kasala *et al*., 2015). In this study, we analyzed the effect of tuibur on *NGALR* expression and found that the mRNA expression of *NGALR* was upregulated dose-dependently in tuibur-treated L132, A549 and NCIH460 cells thus signifying the role of NGALR in mediating tobacco-associated lung carcinogenesis. Further, a complete dose-dependent increase in the mRNA expression of *NGALR* was observed in NNK treated L132, A549 and NCIH460 cells. Similarly, treatment of these cell lines with NNN resulted in a dose-dependent substantial increase of *NGALR* mRNA expression. Additionally, BaP was also found to enhance the expression of *NGALR* in L132 and A549 cells dose-dependently, whereas in the case of NCIH460, BaP significantly increased the *NGALR* expression but was not dose-dependent. Besides these, nicotine treatment enhanced the mRNA level of *NGALR* up to 0.5µM in NCIH460 cells whereas, in A549 and NCIH460 cells, it was increased up to 0.75µM compared to the untreated control. Noteworthy, these tobacco components contribute to the proliferation, survival, and angiogenesis of cancer cells via deregulation of NF-κB, PI3K/Akt, PKC, Wnt, MAPK/ERK, JAK/STAT, IL-8, CCL-2, p21 (RAC1)-activated kinase 6 (PAK6), Myc and other essential signaling molecules/pathways (Ge *et al.*, 2015; Shen *et al.*, 2012; Hirata *et al.*, 2017; Zhang *et al*., 2016; Schaal and Chellappan, 2014; Raja *et al.*, 2016). Aforementioned, nicotine, NNK and NNN exert their tumorigenic effect by activating nAChRs which led to the activation of various molecular pathways involved in carcinogenesis (Zhao *et al*., 2016). Particularly, among the different subunits of nAChRs, nicotine binds to α7nAChR to induce its carcinogenic activity in lung cancer (Wang *et al*., 2018b). NNK also binds to α7nAChR which led to the activation of PKC, the serine/threonine kinase RAF1, ERK1/2, Fos, Jun and Myc, thereby increasing the proliferation of lung cancer

cells (Xue *et al*., 2014). Hence, we determined if α7nAChRs have any effect on NGALR expression in lung cancer cells. Our data showed that knockdown of α7nAChR resulted in substantially decreased expression of NGALR in NCIH460 cells, thus implying that activation of α7nAChR by nicotine and its derivatives plausibly activates NGALR, thereby contributing to tobacco-mediated lung carcinogenesis. Notably, this is the first report that indicates the involvement of NGALR as a key molecule in tobacco-induced lung carcinogenesis. However, further studies are highly imperative to decipher the exact molecular mechanism associated with the alteration of NGALR expression in lung cancer.

Next, we determined the effect of pro-inflammatory cytokines TNF-α and TNF-β on the expression of NGALR in NCIH460 cells. Chronic inflammation has been found to promote lung cancer and alteration in the level of cytokines has been implicated both in smokers and lung cancer patients (Seifart *et al*., 2005). Moreover, a growing body of evidence indicates the crucial role of smoking in inducing pulmonary inflammation which elevates the chance of lung cancer development in smokers (Lee *et al*., 2008). Pro-inflammatory cytokine TNF has been found to promote inflammation-related carcinogenesis through activation of NF-κB (Balkwill *et al*., 2006; Wang and Lin, 2008; Drutskaya *et al*., 2010). In a lung metastasis mouse model, TNFR-1-mediated signals were found to facilitate metastasis by maintaining tumor neovascularization in part via enhancing the expression of hepatocyte growth factor (HGF) expression (Wang and Lin, 2008). Upon treatment of NCIH460 cells with TNF-α and TNF-β, NGALR as well as its ligand NGAL were considerably upregulated from their basal levels. Numerous studies have revealed that TNF members activate NF-κB and its regulated gene products, thereby promoting cancer cell survival and metastasis (Gaur and Aggarwal, 2003; Ooppachai *et al*., 2019). In support of this finding, the expressions of NF-κB and

p-NF-κB^{S536}, as well as its regulated protein cyclin D1, were remarkably upregulated in NCIH460 cells treated with TNF-α and TNF-β. Hence, this is the first evidence of the participation of NGALR in TNF-mediated lung carcinogenesis. Further, promoter analysis is pivotal to have a better understanding of the regulation of NGALR and precise prediction of functional transcription factor binding sites (TFBS) is a prerequisite for successful promoter analysis. Hence, we determine the TFBS at the promoter region of the *NGALR* gene. By using an in-silico approach, we decoded nine transcription factors with the putative binding site at the promoter region of NGALR including NF-κB, STAT-3, AP-4, KLF, NANOG, SOX, CREB, SMAD and NFAT. These transcription factors have been reported to have pivotal roles in lung carcinogenesis (Dutta *et al*., 2014; Hu *et al*., 2016; Liu *et al*., 2017; Cheng *et al*., 2018; Zhu *et al*., 2012; Aggarwal *et al*., 2008; Jeon *et al*., 2010; Guo *et al*., 2015). Interestingly, we have found that upon siRNA-mediated knockdown of the transcription factors STAT-3, AP4, KLF12, NANOG, SOX6, CREB, SMAD3 and NFAT in lung cancer cells, the expression of NGALR was significantly downregulated. However, NF-κB knockdown does not induce downregulation of NGALR expression in lung cancer cells. Hence, this finding indicates that transcription factors STAT-3, AP4, KLF12, NANOG, SOX6, CREB, SMAD3 and NFAT may function as positive upstream regulators of NGALR, potentially regulating the NGALR expression at the transcript level. Nevertheless, further investigation is crucial for conclusive validation.

Next, to ascertain the detailed function of NGALR and its downstream targets, CRISPR/Cas9-mediated knockout of NGALR was carried out in NCIH460 human lung cancer cells and subsequently analyzed the effect of NGALR gene knockout on the different hallmarks of cancer and determined its effect on proteins that are involved in lung cancer development and progression. Aforementioned, numerous studies have

reported that overexpression of NGALR in various human malignancies is positively correlated with poor clinical outcomes and decreased survival rate (Du *et al*., 2011; Liu *et al*., 2011; Zhang *et al*., 2012b; Miyamoto *et al*., 2011). In our study, we observed that NGALR knockout resulted in a considerable decrease in the proliferation of NCIH460 cells compared to scrambled control cells. The inhibition of proliferation of NGALR knockout NCIH460 cells was found to be 75% compared to the scrambled control. This reduction in the proliferation acquired by NGALR knockout was facilitated through cell cycle arrest at the G1 phase as evinced by the cell cycle analysis. Besides enhanced proliferation, increased survivability is another cardinal hallmark of cancer. Based on the analysis using colony formation assay, we have observed that NGALR knockout significantly decreased the clonogenic potential of NCIH460 cells compared to the scrambled control cells, thus indicating the crucial role of NGALR in sustaining the survival of lung cancer cells. Interestingly, our finding is in concordance with previous reports wherein ESCC patients with positive expression of NGALR had a shorter survival time compared to those with negative expression (Du *et al*., 2010). Similarly, gliomas and HCC patients with overexpression of NGALR reportedly had low survival rates (Zhang *et al*., 2012b; Liu *et al*., 2011). Collectively, our data suggest that NGALR has an essential role in enhancing the proliferation and survival of lung cancer cells. Moreover, it is well known that the survival rate of lung cancer patients is extremely low as most of the patients are diagnosed at an advanced stage or metastatic stage of the disease. Preinvasive lung cancer cells tend to migrate across the normal epithelium and invade the surrounding cells due to their high metastatic property (Chanvorachote *et al*., 2016; Millar *et al*., 2017). Thus, by analyzing the effect of NGALR on the migration of lung cancer cells using wound healing assay, we found that disruption of NGALR significantly inhibited the migratory potential of NCIH460 cells with more

than 80% wound area remaining compared to that of the scrambled control. Collectively, this is the first report of NGALR as a positive regulator of lung cancer cell proliferation, survival and migration. Thus, this underpins the invaluable role of NGALR in the development and progression of lung cancer, and hence its immense prospect as a novel molecular target for lung cancer management. Further, to unravel the mechanism of NGALR in inducing the proliferation, survival, and migration of lung cancer cells, we analyzed the expression of its various molecular targets using Western blot. Our results showed that NGALR knockout downregulated the expressions of apoptosis regulatory proteins such as survivin, cIAP-1, procaspase-9 and upregulated the expression of caspase-3. Survivin and cIAP-1 belong to the inhibitor of apoptosis protein (IAP) family and facilitates inhibition of apoptosis (Erkanli *et al*., 2007; Li *et al*., 2018). Procaspase-9 initiates the intrinsic apoptosis pathway and exhibits a caspase activation domain (CARD) motif, by which caspase-9 is activated (Li *et al*., 2017). Caspase 3 is a prototypical apoptotic executioner which upon activation by either caspase 8 or caspase 9, facilitates apoptosis (Zhou *et al*., 2018). Further, in NGALR knockout lung cancer cells, downregulation of LC3B was observed. LC3B is a structural protein found in autophagosomal membranes and is considered a marker for autophagy (Chen *et al*., 2018). Autophagy is a pivotal catabolic process that helps in cell survival in adverse conditions such as hypoxia. Precisely, after tumor development, autophagy aids in the persistence of the tumor. In lung cancer cells, autophagy reportedly contributes to chemoresistance (Lee *et al*., 2015). Additionally, knockout of NGALR altered the expression of various cell cycle regulatory proteins including downregulation of cyclin D1, p-HH3^{S10} and Myt1 and upregulation of G1 specific CDKI such as p27 (Kip1) and p18. Cyclins D1 form active complexes with either CDK4/6, which then phosphorylate the protein Rb and facilitate G1 to S phase

transition of the cell cycle (Qie and Diehl, 2016). Studies also showed that p27 directly inhibits the enzymatic activity of CDK-cyclin complexes and arrests cells in G1 and p18 specifically inhibit the CDK-4/6 activity (Vlach *et al*., 1997; Lloyd *et al*., 1999; Noh *et al*., 1999). Notably, the cell cycle arrest at the G1 phase induced by the NGALR knockout was further established by the downregulation of cyclin D1 and upregulation of p27 (Kip1) and p18. Also, our study showed that loss of NGALR function led to the upregulation of tumor suppressor p53 which is known to induce G1 phase arrest by the transcriptional activation of p21 which binds to cyclin E/CDK-2 and cyclin D/CDK-4 complexes (Chen, 2016). HistoneH3 has been found to facilitate chromosome condensation during mitosis and Myt1 regulates the G2 checkpoint (Dai *et al*., 2005; Booher *et al*., 1997; Liu *et al*., 1999; Wang *et al*., 2004). Besides, proteins involved in cancer cell proliferation, angiogenesis, invasion, and metastasis such as COX-2, MMP-2, MMP-9 and VEGF-A were found to be downregulated upon disruption of NGALR expression in lung cancer cells (Hao *et al*., 2007; Wu *et al*., 2019; Hashemi Goradel *et al*., 2019). Further, we have found that expression of Twist-1 and N-cadherin which contribute to the promotion of EMT and metastasis were considerably downregulated in NGALR knockout lung cancer cells compared to scrambled control cells (Yochum *et al*., 2018; Mrozik *et al*., 2018; Cao *et al*., 2019). Moreover, it has been reported that Wnt pathway transcriptionally regulated 24p3R/NGALR expression leading to the expression of alternative spliced variants of the receptor (Ziegler *et al*., 2007). β-catenin is the vital component of Wnt pathway and nuclear β-catenin induces EMT, cell migration, and invasion in NSCLC (Shang *et al*., 2017). Interestingly, our result revealed the downregulation of β-catenin upon loss of NGALR function in lung cancer cells. Furthermore, increasing lines of evidence have indicated the overexpression of EGFR in about 60% of NSCLC and this overexpression is associated with poor

prognosis, thus making EGFR an oncogenic driver for lung cancer. Increased activation of EGFR was found to result in the hyperactivation of downstream pro-survival pathways including MAPK/ERK, PI3K/Akt/mTOR, and STAT-3. Hence, activation of these pathways is pivotal for lung tumorigenesis (Hsu *et al*., 2019). Notably, our results showed that the expression of p-Akt^{S473}, Akt-1, p-mTOR^{S2448}, mTOR, p-S6$^{S235/236}$ and S6 and were considerably downregulated upon NGALR knockout. Further, PTEN which is an oncosuppressor protein that inhibits the activation of Akt was upregulated (Phadngam *et al*., 2016). Additionally, previous studies have revealed that co-activation of STAT-3 with NF-κB plays a crucial role in tumor maintenance and PI3K/Akt pathway is partially responsible for the constitutive activation of STAT-3 and NF-κB (Han *et al*., 2010). Remarkably, we have also found that NGALR knockout resulted in the downregulations of proteins involved in NF-κB and STAT3 pathways including p-NF-κB^{S536}, NF-κB, p-JAK-2$^{Y1007/1008}$, JAK-2, p-STAT-3^{S727} and STAT-3. Moreover, marked downregulation of EGFR, p-MAPK$^{T202/Y204}$ and MAPK in NGALR knockout cells compared to the scrambled control was observed. Thus, our findings indicate that NGALR induces the proliferation, survival, and migration of lung cancer cells via activation of Akt/mTOR/NF-κB/STAT-3 and EGFR/MAPK signaling pathways. As discussed earlier, we showed that NGALR may be involved in tobacco-induced lung carcinogenesis. To further strengthen this finding, we treated the NGALR knockout NCIH460 cells with the most potent tobacco carcinogen, NNK and subsequently analyzed its effect on the survival and migration of the cancer cells. Further, the effect on the expression of its associated downstream targets was also studied. NNK has been reported to activate PI3K/Akt, NF-κB and other crucial signaling pathways that induce cell proliferation, survival and angiogenesis, thereby promoting the development of smoking-related lung cancers (Ge *et al*., 2015). Additionally, NNK augmented the

proliferation of NSCLC cells in an Akt-dependent manner that was associated with the upregulation of cyclin D1 expression (Tsurutani *et al*., 2005). NNK also inactivated the proapoptotic functions Bax and Bad thereby, contributing to improved survival and chemoresistance in human lung cancer cells (Deng, 2014). Thus, upon examining the effect of NNK on the survivability of NGALR knockout cells using colony formation assay, we have found that NGALR knockout cells treated with NNK showed a marked decrease in the clonogenic potential compared to scrambled control as evinced by the relatively reduced survival fraction in the NGALR knockout cells. Noteworthy, the NNK treated scrambled cells exhibited a comparatively higher survival fraction than untreated scrambled cells possibly due to the enhancement of the survivability instigated by NNK. However, after NGALR knockout, the survival fraction does not increase even after treatment with NNK. This showed that NGALR knockout significantly impedes the survival of NNK treated lung cancer cells. Besides promoting cell proliferation, NNK also promotes tumor metastasis by enhancing migration and invasion in lung cancer cells through activating c-Src/ PKCι/FAK signals (Shen *et al*., 2012). Additionally, NNK-induced migration and invasion of lung cancer cells were found to be facilitated via enhancement of the mRNA levels of N-cadherin and reduction in the mRNA level of E-cadherin (Wang *et al*., 2020b). Interestingly, our wound healing assay showed that the NNK treated NGALR knockout cells exhibited significant inhibition of wound healing with 81% of wound area remaining whereas in the scrambled control there was complete wound healing in 24 h. Also, NNK treated NGALR knockout cells have a larger wound area remaining compared to untreated NGALR knockout cells implying the ability of NNK in enhancing the migratory potential of lung cancer cells. Taken together, it is evident that NGALR is one of the positive regulators of tobacco-associated enhanced survival and migration of lung

cancer cells. Further, upon elucidating the underlined mechanism of NGALR oncogenic effect in tobacco-induced lung carcinogenesis by Western blot, we have found that NNK treated NGALR knockout showed marked downregulation of apoptosis regulatory proteins such as cIAP-1 and procaspase-9 compared to NNK treated scrambled control. Similarly, the downregulation of autophagosomes marker LC3B was also observed. Also, the proteins that promote proliferation, survival, and angiogenesis of cancer cells including cyclin D1, COX-2, VEGF-A were found to be downregulated in NNK treated NGALR knockout cells. Additionally, downregulation of Twist-1, MMP-2 and MMP-9 which contribute to the EMT, migration and metastasis was observed in NNK treated NGALR knockout cells. Mounting evidence has shown the profound implication of the hyperactivated PI3K/Akt/mTOR pathway in the tumorigenesis and progression of NSCLC (Tan, 2020). Also, activated Akt has been detected in NNK-induced lung tumors *in vivo* and human lung cancers obtained from smokers, thus eliciting tobacco-mediated carcinogenesis (West *et al*., 2003). Remarkably, we have observed that NNK treated NGALR knockout cells exhibited downregulation of p-Akt^{S473}, Akt-1, p-mTOR^{S2448} and S6. Additionally, it has been well established that NF-κB regulates several genes involved in lung carcinogenesis (Bordoloi *et al*., 2019). Further, exposure to NNK augmented the proliferation of normal bronchial epithelial and small airway epithelial cells by activating NF-κB, which thereby upregulated the expression of cyclin D1 (Ho *et al*., 2004). Additionally, constitutive activation of STAT-3 contributes to NSCLC progression by promoting proliferation and survival (Njatcha *et al*., 2018). NNK has also been reported to activate JAK/STAT in NSCLC cells (Guo *et al*., 2012). Moreover, the MAPK pathway is another important molecular pathway that has been reported to be involved in the invasion of lung cancer and tobacco smoke-induced EMT (Zhang *et al*., 2018b; Liang

et al., 2018). In our study, we have revealed that NNK treated NGALR knockout cells showed marked downregulation of p-NF-κB^{S536}, p-JAK-2$^{Y1007/1008}$, p-STAT-3^{S727}, STAT-3, p-MAPK$^{T202/Y204}$ and MAPK compared to NNK treated scrambled control cells. Taken together, NGALR manifests its oncogenic effect in NNK-mediated lung carcinogenesis through the activation of Akt/mTOR/STAT-3/NF-κB and MAPK signaling pathways.

Additionally, we have also discussed the possible involvement of NGALR in TNF-associated lung carcinogenesis. To confirm the same, NGALR knockout NCIH460 cells were treated with TNF-α and TNF-β and their effects on the survival and migration as well as their associated downstream targets were studied. TNFs have been reported to induce survival, proliferation, angiogenesis, and metastasis in most cancer cells by binding to its receptor, and activating various molecular pathways such as NF-κB (Wang and Lin, 2008; Subkamkaew *et al*., 2019). TNF-induced NF-κB activation inhibited JNK and upregulated survival factors including XIAP and survivin thereby evading apoptosis (Wang *et al*., 2007). It has been reported that TNFs also support the metastasis of cancer cells. In lung cancer, TNF-α has been found to participate in BaP induced metastasis of lung cancer cells and suppression of TNF-α repressed the induced metastasis (Wang *et al*., 2007). It has also been reported that TNF-β genetic variants may interact with environmental factors and augments susceptibility to NSCLC (Liu *et al*., 2015). In a lung metastasis mouse model, TNFs receptor TNFR-1-mediated signals support tumor neovascularization at least in part by enhancing the hepatocyte growth factor (HGF) expression, which was presumed to facilitate lung metastasis (Wang and Lin, 2008). Upon analyzing the effect of TNF-α and TNF-β on the survival of NGALR knockout lung cancer cells using colony formation assay, we have found a significant decrease in the survival fraction of NGALR knockout cells treated with TNF-α and

TNF-β compared to their respective scrambled control, perhaps due to the increase in survivability induced by the TNFs in the scrambled control cells. Next, we assessed whether NGALR is involved in TNF-α and TNF-β mediated lung cancer migration by wound healing assay. Our results showed significantly large areas of wound remaining in TNF-α and TNF-β treated NGALR knockout cells, whereas complete healing of the wounds was observed in TNF-α and TNF-β treated scrambled control possibly due to the migratory-inducing property of TNFs in the scrambled control cells. However, after depletion of NGALR, the migratory potential of the lung cancer cells significantly reduced even after treatment with the two cytokines. Overall, this proved that NGALR is a key molecule involved in TNF-mediated survival and migration of lung cancer cells. Next, using Western blot, we analyzed the molecular mechanism of NGALR in facilitating TNF-mediated lung cancer pathogenesis. Our results showed that TNF-α treated NGALR knockout cells showed a marked decrease in the expressions of cell cycle regulatory proteins such as CDK-2, CDK-6, cyclin B1, cyclin E2, cyclin D1 and cyclin D3. It is well established that G1 to S phases transition requires the action of CDK-4 and CDK-6 associated with D-type cyclins (D1, D3), and CDK-2 associated with cyclins E and A (Lauper *et al*., 1998). Cyclin B1 is the subunit of the M-phase promoting factor that facilities the G2 to M phase transition (Yuan *et al*., 2004; Ou *et al*., 2013). The deregulation of the CDK-4/6-cyclin D signal is a common aberration implicated in human malignancy (Deng *et al*., 2017). Also, cyclin E expression is frequently altered in lung cancer and results in an unfavorable clinical outcome (Galimberti *et al*., 2010). Moreover, amplification of cyclin D1 has been found to be an adverse prognostic marker for stage I NSCLC patients (Soria *et al*., 2000). Hence, the deregulation of CDKs and their associated cyclins makes them suitable targets for lung cancer therapy. Also, downregulation of p-HH3^{S10} which is involved in chromosome

condensation during mitosis was observed (Goto *et al*.,1999) Further, downregulation proteins involved in proliferation, survival angiogenesis, EMT, invasion and metastasis including, survivin, COX-2, VEGF-A, Twist-1, MMP-2 and MMP-9 was observed in TNF-α treated NGALR knockout cells. Additionally, TIPE and TIPE2 that have been found to facilitate the survival, proliferation, invasion, and migration of lung cancer were also found to be downregulated (Bordoloi *et al*., 2019; Bordoloi *et al*., 2020). Furthermore, TNF has been reported to activate important molecular pathways involved in inflammation-related carcinogenesis including NF-κB and MAPK pathways (Wang and Lin, 2008; Drutskaya *et al*., 2010). Besides, NF-κB and MAPK are key signaling pathways in promoting lung cancer progression and metastasis (Tsao *et al*., 2014). Interestingly, downregulation of p-NF-κB^{S536}, NF-κB, p-MAPK$^{T202/Y204}$ and MAPK was observed in TNF-α treated NGALR knockout cells. Collectively, this is the first report that confirms the involvement of NGALR in TNF-α mediated survival and migration of lung cancer via alteration of MAPK/NF-κB pathway. Similarly, in the case of TNF-β treated NGALR knockout cells, marked downregulation of cyclin D1, cyclin D3, cyclin E, cyclin B1 and p-HH3^{S10} was observed. Moreover, downregulations of survivin, COX-2, VEGF-A, Twist-1, MMP-2 and MMP-9 were also reported in TNF-β treated NGALR knockout cells. Furthermore, the expressions of TIPE and TIPE2 were also found to be downregulated in TNF-β treated NGALR knockout cells. Importantly, TNF-β treated NGALR knockout cells exhibit downregulation of p-Akt^{S473}, Akt-1 and p-mTOR^{S2448}. Thus, this showed that NGALR facilitates TNF-β-associated survival and migration of lung cancer cells via the activation of Akt/mTOR signaling pathway. Additionally, it has been reported that activation of the PI3K/Akt consequently resulted in enhanced activation of STAT-3 and NF-κB (Han *et al*., 2010). Previous studies have revealed that TNF-β activates the NF-κB signaling pathway in

CRC cells, thereby instigating cancer cell proliferation, EMT, invasion and metastasis, and further induces its own expression as well as TNF-α expression (Buhrmann *et al*., 2019a). Interestingly, our results showed that TNF-β treated NGALR knockout cells exhibited downregulation of p-NF-κB^{S536}, NF-κB, p-STAT-3^{S727} and STAT-3 compared to TNF-β treated scrambled control cells. Moreover, downregulation of p-MAPK$^{T202/Y204}$ and MAPK was also observed in TNF-β treated NGALR knockout cells. Taken together, NGALR elicits its tumorigenic effects through the modulation of Akt/mTOR/NF-κB/STAT-3 and MAPK signaling pathways in TNF-β mediated lung carcinogenesis. Altogether, NGALR has been demonstrated as a key molecule that has a profound role in facilitating the development and progression of lung cancer, particularly in tobacco-induced and TNF-induced lung carcinogenesis. Thus, NGALR is a promising biomarker and a potential molecular target for the effective management of lung cancer patients.

Furthermore, it is well established that the effectiveness of chemotherapy is limited due to the acquisition of chemoresistance by the lung cancer cells, leading to a dismal prognosis in patients with an advanced stage (Tao *et al*., 2016). The majority of advanced lung patients relapse after a few cycles of treatment with platinum-based chemotherapy (Hamilton and Rath, 2014). Acquired resistance to various chemotherapeutic agents such as 5-FU, docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib has become a major obstacle for improving the outcome of patients with metastatic and advanced lung cancer (Noro *et al*., 2010; Friboulet *et al*., 2014; Xiao *et al*., 2017; Zhang *et al*., 2018a). Thus, sensitizing chemoresistant cancer cells and enhancing the efficacy of the standard chemotherapeutic drugs is highly critical for the successful management of the disease. In our study, we analyzed if NGALR knockout enhances the therapeutic actions of the standard chemotherapeutic drugs including 5-

FU, docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib in NSCLC cells using MTT assay. Our results demonstrated that loss of NGALR augmented the efficacies of 5-FU, docetaxel, carboplatin, cisplatin, crizotinib, and erlotinib in NSCLC cells as evinced by the marked lower percentage of proliferation in the treated NGALR knockout lung cancer cells compared to the treated scrambled the control cells. The IC_{50} of the chemotherapeutic drugs was found to be considerably lower in the NGALR knockout cells than in the scrambled control cells. In the treated NGALR knockout cells, the IC_{50} of 5-FU, docetaxel, carboplatin, cisplatin, crizotinib and erlotinib were found to be 1.5 µM, 2.75 µM, 25 µM, 4.5 µM, 1.5 µM, and 29 µM respectively, whereas, in the scrambled control cells, the IC_{50} of 5-FU, docetaxel, carboplatin, cisplatin, crizotinib and erlotinib were found to be 3.5 µM, 4.25 µM, 65 µM, 12 µM, 6.5 µM and 45 µM respectively. Hence, these preliminary data indicate that depletion NGALR in lung cancer cells elicits a reduced proliferation phenotype and may potentially increase the sensitivity of lung cancer cells to chemotherapeutic agents.

5.2. Limitations and Future Prospects of the Study

From the current study, we have established the integral role of NGALR in the development and progression of lung cancer, specifically in tobacco and TNF-induced lung carcinogenesis. However, few limitations exist with the study that can be further investigated in the future. Firstly, the lung cancer TMA slide used for the expression analysis of NGALR in human lung cancer tissues does not contain samples of the Indian population. Even though lung carcinogenesis is a multifactorial process induced by genetic or environmental factors, racial and ethnic variances also attribute to the epidemiologic differences in lung cancer incidence, mortality, and patient outcomes as well as the various aspect of cancer hallmarks including proliferation, evasion of

apoptosis, and angiogenesis. Hence, analyzing NGALR expression in tissue samples of different stages, grades and types of lung cancer taken from the Indian population will make the study more epidemiologically relevant. Moreover, the TMA slide contained lung cancer tissue samples up to stage IIIA only. Hence, expression analysis of NGALR in more advanced stages such as stage IIIB and IV of lung cancer would help us predict the role of NGALR at a highly metastatic stage and precisely decipher the stage-wise expression pattern of NGALR. Besides, it is well established that lung cancer development is characterized by a series of sequential preneoplastic changes. The TMA slide also lacks tissue samples of pre-neoplastic lesions, thus warranting the need for assessing the NGALR expression in various pre-neoplastic lesions for the meticulous elucidation of the role of NGALR in the developmental process of lung cancer. Further, the TMA slide does not come with detailed information about the smoking status of the patients or their use of other forms of tobacco. As tobacco is the main risk factor for lung cancer, information about patients' tobacco consumption history would have rendered a stronger correlation of NGALR with tobacco-mediated lung carcinogenesis. Moreover, a study of NGALR expression on lung cancer tissues of different subtypes, pathologies, stages, grades, age groups and sexes in larger cohorts is essential to attain a significant prognostic value of NGALR in lung cancer.

Secondly, we studied the association of NGALR in tobacco-mediated lung carcinogenesis using tobacco extract in the form tuibur, and four tobacco components NNK, NNN, BaP and nicotine. As tobacco contains more than 20 known lung carcinogens, additional studies on the expression of NGALR with other lung carcinogens besides NNK, NNN, BaP and nicotine will deliver a comprehensive understanding of the involvement of NGALR in lung cancer induced by tobacco. Also,

among the states of India, the highest lung cancer incidence was reported from Mizoram. Approximately 58.7% of the population of Mizoram are consuming tobacco, either in smoke or smokeless form. Although we studied the effect of tuibur, a locally prepared and frequently consumed smokeless tobacco in Mizoram, the effect of other tobacco preparations used by the Mizo people such as 'zozial', a local roll-up cigarette without filters, can be further analyzed. Additionally, we have used an in silico-based approach to analyze the transcription factors that have potential binding sites in the NGALR promoter region which infer the binding potential, but not the functionality of a site. Further *in vitro* experiments to prove the functionality is highly imperative to confirm the transcription factors that serve as upstream regulators of NGALR expression in lung cancer.

Thirdly, we performed CRISPR/Cas9 mediated knockout of the *NGALR* gene in NSCLC cells and studied its detailed role in lung carcinogenesis, with emphasis on NNK and TNF-mediated lung carcinogenesis *in vitro*. The role of NGALR in lung cancer can further be evinced by silencing NGALR and elucidating its function in the SCLC subtype. Importantly, as the crystallographic structure of NGALR has yet to be elucidated, determining the NGALR structure is of prime importance to provide us comprehensive information on the physical property of the protein and help us in better understanding of NGALR function in various malignancies. Apart from this, we have elucidated the chemosensitizing action of NGALR knockout to the standard chemotherapeutic agents in lung cancer cells using MTT assay. This particular aspect of the study requires further *in vitro* studies for validation and to decipher the role of NGALR in the regulation of chemoresistance in lung cancer cells. Additionally, global gene and protein expression profiling of NGALR knockout cells by microarray analysis

would give us greater insights into the complex network of interactions among various genes/proteins involved in the pathogenesis of lung cancer. Importantly, the tumorigenic role of NGALR in lung cancer that we have elucidated *in vitro* needs to be further evinced *in vivo* to wholly establish NGALR as a novel and promising molecular target which will enable the development of efficacious therapeutic strategies for the better management of this aggressive malignancy.

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Publications and Presentations

Articles and Book chapters

1. **Sailo BL**, Banik K, Bordoloi D, Harsha C, Kunnumakkara AB. NGALR enhances the proliferation, survival and migration of lung cancer cells via modulating Akt/mTOR/NF-κB/STAT3 and EGFR/MAPK signaling pathways. (Submitted)
2. Bordoloi D, Banik K, Vikkurthi R, Thakur KK, Padmavathi G, **Sailo BL**, Girisa S, Chinnathambi A, Alahmadi TA, Alharbi SA, Buhrmann C, Shakibaei M, Kunnumakkara AB. Inflection of Akt/mTOR/STAT-3 cascade in TNF-α induced protein 8 mediated human lung carcinogenesis. Life Sci. 2020 Sep 22;262:118475.
3. Khatoon E, Banik K, Harsha C, **Sailo BL**, Thakur KK, Khwairakpam AD, Vikkurthi R, Devi TB, Gupta SC, Kunnumakkara AB. Phytochemicals in cancer cell chemosensitization: Current knowledge and future perspectives. Semin Cancer Biol. 2020 Jun 28:S1044-579X(20)30150-4.
4. Kunnumakkara AB, Shabnam B, Girisa S, Harsha C, Banik K, Devi TB, Choudhury R, Sahu H, Parama D, **Sailo BL**, Thakur KK, Gupta SC, Aggarwal BB. Inflammation, NF-κB, and Chronic Diseases: How are They Linked? Crit Rev Immunol. 2020;40(1):1-39.
5. Henamayee S, Banik K, **Sailo BL**, Shabnam B, Harsha C, Srilakshmi S, Vgm N, Baek SH, Ahn KS, Kunnumakkara AB. Therapeutic Emergence of Rhein as a Potential Anticancer Drug: A Review of Its Molecular Targets and Anticancer Properties. Molecules. 2020 May 12;25(10):2278.
6. **Sailo BL**, Banik K, Girisa S, Bordoloi D, Fan L, Halim CE, Wang H, Kumar AP, Zheng D, Mao X, Sethi G, Kunnumakkara AB. FBXW7 in Cancer: What Has Been Unraveled Thus Far? Cancers (Basel). 2019 Feb 19;11(2). pii: E246.
7. Kunnumakkara AB, Harsha C, Banik K, Vikkurthi R, **Sailo BL**, Bordoloi D, Gupta SC, Aggarwal BB. Is curcumin bioavailability a problem in humans: lessons from clinical trials. Expert Opin Drug Metab Toxicol. 2019 Sep;15(9):705-733.
8. Kunnumakkara AB, Bordoloi D, **Sailo BL**, Roy NK, Thakur KK, Banik K, Shakibaei,M, Gupta SC, Aggarwal BB. Cancer drug development: The missing links. Exp Biol Med (Maywood). 2019 May;244(8):663-689.
9. Banik K, Ranaware AM, Deshpande V, Nalawade SP, Padmavathi G, Bordoloi D, **Sailo BL,** Shanmugam MK, Fan L, Arfuso F, Sethi G, Kunnumakkara AB. Honokiol for cancer

therapeutics: A traditional medicine that can modulate multiple oncogenic targets. Pharmacol Res. 2019 Jun;144:192-209.

10. Kunnumakkara AB, Thakur KK, Rana V, Bora B, Banik K, Khatoon E, **Sailo BL**, Shabnam B, Girisa S, Gupta SC, Aggarwal BB. Upside and Downside of Tumor Necrosis Factor Blockers for Treatment of Immune/Inflammatory Diseases. Crit Rev Immunol. 2019;39(6):439-479.
11. Haque MA, **Sailo BL**, Padmavathi G, Kunnumakkara AB, Jana CK. Nature-inspired development of unnatural meroterpenoids as the non-toxic anti-colon cancer agents. Eur J Med Chem. 2018 Dec 5;160:256-265.
12. Kunnumakkara AB, Banik K, Bordoloi D, Harsha C, **Sailo BL**, Padmavathi G, Roy NK, Gupta SC, Aggarwal BB. Googling the Guggul (Commiphora and Boswellia) for Prevention of Chronic Diseases. Front Pharmacol. 2018 Aug 6;9:686.
13. Banik K, **Sailo BL**, Thakur KK, Jaiswal A, Bordoloi D and Kunnumakkara AB, 'Potential of Different Chemosensitizers to Overcome Chemoresistance in Cervical Cancer', In Cancer Cell Chemoresistance and Chemosensitization. World Scientific Publications (2018).
14. Bordoloi D, Banik K, Khwairakpam AD, Sharma A, **Sailo BL** and Kunnumakkara AB, 'Different Approaches to Overcome Chemoresistance in Esophageal Cancer', In Cancer Cell Chemoresistance and Chemosensitization. World Scientific Publications (2018).
15. **Sailo BL**, Monisha J, Jaiswal A, Prakash J, Roy NK, Thakur KK, Banik K, Bordoloi D and Kunnumakkara AB, 'Molecular Alterations Involved in Pancreatic Cancer Chemoresistance and Chemosensitization Strategies', In Cancer Cell Chemoresistance and Chemosensitization. World Scientific Publications (2018).
16. **Sailo BL**, Bordoloi D, Banik K, Prakash J and Kunnumakkara AB, 'Therapeutic Strategies for Chemosensitization of Renal Cancer', In Cancer Cell Chemoresistance and Chemosensitization. World Scientific Publications (2018).
17. Bordoloi D, **Sailo BL**, Mantegh N, Padmavathi G and Kunnumakkara AB,'Introduction and Basic Concepts of Cancer , In Cancer Cell Chemoresistance and Chemosensitization. World Scientific Publications (2018).
18. **Sailo BL**, Banik K, Padmavathi G, Javadi M, Bordoloi D, Kunnumakkara AB. Tocotrienols: The promising analogues of vitamin E for cancer therapeutics. Pharmacol Res. 2018 Apr;130:259-272.

19. Banik K, Harsha C, Bordoloi D, **Lalduhsaki Sailo B**, Sethi G, Leong HC, Arfuso F, Mishra S, Wang L, Kumar AP, Kunnumakkara AB. Therapeutic potential of gambogic acid, a caged xanthone, to target cancer. Cancer Lett. 2018 Mar 1;416:75-86.
20. Bordoloi D, Banik K, **Sailo BL**, Kunnumakkara AB. Nutraceuticals in Cancer Prevention and Treatment. North-East Bioline. 2017; 3:6-9.
21. Kunnumakkara AB, **Sailo BL**, Banik K, Harsha C, Prasad S, Gupta SC, Bharti AC, Aggarwal BB. Chronic diseases, inflammation, and spices: how are they linked? J Transl Med. 2018 Jan 25;16(1):14.
22. Khwairakpam AD, Shyamananda MS, **Sailo BL**, Rathnakaram SR, Padmavathi G, Kotoky J, Kunnumakkara AB. ATP citrate lyase (ACLY): a promising target for cancer prevention and treatment. Curr Drug Targets. 2015;16(2):156-63.

Conferences, workshops and trainings attended

1. Presented oral presentation in 1st Department Retreat (Biotech Express), titled "Role of SLC22A17 in lung cancer", organized by BSBE Department, IIT Guwahati, held on 21st December 2019.
2. Presented oral presentation in “International Conference on Nutraceuticals and Chronic Diseases (INCD) 2019”, held on September 23rd-25th, 2019 organized by Society for Nutraceuticals and Chronic Diseases and Indian Institute of Technology Guwahati, at IIT Guwahati, India, 2019.
3. Presented poster presentation in “INSPIRE Fellowship Review Meet” titled, " An Investigation on the Role of NGALR in the Development and Progression of Lung Cancer" organized at the IASST, Guwahati, Assam 781035 during 30th May-1st June 2019.
4. Presented poster presentation in Research Conclave, titled "Role of Lipocalin Receptor in the Development of Lung Cancer”, organized by Student Activity Board, Indian Institute of Technology Guwahati, Assam, India, on 2019.
5. Presented poster in 10days in 6th AIST Imaging Workshop, DAICENTER, at Biomedical Research Institute, Tsukuba Science City, Japan, January 2019
6. Participated in a 10 days advanced research training workshop on ‘Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches’ sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by

National Institute of Biomedical Genomics, Kalyani, Kolkata, held on 23rd July-1st August, 2018.

7. Presented poster in the International Conference on Translational Cancer Research-2018, held on February 8th-11th, 2018, organized by IIT Madras, Chennai, India, 2018.
8. Presented poster in the national conference, "Ethno-medicine and Traditional Health Practices In North-East Region of India" organized by National Institute of Pharmaceutical Education and Research (NIPER) Guwahati, Assam, India, 25th August, 2018.
9. Participated in oral presentation at 3rd International Conference on "Nutraceuticals and Chronic Diseases" organized by Sri Rama Himalaya University and Society for Nutraceuticals and chronic diseases in Rishikesh, Uttarakhand, India, 2018.
10. 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.
11. Participated in a 5 day research training workshop on 'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches' sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kolkata and Department of Biotechnology, Mizoram University, held during November 19th-24th, 2017, and selected for the advanced level workshop.
12. Presented poster in "International Conference on Nutraceuticals and Chronic Diseases (INCD) 2017", held on September 1st-3rd, 2017 organized by Society for Nutraceuticals and Chronic Diseases and Indian Institute of Technology Guwahati, at Goa, India, 2017.
13. Presented poster in Research Conclave, organized by Student Activity Board, Indian Institute of Technology Guwahati, Assam, India, 2017.
14. Presented poster in the International Conference on Translational Cancer Research-2016, held on February 4th-7th, 2016, organized by Gujarat Cancer and Research Institute, Ahmedabad, India, 2016.
15. Presented poster in Research Conclave, organized by Student Activity Board, Indian Institute of Technology Guwahati, Assam, India, 2016.
16. Participated in National Conference on Recebt Development in Medical Biomedical Biotechnology and Structure Based Drug Designing {RDMBSBDD-2015} held on December 6-7, 2015, organized by the Department of Biotechnology, Indian Institute of Technology Guwahati, 2015

17. Participated in the National course on "Theoretical and Practical Aspect of Cancer Research" conducted by the Technical Educational Quality Improvement Programme, sponsored by the Ministry of Human Resource Development, Govt. Of India, held on February 4th - 8th, 2015 at Indian Institute of Technology Guwahati, India.
18. Participated in "Flow Cytometry Data Analysis" held on January 23th - 24th, 2015, conducted by Department of Biotechnology, Indian Institute of Technology Guwahati
19. Participated in the National Conference on "Recent Advances in Cancer Biology and Therapeutics- 2014 (RACBT- 2014)", held on December 5th, 2014, organized by the Department of Biotechnology, Indian Institute of Technology Guwahati, 2014.
20. Participated in "International Conference on Disease Biology and Therapeutics (ICDBT 2014)", held from December 3rd – 5th, 2014, organized by Institute of Advance study in Science and technology, Guwahati, 2014
21. Participated in the National Conference on "Advances of Cancer Genomics", held on May 30th – 31st, 2014, organized by Department of Biotechnology, Mizoram University.
22. Participated in "Next Generation Sequencing and Data Analysis" Workshop, held on May 14th – 17th, 2014, organized by Biotech Hub, Centre for the Environment, Indian Institute of Technology Guwahati.

Awards and achievements

1. Received "**Best Oral Presentation Award**" for "An Investigation on the Role of Lipocalin Receptors in the Development of Lung Cancer at the Indo-Japan Symposium on "Recent Advances in Biomedical Research-2019" jointly organized by IIT Guwahati, India and AIST, Tsukuba Japan, sponsored by the DBT, Govt. of India from 26th-27th March, 2019
2. Selected for participating at the 6th AIST International Imaging Workshop to be held in January 20-27, 2019 at DAICENTER, AIST Tsukuba, Japan.
3. Received "**First Prize in Poster Presentation Category**" at National conference on "Ethno-medicine and Traditional Health Practices in North-East Region of India" organized by National Institute of Pharmaceutical Education and Research (NIPER) Guwahati, Assam, India, August 2018.
4. Received **'Best Poster Award'** at the Second International Conference on Nutraceuticals and Chronic Diseases 2017 (INCD-2017), Goa, India, September 2017.