An Investigation on the Role of Tumor Necrosis Factor-α Induced Protein 8 (TNFAIP8) Family in the Development and Progression of Oral Cancer

A thesis submitted for the degree of

Doctor of Philosophy

To

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

By

PADMAVATHI G

Department of Biosciences and Bioengineering

Indian Institute of Technology Guwahati

Guwahati, Assam-781039, India

February, 2019

Dedicated to

My dear family, friends and teachers

For motivating and supporting me in all my endeavours

DEPARTMENT OF BIOSCIEENCES AND BIOENGINEERIG
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
GUWAHATI-781039

DECLARATION

I hereby declare that the contents of the research work described in this thesis titled **"An investigation on the role of tumor necrosis factor-α induced protein 8 (TNFAIP8) family in the development and progression of oral cancer"**, is a presentation of my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Ajaikumar B. Kunnumakkra.

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

February, 2019

Padmavathi G
Roll No. 136106007
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Guwahati, India.

DEPARTMENT OF BIOSCIEENCES AND BIOENGINEERIG
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
GUWAHATI-781039

CERTIFICATE

This is to certify that the work described in the thesis titled **"An investigation on the role of tumor necrosis factor-α induced protein 8 (TNFAIP8) family in the development and progression of oral cancer"**, submitted by Padmavathi G (Roll no: 136106007) 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.

February, 2019

Dr. Ajaikumar B Kunnumakkara
Associate professor
Cancer Biology Laboratory &
DBT-AIST International Laboratory for Advanced Biomedicine (DAILAB)
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Guwahati, India.

ACKNOWLEDGEMENTS

It would have been impossible for me to successfully complete my PhD without the support of so many good souls who have been a great inspiration and guidance during the journey of my PhD and life. I would take this opportunity to acknowledge all of them without which my thesis would remain incomplete.

First and foremost, I would like to express my sincere gratitude to my thesis supervisor Dr. Ajaikumar B. Kunnumakkara for giving me this opportunity and guiding me throughout my PhD. His words have always been a true inspiration and motivation which made me realise and improvise my real potential.

I am extremely grateful to my doctoral committee members Prof. R. Swaminathan, Dr. Satpati Priyadarshi, Dr. Ashish Anand and Dr. Piruthivi Sukumar for their timely suggestions which enormously helped me to improve the quality of my thesis.

I am thankful to the Department of Biosciences & Bioengineering, IIT Guwahati for providing me the facilities and Ministry of Human Resource Development (MHRD), Govt. of India for the financial support.

I am grateful to the present and past Heads of the Department of Biosciences & Bioengineering, Prof. Veeranki Venkata Dasu, Prof. Kannan Pakshirajan and Prof. Latha Rangan for availing me the departmental facilities.

I would also like to thank all the teaching and non-teaching staffs of the department and institute, janitors, and securities for their support and timely helps during the course of my PhD.

I sincerely acknowledge the immense help received from my fellow colleagues and friends Dr. J. Monisha, Dr. NK Roy, Devivasha, Dr. A Singh, Amrita, Bethsibie,

Kishore, Harsha, Krishan, Shabnam, Sosmitha, Rajesh, Babita, Amit, Ajit Akela, Shyamananda without whom completion of this work would have been really difficult.

I also thank all the present and past JRFs and trainees of our lab for their immeasurable assistance and support.

I am highly indebted to the most important persons of my life, my dear family, Appa, Amma, Anna, Akka, Athai and Mama for their unconditional love, support, patience and motivation throughout my life and career.

It definitely requires a special mention to this person who have always been a part of my lows and highs- my better half Mr. S. Gunasekaran for his never-ending love, care and support without whom it wouldn't have possible for me to join PhD in the first place. With immense patience, he has always been my PhD stressbuster because of which I could successfully complete the hard journey of PhD.

I would also like to thank all my dear friends Vanitha, Janani, Sumitha, Anujaa, Sharan, Amrutha, Sajitha, Shiva, Vicky Babu, Johnny Bhai, Anil, Philip, Vignesh, Nithin, Gowtham, Boopathy, Sheeba, Vartika and all my batch mates for always being a family away from home.

I would also like to express my gratitude to all my teachers who groomed me to the person I am now by sharing their precious knowledge and ethical values.

Last but not the least, I sincerely thank the Almighty God for giving me good health and opportunities to establish and prove myself.

Padmavathi G

TABLE OF CONTENTS

CHAPTER 1

Introduction and Review of Literature

Chapter 1

1.1 Introduction

Oral cancer stands one among the top ten most common cancers worldwide and one of the major life threatening diseases in India (Scully et al., 2005). According to the Globocan survey, in 2018 alone approximately 1.2 lakh new cases have been reported contributing to 10.4% of all cancer incidences in India. Further, cancers of lip and oral cavity have been found to be the second most common cause of cancer related mortality in India with 72,616 deaths reported in 2018. Notably, global oral cancer burden is expected to increase exponentially in the coming years with an estimated increase of 53% by 2040 (GLOBOCAN 2018). Adding to this, cancer of gingivo-buccal complex (cancers of lower alveolus, buccal mucosa and the retro molar trigone) is known as Indian oral cancer as it constitutes around 60% of all oral cancer incidences in India (Byakodi et al., 2012). The increase in the rate of oral cancer incidences in India can be attributed to the increased exposure to tobacco. Supporting this, more than 90% of oral cancer cases in India have been found to be associated with the history of tobacco consumption (https://vovindia.org/tag/mawa/, Gupta et al., 2013). Further, tobacco consumption in any form, with or without alcohol was also found to be correlated with precancerous lesions of oral cancer such as leukoplakia and erythroplakia, which possess high risk of developing into a malignant disease (Goel et al., 2014). Besides tobacco, age, gender, viral infections like human papilloma virus (HPV), air pollution and immune deficiency syndromes are also believed to be the potential risk factors for oral cancer (Oral cancer foundation, Simard et al., 2014). In addition, a report has shown that the median age of oral cancer diagnosis is 62 years supporting the fact that oral cancer is more prevalent in elderly people (Macek, 2010). Oral cancer development is a multistage process involving various molecular alterations such as activation of oncogenes, loss of tumor

suppressor genes and alterations in the genes regulating various cellular processes like cell survival, proliferation, angiogenesis, tumor invasion and progression etc. (Pérez-Sayáns et al., 2009). Like any other cancers, the most common treatment strategies for OSCC are surgery, radiotherapy and chemotherapy or combination of these. Modality of treatment depends on the severity of disease (Das and Nagpal, 2002). The most commonly used chemotherapeutic drugs for the treatment of OSCC are cisplatin, 5-fluorouracil, docetaxel and cetuximab etc. (Lyu et al., 2014, Petrelli et al., 2014). However, most of these chemotherapeutic drugs were found to be associated with adverse side effects affecting the patient's physiology and causing myelosuppression, difficulty in speaking, swallowing etc. (Liu et al., 2014a, Tahover et al., 2015, Das and Nagpal, 2002). Moreover, development of chemoresistance and radioresistance through the activation of different survival proteins like survivin, worsen the scenario reducing the efficacy of the drug (Su et al., 2010). Further, the age factor was also found to play a significant role in deciding the fate of treatment and prognosis. In line with this, OSCC patients below 40 years of age had significantly higher 5 year survival rate compared to that of patients above 40 years (Macek, 2010). Above all, detection in early stages can give better treatment options and better prognosis. However, in majority of the cases, oral cancer is diagnosed only in the advanced stages which narrow down the chances of recovery (Mignogna et al., 2004). Therefore, it is essential to develop novel biomarkers as early diagnostic, therapeutic and prognostic targets for this deadly disease.

Recent reports have shown that tumor necrosis factor (TNF)-α- induced protein 8 (TNFAIP8 or TIPE), a newly identified protein family, comprising of four proteins namely TNFAIP8 (TIPE), TNFAIP8L1 (TIPE1), TNFAIP8L2 (TIPE2) and TNFAIP8L3 (TIPE3), plays major roles in inflammation, immunity and

tumorigenesis (Padmavathi et al., 2018, Kumar et al., 2004, Sun et al., 2008, Kumar et al., 2000, Fayngerts et al., 2014). Interestingly, TNFAIP8 is found to be overexpressed in different cancers like chronic myelogenous leukemia, lymphoblastic leukemia and lung carcinoma etc. However, little is known about the actual molecular mechanism behind TNFAIP8 induced carcinogenesis (Kumar et al., 2000, Lou and Liu, 2011, Padmavathi et al., 2018). Likewise, reports showed that TNFAIP8L2 inhibits MAPKs and NF-κB signaling pathways further explaining the importance of this protein in cancer development (Sun et al., 2008, Freundt et al., 2008, Padmavathi et al., 2018). Further, the other two proteins of this family, TNFAIP8L1 and TNFAIP8L3 were also found to differentially regulate oncogenesis. However, only very few reports are available on these two proteins (Hitomi et al., 2008, Fayngerts et al., 2014, Padmavathi et al., 2018). Above all, the first member of this family, TNFAIP8 was initially identified to be differentially expressed in primary human head and neck squamous cell carcinoma (HNSCC) and metastatic head and neck squamous cell carcinoma (HNSCC) cell lines (Patel et al, 1997, Kumar et al., 2000, Padmavathi et al., 2018). Despite being identified in HNSCC cells, the actual role of TNFAIP8 in the development and progression of HNSCC remains elusive (Padmavathi et al., 2018). Therefore, the current study aimed at deciphering the role of TNFAIP8 and its other family members in the development and progression of oral cancer, a predominant HNSCC which would help us to develop novel biomarkers for the better management of this disease.

1.2 Oral Cancer

Cancers of oral cavity, oropharynx, lips, cheeks, roof of mouth (hard palate), back of the mouth (soft palate and uvula), floor of the mouth (area under the tongue), gums, teeth, tongue, and tonsils are classified as oral cancers and the oral squamous cell

carcinoma is the sixth most common cancer among all head and neck malignancies (Scully et al., 2005, Elango et al., 2009). Also, among all types of oral cancers, the squamous cell carcinoma constitutes around 90%, marking it as the most common neoplasm of oral cavity (Pereira et al., 2007, Joseph et al., 2018). Regions of Melanesia, South-Central Asia, Australia and Eastern Europe are the hit regions of oral cancer both in males and females (Jemal et al., 2008, Bray et al., 2018). However, in India, incidence of oral cancer has been found to be more common in males with the male to female ratio of 4.18:1 (Shenoi et al., 2012). Moreover, the age standardized oral cancer incidence in India is reportedly approximately 12.6 per 100,000 population contributing to 30-40% of all cancer incidences (Byakodi et al., 2012, Petersen, 2005). Further, the 5-year survival rate for oral cancer remains as low as 50–55% despite the therapeutic advancements, which is mainly due to late stage diagnosis. However, if diagnosed at the earlier stages, oral cancer will have the highest survival rate compared to other malignancies (Joseph et al., 2018; Irani, 2016).

1.2.1 Types of oral cancer:

Based on the type of tissues affected, oral cancer can be classified as follows (Montero and Patel, 2015, Cancer Treatment Centers of America® (CTCA)):

(i) Squamous cell carcinoma: cancers of squamous cells lining the mouth and throat which contributes to more than 90% of oral cancer.

(ii) Verrucous carcinoma: unlike the common squamous cell carcinoma, verrucous carcinoma is slow-growing cancers of squamous cells which contributes to only 5% of oral cancers and very rarely metastasize to a distant organ.

(iii) Salivary gland tumors: cancers arising at the small glandular tissues found near oral linings which are further categorized as adenoid cystic carcinoma, mucoepidermoid carcinoma, and polymorphous low-grade adenocarcinoma etc.

(iv) Lymphomas: cancers of lymphoid tissues of oral cavity.

1.2.2 Preneoplastic lesions:

Development of oral squamous cell carcinoma, the most common type of oral cancer, has been found to be associated with various precancerous lesions such as leukoplakia, erythroplakia, oral lichen planus (OLP), and oral submucous fibrosis (OSF) (Montero and Patel, 2015).

Leukoplakia: The WHO defines leukoplakia as 'a white patch or plaque that cannot be easily removed and characterized clinically or pathologically as any other disease' (Kramer et al., 1978, Summerlin, 1996, Irani, 2016). Oral leukoplakia incidence ranges from 1-5% (Awadallah et al., 2018). Initially, leukoplakia starts as whitish grey plaques mostly at alveolar mucosa, buccal mucosa, lower lip, floor of mouth and lateral tongue and progresses into thicker, whiter and leathery with surface fissures (Neville and Day, 2002). Also, it is clinically manifested in various forms like thin, thick or homogeneous, granular or nodular and proliferative verrucous leukoplakia (Irani, 2016). Risk of malignant transformation of leukoplakia varies from 3% to 17% (Awadallah et al., 2018). Further, the risk of developing into a malignant disease increases in people of 60-70 years of age and people with dysplasia (mild, moderate and severe). In addition to age and dysplasia, other factors such as gender, idiopathic leukoplakia, size of leukoplakia, non-homogenous type and Candida infection were also found to contribute to the malignant transformation (Irani, 2016). At the molecular levels, leukoplakia has been shown to be associated with increased levels

of TP53, MDM2, human telomerase reverse transcriptase (hTERT), retinoblastoma (Rb) and decreased levels of p27. Further, the upregulation of SMAD4, cyclin D1, Cox-2, Ki-67, p63, vascular endothelial growth factor (VEGF), MMP-1, -9, and -11 were reported to increase the degree of dysplasia and the risk of malignant transformation (Irani, 2016).

Erythroplakia: Well distinguished lesions that are red in colour, with soft velvety texture and arise mainly at the floor of mouth, lateral tongue, retro molar pad, and soft palate are considered as erythroplakia. In certain cases they are intermixed with white patches which are known as erythroleukoplakia. Unlike leukoplakia, these are not common, and often progress into squamous cell carcinoma (Neville and Day, 2002, Summerlin, 1996). It is more common in males older than 50 years. Erythroplakia are often manifested as flat to slightly raised uniform red lesions with irregular borders. Remarkably, more than 90% erythroplakia are developed into severe dysplasia, carcinoma *in situ* or carcinomas defining it to be a universal premalignant lesion thus considered as a first clinical sign of OSCC (Summerlin, 1996, Irani, 2016, Awadallah et al., 2018). At the molecular level, more than 46% of erythroplakia were found to possess TP53 mutation (Irani, 2016). Surgical removal is a treatment of choice for erythroplakia with dysplasia and carcinoma *in situ*. However, it also depends on the histopathologic diagnosis and clinical staging as recurrence and progression of erythroplakia are common in case of invasive diseases (Summerlin, 1996).

Oral lichen planus (OLP) is a T-cell mediated chronic autoimmune disease affecting the mucous membrane of the oral cavity. It is suggested to be developed due to CD8+ T cells induced apoptosis (Irani, 2016, Neville and Day, 2002, Fang et al., 2009). It has been found to be more prevalent in females and onset occurs at the age of 30-70 years (Awadallah et al., 2018, Irani, 2016). Buccal mucosa, tongue and gingiva are

the most commonly affected regions. The lesions present in various forms such as reticular, papular, plaque-like, erosive, atrophic or bullous appearance and the reticular form is the most common one (Neville and Day, 2002, Awadallah et al., 2018). Average risk of developing into a malignant disease is 1%. However, the rate of malignant transformation was found to be more in erosive and atrophic types and in smokers, alcoholics, *Candida albicans* and hepatitis C virus infected patients and in presence of dysplasia. Additionally, infiltration of chronic inflammatory cells also strongly correlated with cancer development (Awadallah et al., 2018, Irani, 2016). Further, downregulation of E-cadherin, β-catenin, epidermal growth factor receptor (EGFR) and ANXA1 are some of the molecular alterations observed in OLP (Irani, 2016). Topical corticosteroids, and other topical agents, such as retinoid, cyclosporine, calcineurin inhibitors, and photodynamic therapy are the first and second line therapeutics for localized OLP (Awadallah et al., 2018).

Oral submucous fibrosis (OSF) is a progressive fibrotic premalignant condition that is very common in Indians and characterized by disrupted collagen metabolism, opaque, blanched and hardened oral mucosa and burning sensation. When progresses OSF leads to several severe clinical manifestations such as xerostomia, restriction of tongue movement and reduced elasticity of lips, floor of the mouth and buccal mucosa finally resulting in fibrosis and trismus. OSF onset occurs between 20-30 years of age. Areca nut consumption has been suggested to be closely associated with OSF as it induces transforming growth factor beta (TGF-β) signaling in oral epithelial cells ultimately leading to inflammation and fibrosis in the underlying connective tissue. In addition to areca nut and its derivatives, other factors such as nutritional deficiencies, autoantibodies and capsaicin were also suggested to contribute to OSF development (Irani, 2016, Awadallah et al., 2018). Sudden intolerance to spicy foods, vesicle and

ulcer formation on hard palate and buccal mucosa are some of the initial clinical signs and symptoms. The rate of malignant transformation was observed to be 7-30 % for OSF (Awadallah et al., 2018). At the molecular levels, OSF has been found to be associated with upregulation of certain cytokines viz. IL-1, IL-6, IL-8 and fibroblast growth factor (FGF); and overexpression of p53, p63, plasminogen activator inhibitot-1 (PAI-1), and tissue inhibitor metalloproteinase-1 (TIMP-1) (Irani, 2016). OSF is regarded as almost irreversible once it reaches the trismus stage. Therefore, it is essential to diagnose and treat at earliest to avoid complications and malignant transformation (Awadallah et al., 2018). Prohibiting the consumption of areca nut/betel nut is the immediate necessity in the treatment of OSF followed by halting the disease progression through enzymatic degradation by hyaluronidase, collagenase, and chymotrypsin, anti-inflammatory/immunomodulators, antioxidant therapy and micronutrient supplementation etc. (Awadallah et al., 2018). Further, reduction in vasculature is one of the characteristic features of OSF which results in the reduced nutrient supplement to the affected tissues. Therefore, improving the vascular integrity using polyphenols is another method of treating OSF. In addition, surgical therapy is also used in order to suppress trismus by releasing the fibrotic bands or tissue (Rajalalitha and Vali, 2005, Awadallah et al., 2018).

Epithelial dysplasia: It is the most common premalignant disease of OSCC characterized by cellular atypia, loss of stratification and normal maturation of stratified squamous epithelium and basal cell hyperplasia etc. (Awadallah et al., 2018, Shirani et al., 2014). In general, the dysplastic changes starts at the basal or parabasal epithelium. Depending on the extent of epithelial layers involved, dysplasia is graded as mild, moderate, severe dysplasia and carcinoma *in situ*. Dysplastic changes restricted to the basal or parabasal layer are considered as mild dysplasia. Changes

that extended to the middle of granular layer are regarded as moderate dysplasia and changes extended from basal layer to upper and middle layer of epithelium are considered as severe dysplasia. Dysplasia that spread from the basal epithelial layer to the surface of the mucosa is called as carcinoma *in situ* (Shirani et al., 2014). Presence of different grades of dysplasia (mild, moderate and severe) in the aforementioned precancerous lesions is known to significantly increase the risk of malignant transformation. However, not all dysplasias turn into a malignant disease. Supporting this, the rate of malignant transformation of dysplasia was found to range between 6-36%. Surgical removal using cold knife or CO_2 laser, immunomodulatory or antioxidant therapies are used alone or in combination for treating different grades of dysplasia (Awadallah et al., 2018).

1.2.3 Molecular alterations associated with oral cancer:

Several mutations that remarkably alter the survival, proliferation, morphology, angiogenesis and other properties of a cell have been reported in the genome of oral cancer cells. For instance, loss of heterozygosity of the p53 gene has been identified in 20% of OSCCs, and 22% of oral premalignant lesions (Mehrotra and Yadav, 2006). Likewise, inactivation of several other tumor suppressor genes including, BARD1, CASP8, CASP10, ILKAP, ING5, LRP1B and PPP1R7 by genomic instabilities and deletions at the regions of 2q21-24, 2q33-35, and 2q37 were also reported in different OSCC cell lines (Yanamoto et al., 2007). Similarly, allelic loss of cyclin dependent kinase inhibitor genes p16 and p14 and chromosomal aberrations in the regions of 4q, 5q21-22, 11q, 18q, 21q and 22q13 were also observed in the developmental stages of oral cancer (Ohta et al., 2009, Moles et al., 2008) (Figure 1.1). In addition to inactivation of tumor suppressors, activation and overexpression of oncogenes such as

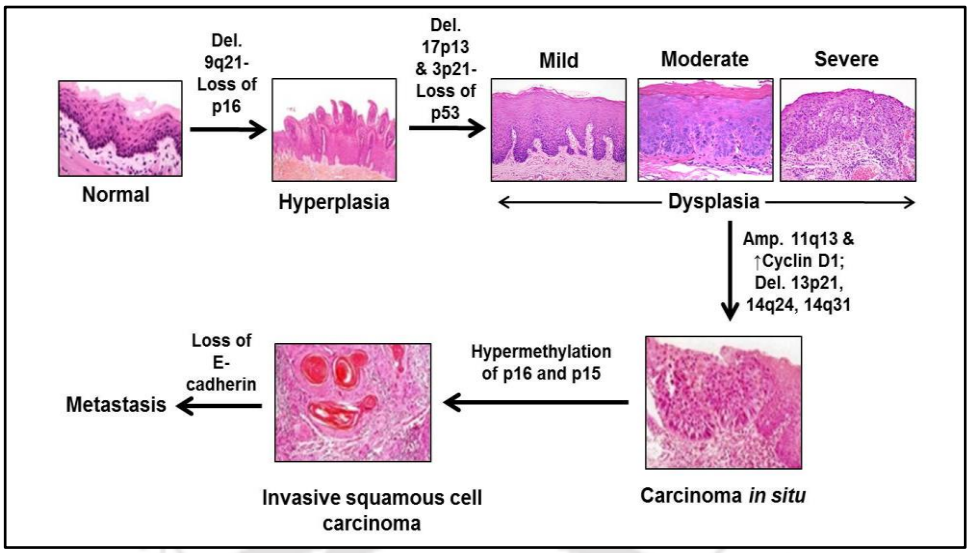


Figure 1.1: Genetic and molecular alterations associated with the development of OSCC (*Adapted from*: Pérez-sayáns et al., 2009, Tanaka and Ishigamori, 2011).

Bcl-2, c-Myc, cyclin B, cyclin D, EGFR, Ras genes, int-2, hst-1 and PRAD-1 were also found to be associated with the development of oral cancer (Moles et al., 2008, Tsantoulis et al., 2007, Kushner et al., 1999). Further, let-7b downregulation mediated DICER overexpression was shown to increase cell proliferation in oral cancer cells (Jakymiw et al., 2010). Additionally, deregulation of different angiogenesis related molecules and growth factors such as VEGF, VEGF-R, PGF-3, PD-ECGF, NOS2, FGF-2, HIF1α and Cox-2 was shown to induce the progression and aggressiveness of the disease (Tsantoulis et al., 2007, Schliephake, 2003, Wakulich et al., 2002, Sudbø, 2004). In addition, matrix metalloproteinases (MMP)- 1, 2, 3, 9, 10 and 13 were also shown to play essential role in tumor invasion and metastasis of OSCC (Thomas et al., 1999). Altogether, like any other malignancies, development of oral cancer is also a result of cumulative effect of numerous molecular alterations with novel targets added to the list every time.

1.2.4 Staging and grading of oral cancer:

TNM staging:

The TNM system of classification of malignant tumors was first described by Pierre Denoix and was later adapted by the International Union against Cancer. TNM staging system is mainly based on the assessment of three main components: (i) T, surface diameter of the primary tumor, (ii) N, presence or absence and extent of regional/cervical lymph node metastasis, and (iii) M, presence or absence of distant metastasis (Mupparapu and Shanti, 2018). Table 1.1 summarizes the TNM staging of oral cancer.

Table 1.1: TNM staging (Mupparapu and Shanti, 2018)

T (Primary tumor)	N (Lymph node metastasis)	M (Distant metastasis)
TX- cannot be assessed	**Nx**- cannot be assessed	**Mx**- cannot be assessed
T0- No evidence of primary tumor	**N0**- No regional lymph node metastasis	**M0**- No distant metastasis
Tis- Carcinoma *in situ*	**N1**- Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension	**M1**- Distant metastasis
T1- Tumor ≤2 cm in greatest dimension	**N2**- Metastasis in a single ipsilateral lymph node, 3-6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, <6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, <6 cm in greatest dimension	-
T2- Tumor 2-4 cm in greatest dimension	**N2a**- Metastasis in a single ipsilateral lymph node, 3-6 cm in greatest dimension	-
T3- Tumor >4 cm in greatest dimension	**N2b**- Metastasis in multiple ipsilateral lymph nodes, none >6 cm in greatest dimension	-

T4a- Lip Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face (i.e., chin or nose) Oral Tumor invades through cortical bone, into deep [extrinsic] Cavity Muscle of tongue, maxillary sinus, or skin of face	**N2c-** Metastasis in bilateral or contralateral lymph nodes, none >6 cm in greatest dimension	-
T4b- Tumor involves masticator space, pterygoid plates, or skull base and/or encases internal carotid artery	**N3-** Metastasis in a lymph >6 cm in greatest dimension	-

Histopathologic grading:

In addition to TNM staging, the head and neck tumors are also categorized into different pathological grades based on the differentiation status. Table 1.2 describes the 'G' grading system of head and neck malignancies except thyroid (Mupparapu and Shanti, 2018).

Table 1.2: Histopathologic grading (Mupparapu and Shanti, 2018)

Grades	**Differentiation status**
Gx	Grade of differentiation cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Staging:

Based on the TNM assessment, the head and neck tumors are further grouped into different stages. Table 1.3 represents the stage grouping of all head and neck tumors except nasopharyngeal and thyroid tumors (Mupparapu and Shanti, 2018).

Table 1.3: Stage grouping (Mupparapu and Shanti, 2018)

Stage	T status	N status	M status
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T4b	Any N	M0
	Any T	N3	M0
IVC	Any T	Any N	M1

R classification:

Further, the oral cancers have also been classified based on presence of tumor post-treatment i.e. residual tumor (R) which is denoted as R classification. Table 1.4 describes the R classification of all head and neck tumors (Mupparapu and Shanti, 2018).

Table 1.4: R classification of all head and neck tumors (Mupparapu and Shanti, 2018)

	Residual tumor post treatment (R)
Rx	Presence of residual tumor cannot be assessed
R0	No residual tumor
R1	Microscopic residual tumor
R2	Macroscopic residual tumor

1.2.5 Risk factors:

It is well known that the main causative factor for oral cancer is consumption of tobacco and tobacco related components, alcohol, betel leaf/nut etc. (Gupta et al.,

2013, Mignogna et al., 2004, Goel et al., 2014). Besides tobacco, age, gender, viral infections like human papilloma virus (HPV), air pollution and immune deficiency syndromes are also believed to be the potential risk factors for oral cancer (Oral cancer foundation) (Figure 1.2).

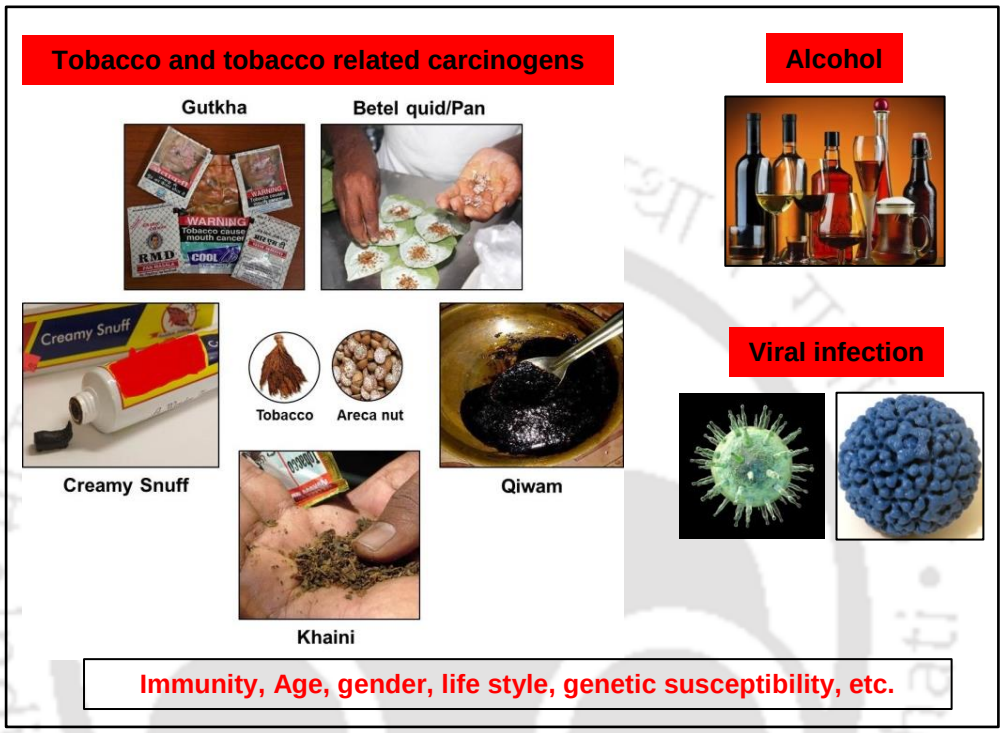


Figure 1.2: Risk factors associated with oral cancer

Tobacco: In India alone more than 275 million people are reported to consume tobacco out of which around 164 million consume smokeless tobacco, 69 million are smokers, and 42 million consume both smoke and smokeless tobacco thus directly exposing to high risk of developing oral cancers (Barik et al., 2016). Tobacco is consumed in various forms like chewing tobacco, oral use of snuff, smoking of cigars, cigarettes, bidis, pipes etc. in different parts of India (Radhakrishnan et al., 2012). In addition, it has been identified that tobacco and tobacco smoke contains more than 8,000 chemicals including 70 potent carcinogenic chemicals that can induce the development of more than 14 types of cancers by altering several cellular signaling cascades such as PI3K/Akt, STAT3, MEK/ERK, NF-κB etc. and inducing mutations

in tumor suppressor genes (Hecht and Szabo, 2014, Hecht, 2003, Xue et al., 2014). Benzo[a]pyrene (BaP) and tobacco specific N'-nitrosamines (TSNAs) such as N-Nitrosonornicotine (NNN) and 4 [methylnitrosoamino]-1-[3-pyridyl]-1-butanone (NNK) etc. are some of the carcinogens identified and isolated from tobacco. These are known to induce debilitating mutations in the genetic material including formation of DNA adducts and free radical formation which give raise to cancers of oral cavities (Johnson, 2001, Bartsch et al., 1999). Moreover, intake of tobacco in any form, conjunction with or without alcohol was also found to be generally correlated with leukoplakia and erythroplakia, the initial stages of oral cancer which possess high risk of developing into a malignant disease (Goel et al., 2014).

Alcohol: In case of non-smokers, alcohol was shown to be a main risk factor for oral cancer (Radhakrishnan et al., 2012). Consumption of alcohol damages the oral mucosa, by removing lipids and increasing the membrane permeability to carcinogenic compounds. Further, alcohol consumption is also known to damage the liver, thus impeding the detoxification of different mutagens and increasing the oncogenic effect of carcinogens particularly in oral mucosa (Radhakrishnan et al., 2012). In addition, alcohol was shown to act synergistically with the tobacco components in increasing the risk of OSCC by acting as a solvent for tobacco carcinogens. Moreover, generation of free radicals and acetaldehyde, an immediate metabolite of ethanol, were also known to contribute to alcohol mediated oral carcinogenesis through DNA damage (Radhakrishnan et al., 2012, Feller et al., 2013).

Viruses: In addition to the chemical carcinogens, viral infection is also known to cause oral cancer. Both DNA and RNA viruses have been shown to possess tumorigenic properties. Although, many viruses like Epstein- Barr virus (EBV), Herpes Simplex viruses (HSV), Retroviruses and human papillomavirus (HPV) have

been implicated in the development of oral cancer, only HPV is regarded as the major risk factor as around 42% oral cancer patients were found to be positive for HPV infection (Radhakrishnan et al., 2012). HPV is a DNA virus that affects the epithelial cells of mucosa. Several reports have shown a strong correlation of HPV infection with OSCC. For instance, Jalouli et al., 2010 identified around 24% of OSCC patients samples to have HPV DNA and 16% to have HPV 16 and 18 (Jalouli et al., 2010, Chen and Zhao, 2017). Similarly, in another study sampling of 118 OSCC samples showed that 43% of them contained HPV DNA and 35% contained HPV 16 and 18 DNAs (Ostwald et al., 2003, Chen and Zhao, 2017). HPV viral genome consists of early (E) and late (L) genes that encode E1-E7 proteins and L1 and L2 capsid proteins respectively. Among the early proteins, E6 and E7 were known to have malignant potential. For example, E6 protein was shown to bind with E6-associated ubiquitin ligase and induce p53 degradation. E7 binds to retinoblastoma protein (pRb) and inhibit its association with E2F. However, there are no clear evidences that explain the mechanism of HPV mediated oral carcinogenesis. Nevertheless, presence of HPV DNAs is regarded as a potential risk factor for the development of OSCC (Chen and Zhao, 2017).

Other risk factors: Apart from the above mentioned causes, many other factors such as immunosuppression by chemotherapeutic drugs, graft-versus-host disease (GVHD) during stem- cell transplantation, and radiation are also known to induce oral carcinogenesis (Cancer treatment centers of America). In addition, poor oral hygiene, faulty restorations, sharp teeth and ill-fitting dentures may also play a key role in etiopathogenesis of oral cancer (Radhakrishnan et al., 2012).

1.2.6 Therapeutic strategies:

The most commonly used treatment strategies for OSCC are surgery, radiotherapy and chemotherapy or combination of these. Selection of treatment strategy relies on the severity of the disease, age and health of the patients (Das and Nagpal, 2002). Detection in early stages can give better treatment options and better prognosis. However, in majority of the cases, it is diagnosed only in the advanced stages which narrow down the chances of recovery (Mignogna et al., 2004).

Surgery: Surgery is the prime and preferred treatment modality for small accessible tumors (Stage I and Stage II OSCCs) in which the tumor along with a thin lining of tumor adjacent normal tissues is removed. Excision of a part or all of the jaw, lymph nodes and other tissue in the neck, maxillectomy, tracheotomy to help in breathing for patients with large tumors or after surgical removal of the tumor, dental surgery to remove teeth or assist with reconstruction are the common surgical methods in practise. However, for the cancers that have invaded the bone, tumor should be excised along with the segment of the bone (Alvi et al., 1996).

Adjuvant therapy: As the surgical removal of advanced tumors often associated relapse, adjuvant therapy is used where the primary site and neck with positive nodes are treated with a total dose of 60 Gy radiation within 6 weeks after surgery to avoid any relapse or metastasis. With recent development of novel therapeutic drugs, chemotherapy is also used before and after surgery or in combination with radiation (chemoradiotherapy) which has emerged as a standard method of treating oral cancers (Omura, 2014).

Radiation therapy: Radiation therapy is also usually recommended for small tumors or in the initial stages of tumor. In radiation therapy high energy rays like X-rays or γ-rays are used to kill or shrink the size of tumors. Fractionation of radiation should be

used to prevent the surrounding normal cells from damage. Two types of fractionations can be done: (i) hyper-fractionation in which small doses of radiation are given at different time points in a single day; (ii) in accelerated fractionation, rate of weekly dose accumulation that is 20 to 50 percent faster than that of standard fractionation is set. This will reduce the treatment time duration and tumor recurrence that most likely to happen between fractions (Budach et al., 2006, Awwad et al., 2002). National Comprehensive Cancer Network suggested that 66-74 Gy is the best sited dose for the treatment of OSCC. Radiation therapy can also be combined with chemotherapy for better prognosis (David et al., 2011, Kerawala et al., 2016). Also, it is used as adjuvant therapy after the surgical removal of tumors in order to prevent recurrence (Omura, 2014).

Chemotherapy: Several decades of cancer research developed numerous natural and synthetic chemotherapeutic drugs which are most often used before surgery or in combination with radiation to inhibit tumor growth, progression, and invasion and induce cell death in cancer cells. Cisplatin, carboplatin, ifosfamide, methotrexate, bleomycin, cetuximab, fluorouracil, paclitaxel, and docetaxel etc. are some of such chemotherapeutics commonly used in the management of oral cancer (Fotedar et al., 2013, Vermorken and Specenier, 2010). Aforementioned, chemotherapy can be either neoadjuvant therapy where chemotherapeutic drug is used before any other therapy or integrated chemoradiation where the drug and radiation are given simultaneously (Jain et al., 2008, Kerawala et al., 2016).

However, none of these treatment methods are 100% successful as all these are associated with their own complications. For example, surgery cannot be used for large tumors. In radiotherapy, along with the tumor cells it also affects the neighbouring normal tissues. Chemotherapy is also associated with adverse side

effects such as nephrotoxicity, fatigue, vomiting, diarrhea, bone marrow suppression, myelosuppression, hepatic dysfunction, ischemic heart disease, hypertension, malaise etc. (Liu et al., 2014a, Tahover et al., 2015, Sindhu and Bauman, 2019). Besides the side effects, in most cases, cancer cells develop resistance against the treatment and tumor recurrence also pose a great difficulty in managing this aggressive disease (Wang et al., 2012a). The complications in the management of oral cancer are mainly attributable to diagnosis at the advanced stage of the disease and lack of proper therapeutic targets. Therefore, it is mandatory to discover novel therapeutic, diagnostic and prognostic molecular targets for the better management of OSCC.

1.3 TNFAIP8 Family

Tumor necrosis factor (TNF)-α- induced protein 8 (TNFAIP8) or TIPE family of proteins comprises of four proteins namely TNFAIP8 (TIPE), TNFAIP8L1 (TIPE1), TNFAIP8L2 (TIPE2), and TNFAIP8L3 (TIPE3) (Padmavathi et al., 2018; Sun et al., 2008, Freundt et al., 2008, Lou and Liu, 2011). All four proteins of this family were found to have a remarkable structural homology among themselves and share no significant sequence or structural homology with any other protein families except a minor similarity in their death effector domain (DED) thus the TIPE family is regarded as a novel subfamily of DED containing proteins (Padmavathi et al., 2018; Kumar et al., 2000, You et al., 2001, Kumar et al., 2004, Freundt et al., 2008, Lou and Liu, 2011, Fayngerts et al., 2014). Further, the TIPE proteins were found to have a highly conserved TIPE2 homology (TH) domain with seven α helices making a hydrophobic cavity. Interestingly, the TIPE3 protein was found to be a lipid carrier that interacts and transports lipid secondary messengers through this hydrophobic cavity (Fayngerts et al., 2014). In spite of the structural homology, TIPE proteins were found to differentially regulate various cellular processes. For example, TIPE

was shown to regulate cell growth and apoptosis and promote tumor metastasis; TIPE1 was found to regulate autophagy; TIPE2 was shown to regulate immune homeostasis; and TIPE3 was found to be a transporter of lipid secondary messengers (Padmavathi et al., 2018, Lou and Liu, 2011, Fayngerts et al., 2014, Sun et al., 2008, Zhang et al., 2012a). With their immune and death regulatory activities the TIPE proteins were found to modulate the expression of various signalling molecules and to play crucial roles in the tumorigenesis of different cancers (Padmavathi et al., 2018).

1.3.1 TNFAIP8 (TIPE):

Tumor necrosis factor (TNF)-α- induced protein 8 is the first discovered member of TIPE family which is also called as SCC-S2, GG2-1, NF-κB-inducible DED-containing protein (NDED), MDC-3.13 and TIPE (Patel et al., 1997, Kumar et al., 2000, Lou and Liu, 2011). Patel et al. (1997) first identified TIPE as a differentially expressed transcript in primary and matched metastatic cell lines derived from metastatic radioresistant head and neck squamous cell carcinoma patient and named it as SCC-S2 (Patel et al., 1997). It is a 23 kDa cytosolic protein containing 198 amino acids encoded by *TNFAIP8* gene positioned at 5q23.1 chromosomal region (Gene ID: 25816, UniProtKB - O95379 (TFIP8_HUMAN)). Further, TIPE was found to have a small DED homologous to the DED II of Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (FLIP) at the amino terminus which contributes to the protein's cell death regulatory function (Kumar et al., 2000, Kumar et al., 2004, Padmavathi et al., 2018). BLAST search of this DED domain showed sequence similarities: mouse CASH α/β, 58%; mouse FLIP (L), 53%; human CASH α/β, 50%; and human FLIP(L), 58% (Kumar et al., 2000). Expression of TIPE was found in both normal as well as tumor cells, levels of which can be induced by TNF-α mediated activation of NF-κB (Padmavathi et al., 2018, Kumar et al., 2000). In

general, a moderate to low level of TIPE expression was found in the tissues of adrenal glands, heart, lung, ovary, skeletal muscle, spinal cord and testis. In contrast, expression of TIPE was observed to be relatively high in the tissues of bone marrow, lymph node, placenta, spleen, thymus and thyroid. Similarly, increased TIPE expression was also found in foetal liver, lung, and kidney tissues. In addition, TIPE expression was also detected in both cytokine-activated and unstimulated endothelial cells. However, its expression could not be detected in brain tissues (Kumar et al., 2000, Horrevoets et al., 1999).

Functions of TIPE:

TIPE is considered as a negative regulator of apoptosis as it inhibited the TNF-α induced cell death in HeLa cells (Kumar et al., 2000; Luan et al., 2013). Further, the anti-apoptotic activity of TIPE was found to be regulated by NF-κB. Surprisingly, overexpression of TIPE was shown to supress TNF-mediated apoptosis in NF-κB null cells as well. In addition, IκBα, a natural inhibitor of NF-κB was also found to inhibit the expression of TIPE. Taken together, TIPE is a NF-κB-inducible anti-apoptotic molecule which may possess a potential role in NF-κB-related tumors (You et al., 2001). Additionally, it was also reported that interaction of TIPE with Gαi, an inhibitory G-protein, is essential for the oncogenic transformation of Gi/Go-coupled D2S receptor (dopamine D2 receptor short form) transfected mesenchymal Balb/c-3T3 fibroblast cells as the constitutive activation of TIPE and Gαi resulted in the initiation of D2S receptor signaling which in turn inhibited TNF-α-induced cell death (Laliberté et al., 2010). Furthermore, TIPE was found to exert its anti-apoptotic activity through the inhibition of caspase-8 and caspase-3 activities. However, it failed to inhibit the etoposide-mediated apoptosis of fibrosarcoma cells as it was caspase-8 independent mechanism thus further assuring the importance of caspases in

the anti-apoptotic activity of TIPE (You et al., 2001, Kumar et al., 2000, Kumar et al., 2004, Zhang et al., 2006). Interestingly, TIPE was also found to exhibit pro-apoptotic activity in glucocorticoid treated thymocytes signifying the fact that effect of TIPE on the regulation of apoptosis may depend on the stimulator (Woodward et al., 2010). Additionally, TIPE expression was also found to contribute to the genetic susceptibility of A/J mice to *Staphylococcus aureus* sepsis (Ahn et al., 2010). Further, TIPE was found to play essential role in immune regulation, suppression of which resulted in elevated production of inflammatory cytokines, leukocyte infiltration, and bacterial invasion leading to colitis in dextran sodium sulfate (DSF)-induced mice. Also, suppression of TIPE reduced proliferation and increased cell death of intestinal epithelial cells (Sun et al., 2015). In support to the immune regulatory function of TIPE, Xin et al. (2016) showed that knockdown of TIPE reduced the proliferation of CD4+ T lymphocyte, and the levels of IL-2 and soluble IL-2R in thermal injured mice model (Xin et al., 2016). In addition to all, TIPE was also found to play essential role in the regulation of various processes of tumorigenesis such as cell survival, cell cycle progression, invasion, metastasis and angiogenesis (Padmavathi et al., 2018).

TIPE in cancer:

Aforementioned, TIPE was proved to regulate various hallmarks of cancer endorsing the importance of this protein in cancer development and progression (Figure 1.3A). In support to this, overexpression of TIPE was observed in different cancers like breast cancer, chronic myelogenous leukemia, colon cancer, hepatocellular carcinoma (HCC), lymphoblastic leukemia, lung carcinoma, renal cell carcinoma and thyroid carcinoma etc. (Kumar et al., 2000, Kumar et al., 2004, Dong et al., 2010, Dong et al., 2017, Miao et al., 2012, Duan et al., 2014). Further, overexpression of TIPE was found to be associated with increased invasion and metastasis of breast cancer through

the regulation of vascular endothelial growth factor receptor-2 (VEGFR-2), MMP-1, and MMP-9 (Kumar et al., 2004, Zhang et al., 2006). In line with this, overexpression of TIPE was also shown to be positively associated with advanced TNM stage, axillary lymph node metastasis, postoperative recurrence and poor survival of invasive ductal breast carcinoma (IDC) patients suggesting the prognostic value of TIPE in IDC (Xiao et al., 2017). Similarly, increased levels of TIPE was correlated with increased proliferation and lymph node metastasis of colon cancer as well, downregulation of which significantly reduced proliferation and colony forming ability of Caco-2 and HCT116 colon cancer cells by downregulating cyclin D1 and pRb (Miao et al., 2012). In addition to breast and colon cancers, TIPE overexpression was also correlated with lymph node metastasis and poor survival of lung cancer and silencing of the same resulted in reduced proliferation, migration and invasion of A549 and H1299 lung cancer cells (Dong et al., 2010). Further, the TIPE associated increase in proliferation and invasion of lung cancer cells was found to be mediated through deregulation of Hippo pathway as TIPE inhibited the negative regulator of Yes associated protein 1 (YAP1) i.e. large tumor suppressor kinase 1 (LATS1) (Han et al., 2018). Likewise, TIPE overexpression was found to modulate the Hippo pathway by inhibiting LATS1 and YAP phosphorylation in HCC as well which resulted in increased tumor growth, cell proliferation, invasion and migration of HCC cells. Also, the increased TIPE level in HCC was correlated with increase in TNM stage, tumor recurrence, and poor prognosis (Dong et al., 2017). In addition, the pancreatic patients with increased TIPE levels had increased EGFR levels, disease progression and lymph node metastasis suggesting the importance of TIPE in pancreatic cancer as well (Liu et al., 2012). Furthermore, TIPE was also found to be upregulated in endometrial cancer (EC), gastric cancer, intestinal-type gastric

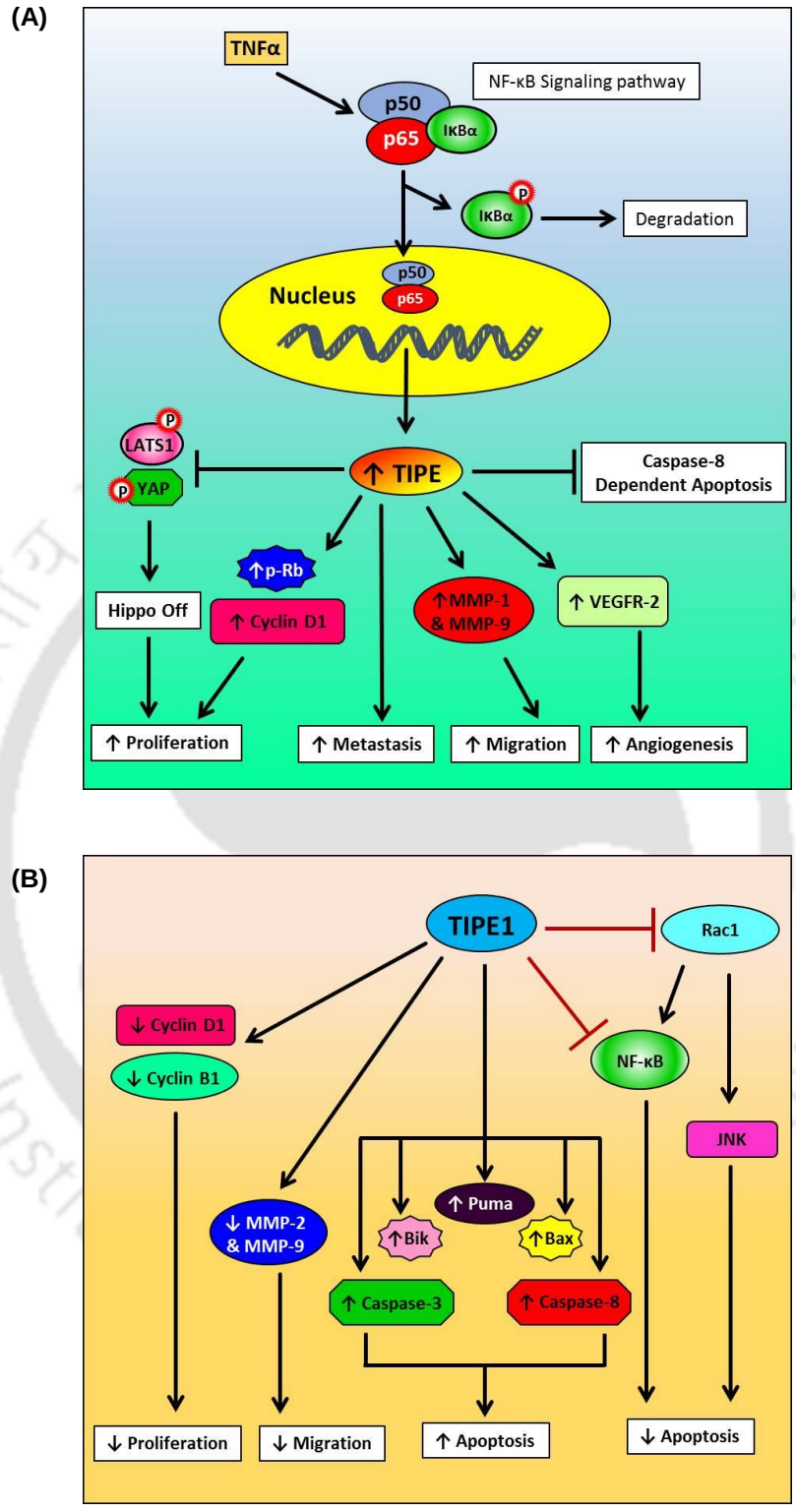


Figure 1.3: (A) TIPE induces tumorigenesis through deregulation of multiple cellular events and signaling cascades; (B) TIPE1 negatively regulates the genes involved in cell survival, proliferation, and migration, and positively regulates the genes involved in apoptosis. ↑- upregulation; ↓- downregulation. (*Adapted from*: Padmavathi et al., 2018)

adenocarcinoma (IGA), platinum-resistant epithelial ovarian cancer (EOC) and esophageal squamous cell carcinoma (ESCC) and associated with lymph node metastasis, tumor recurrence, poor survival and prognosis (Liu et al., 2013, 2014b, 2014c, Li et al., 2015a, Yang et al., 2014, Chen et al., 2016, Hu et al., 2016, Hadisaputri et al., 2012). Adding to this, silencing of TIPE also resulted in the decreased cell growth, proliferation, invasion and migration in both gastric cancer and ESCC *in vitro* (Li et al., 2015a, Hadisaputri et al., 2012, Sun et al., 2016). Moreover, it was also reported that increased nuclear levels of TIPE is associated with prostate cancer recurrence (Zhang et al., 2013a). Further, studies on osteosarcoma revealed overexpression of TIPE to be a contributing factor for the development and progression of osteosarcoma and miR-99a and miR-138 to be the negative regulators of TIPE in osteosarcoma (Xing and Ren, 2016, Zhou et al., 2017). Besides the elevated expression, single nucleotide polymorphism (SNP) in the TIPE gene was also found to be associated with cancer development. For instance, one such SNP 1045241C>T was observed in significant number of Non-Hodgkin's lymphoma (NHL) patients marking it as a susceptibility factor for NHL development (Zhang et al., 2012a). Another SNP TIPE-rs11064 was identified in cervical cancer patients, which was found responsible for platinum resistance, and poor survival (Shi et al., 2013).

TIPE in other pathological conditions:

Apart from cancer, TIPE proteins were also found to be involved in other chronic diseases such as diabetic nephropathy (DN). All the four proteins of the TIPE family were found to be differentially expressed in kidney and renal cells. However, not all the four but only TIPE and TIPE2 were observed to be overexpressed in the glomeruli of streptozotocin (STZ)-induced diabetic rats and in renal biopsies of DN patients

(Bordoloi et al., 2018). Furthermore, expression of TIPE was upregulated in response to high glucose or TNF-α in mesangial cells and there was a strong association between high glucose induced TIPE overexpression and mesangial cell proliferation. Also, NADPH oxidase activity was shown to be a mediator of this high glucose or TNF-α induced overexpression of TIPE. All these findings indicated that TIPE is an important molecule in the signal transduction that correlates mesangial cell proliferation with diabetic renal injury thus inhibition of TIPE could be a possible therapeutic strategy for the treatment of DN (Zhang et al., 2010a). Further, TIPE was also proved to regulate the pathogenesis of listeriosis by modulating pathogen invasion and host cell apoptosis during *Listeria monocytogenes* infection. Silencing of TIPE increased the host cell resistance towards the lethal *L. monocytogenes* infection and reduced the pathogen invasion and bacterial load in the spleen and liver of the mice in a Rac1 GTPase-dependent manner (Porturas et al., 2015). It was also found to facilitate the insulin-mediated inhibition of autophagy in hepatocytes during conditions like obesity and diabetes by forming a ternary complex with phosphatidylethanolamine (PE) and Gαi3 (Kim et al., 2017). Further, TIPE was also shown to be involved in the pathogenesis of cecal ligation and puncture (CLP) induced immune dysfunction in splenic T lymphocytes (Yu et al., 2018). Besides, a very recent report identified an indel (chr5:118704153:D) mutation in TNFAIP8 locus to be present in the patients with plantar fascial disorders suggesting its role in the disease susceptibility (Kim et al., 2018). Also, as mentioned earlier, loss of TIPE increased the susceptibility to colitis in dextran sodium sulfate (DSF)-induced mice model (Sun et al., 2015).

1.3.2 TNFAIP8L1 (TIPE1):

The next identified member of the TIPE family is tumor necrosis factor-α-induced protein 8-like 1 (TNFAIP8L1/TIPE1/Oxi-β). It is also a cytosolic protein, first discovered in 2008 by Sun et al. (Sun et al., 2008, Luan et al., 2013, Ha et al., 2014). Unlike TIPE, it has been shown to have anti-survival activity (Hitomi et al., 2008, Galluzzi et al., 2008). Further, expression of TIPE1 was detected in various murine tissues and human cell lines including germ cells, hepatocytes, muscular tissues, neurons, secretary cells of the epithelial origin, human B lymphoblast and murine T cells. However, mature B and T lymphocytes were found to lack TIPE1 expression (Cui et al., 2008).

Functions of TIPE1:

TIPE1 is suggested to have an anti-survival and pro-apoptotic function as downregulation of TIPE1 resulted in the inhibition of TNF-α and Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) a pan-caspase inhibitor mediated necroptosis of murine fibroblast cells (Hitomi et al., 2008, Galluzzi et al., 2008, Lou and Liu, 2011). Similarly, TIPE1 was also found to positively regulate autophagy through modulation of mTOR signaling. For instance, oxidative stress generated in a 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease model was shown to increase the levels of TIPE1 which binds to FBXW5 and stabilizes the tuberous sclerosis complex 2 (TSC2), a negative regulator of mTOR thus ultimately resulting in increased autophagy of dopaminergic neurons (Ha et al., 2014).

TIPE1 in cancer:

Unlike TIPE, the role of TIPE1 in cancer is largely unknown. However, an elevated mRNA levels were observed in cervical carcinoma, tongue carcinoma, ovarian carcinoma and melanoma cells suggesting a possible involvement in cancer

development (Cui et al., 2008). In contrast to this, a decreased TIPE1 protein expression was observed in HCC tissue samples compared to the adjacent normal tissues indicating the anti-cancer effect of TIPE1. Further supporting this fact, TIPE1 expression was found to remarkably reduce the liver tumor growth *in vivo*. And adding to its pro-apoptotic effect, upregulation of TIPE1 was found to be associated with decreased cell growth and increased cell death of HCC cells *in vitro*. Further, the TIPE1 mediated apoptosis of HCC cells was identified to be caspase-independent and achieved through interactive inhibition of Rac1 and its targets such as c-Jun N-terminal kinase (JNK) and NF-κB pathways (Zhang et al., 2015a). In addition, Wang et al. (2016) also further confirmed the direct involvement of TIPE1 in apoptosis induction of RAW264.7, a murine macrophage cell line in which they have shown increased levels of TIPE1 to significantly increase apoptosis and cisplatin-induced cell death of RAW264.7 cells *in vitro* and suppress tumor growth *in vivo* through upregulation of Bax, Bik, and Puma (Wang et al., 2016a). Further supporting the antitumorigenic effect, reduced levels of TIPE1 was found in lung cancer as well, recovery of which resulted in the activation of pro-apoptotic genes caspase-3 and -8 and downregulation of cyclin B1, cyclin D1, MMP-2, and -9 leading to increased apoptosis and decreased proliferation, invasion and tumor growth (Wu et al., 2017a). All these findings suggested TIPE1 to be a pro-apoptotic and antitumor protein (Figure 1.3B).

1.3.3 TNFAIP8L2 (TIPE2):

Tumor necrosis factor-α-induced protein 8-like 2 (TNFAIP8L2/TIPE2), the third member of TIPE family, is a cytosolic protein with 53% sequence similarity to human TNFAIP8/TIPE and encoded by *TNFAIP8L2* gene (Sun et al., 2008, Freundt et al., 2008, Zhang et al., 2010b, Padmavathi et al., 2018). TIPE2 was initially identified in

experimental autoimmune encephalomyelitis (EAE) where the expression was several fold increased in the inflamed spinal cord and completely absent in normal spinal cord suggesting a strong correlation with inflammation. In line with this, it was found to be expressed in lymphoid and myeloid tissues, macrophages, B and T lymphocytes and induced by the cytokine TNF-α in other tissues (Sun et al., 2008, Lou and Liu, 2011). Further, it was also found to be expressed in hepatocytes, glandular epithelial cells of the stomach, colon, and appendix, germ cells, squamous epithelial cells of esophagus and cervix, transitional epithelial cells of bladder and ureter, and neurons of the brain and brainstem. Moreover, expression of TIPE2 is expected to be tissue specific (Lou and Liu, 2011, Zhang et al., 2010b, 2011, Li et al., 2018, Carmody et al., 2002).

Structural features of TIPE2:

Zhang et al., (2009) solved the crystal structure of human TIPE2 protein and discovered some novel interesting facts about this protein. First of all, TIPE2 was found to be made of a helix bundle comprising six antiparallel α-helices and the α5 was split into two parts α5a (long) and α5b (short) by a small kink created by Pro153. This α5b formed the base of the bundle. Interestingly, the crystal structure of TIPE2 revealed that it does not share any structural similarity with DED or any other functional proteins suggesting a novel uncharacterized fold. However, a detailed analysis of the topology diagram of TIPE2 showed that TIPE2 topology is the mirror image of DED topology. Another interesting finding is the central highly hydrophobic cylindrical cavity. Moreover, the hydrophobic residues of this cavity were found to be highly conserved among the other members of TIPE family across species suggesting an important cofactor binding possibility and this domain is called as TIPE2 homology (TH) domain (Zhang et al., 2009, Fayngerts et al., 2014).

Functions of TIPE2:

TIPE2 has been proved to play crucial roles in the regulation of inflammation and immune homeostasis. For instance, TIPE2 was shown to negatively impact the Toll-like receptor (TLR) and T cell receptor (TCR) signaling pathways (Sun et al., 2008, Freundt et al., 2008). Further exemplifying its significance in the maintenance of immune response, knockout of TIPE2 was found to be associated with fatal inflammatory diseases and systemic autoimmunity in mice and human respectively (Zhang et al., 2010b). Also, TIPE2 knockout mice were found to develop multi-organ inflammation, splenomegaly and premature death. In addition, loss of TIPE2 increased the CD4+ and CD8+ T cell immune responses resulting in hypersensitivity reactions, and auto-inflammatory diseases. Further, the loss of TIPE2- mediated inflammatory diseases were associated with accumulation of inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, and TNF-α in the serum (Sun et al., 2008, Freundt et al., 2008, Lou and Liu, 2011, Padmavathi et al., 2018). Likewise, overexpression of TIPE2 was found to inhibit the activation of T lymphocytes and induction of CD4+CD25+ Treg-mediated immune suppressive functions (Sun et al., 2008, Luan et al., 2011). Furthermore, TIPE2 was proved to contribute to the leukocyte chemotaxis thus critically regulating the process of inflammation (Fayngerts et al., 2017). In addition to immune homeostasis, TIPE2 was also suggested to regulate cell division (Sun et al., 2008). Further, it was also found to have a pro-apoptotic effect as it interacted with caspase-8 and stimulated Fas-induced apoptosis of immune cells through inhibition of AP-1 and NF-κB (Sun et al., 2008). Moreover, TIPE2 was shown to maintain the balance between cell survival and cell death through inhibition of Ral and Akt activation (Gus-Brautbar et al., 2012). In addition to the NF-κB and Akt pathways, TIPE2 was also found to negatively regulate JNK and p38 MAPK pathways (Sun et al., 2008, Lou and Liu, 2011). Interestingly,

microRNA-21, a direct target of NF-κB is considered to regulate the expression of TIPE2 in a coding region-dependent manner. For example, in activated T cells and macrophages, miR-21 was highly expressed, whereas TIPE2 was downregulated and in contrary to miR-21 deficient cells, the TIPE2 deficient T cells were found insensitive to apoptosis. Therefore, it is clear that miR-21 inhibits apoptosis of T cells through the regulation of tumor suppressor gene TIPE2 (Ruan et al., 2014). Further, TIPE2 was also proved to prevent stroke and act as a neuroprotectant (Zhang et al., 2012b). Moreover, it was also found to control phagocytosis thus modulating the susceptibility of animals to bacterial infections (Wang et al., 2012b).

TIPE2 in cancer:

Exemplifying the involvement of TIPE2 in tumorigenesis, reports have shown TIPE2 to negatively regulate the activation of Ras oncogene which has been well known to induce several hallmarks of cancer such as cell survival, proliferation, migration, and malignant transformation. TIPE2 was shown to bind with Ras-interacting domain of the RalGDS family of proteins thus preventing the complex formation of Ras and its effector proteins ultimately resulting in the inhibition of Ral and Akt activation. Further assuring its inhibitory effect on Ras signaling, knockdown of TIPE2 reversed the inhibition on Ras pathway leading to the increased activation of Ral and Akt which in turn reduced cell death and increased the migration of 293T cells. Thus, TIPE2 was expected to have an antitumorigenic effect (Padmavathi et al., 2018, Gus-Brautbar et al., 2012). In support to this antitumorigenic activity, downregulation of TIPE2 observed in primary HCC patient samples was associated with increased tumor invasion and metastasis and forced upregulation of the same significantly decreased the invasion and migration of HCC cells through suppression of Rac1, MMP-9, urokinase plasminogen activator (uPA) and F-actin polymerization (Cao et al., 2013).

Further, the TIPE2 upregulation was also shown to inhibit TNF-α-induced metastasis of HCC cells through inhibition of LPS induced TNF-α secretion thus inactivating the TNF-α mediated upregulation of MMP-13/MMP-3, ERK1/2 and NF-κB (Zhang et al., 2015b). Further implicating the importance of TIPE2 in the development of hepatitis C virus (HCV)-mediated HCC, the non-structural protein, NS5A of HCV induced the TIPE2 degradation and led to the development of HCC (Wang et al., 2016b). Likewise, decreased levels of TIPE2 were also observed in glioma and upregulation of the same inhibited the invasion, migration and epithelial to mesenchymal transition (EMT) of glioma cells via modulation of Wnt/β-catenin cascade and downregulating cyclin D1, and c-Myc (Liu et al., 2016). Similarly, in prostate cancer also downregulation of TIPE2 was observed and restoring the same resulted in the suppression of cell proliferation, migration, invasion and EMT. However, the mechanism was different, as in prostate cancer TIPE2 targeted the PI3K/Akt signaling (Lu et al., 2016). In line with prostate cancer, TIPE2 was found to exert its anticancer effect through modulation of PI3K/Akt pathway in gastric cancer as well, where the recovery of lost TIPE2 expression in gastric cancer cells inhibited the Akt signaling thereby activating GSK3β and suppressing β-catenin which resulted in reduced cell migration and invasion. Additionally, it was also found to target the EMT markers such as Snail1, Snail2/Slug, and Zeb1 contributing to the anti-metastatic effect (Wu et al., 2016, Yin et al., 2017). Also, an almost undetectable expression of TIPE2 was witnessed in gastric cancer cells in comparison to the normal gastric mucous epithelial cells (Wu et al., 2016). Further, it was also reported that TIPE2 expression is negatively correlated with the development of gastric cancer from gastritis and p27 is another downstream target of TIPE2 that contributes to its antitumor activities (Zhao et al., 2015, Peng et al., 2016). Moreover, induction of TIPE2 expression in gastric

cancer resulted in reduced tumor growth and increased apoptosis through inhibition of Akt and ERK1/2 pathways and activation of intrinsic apoptotic pathway (Zhu et al., 2016). Furthermore, downregulation of TIPE2 was also detected in non-small cell lung cancers (NSCLC), expression of which was negatively associated with advanced clinical stage and lymph node metastasis. Similar to other cancers, TIPE2 overexpression was associated with reduced invasion, migration and angiogenesis of lung cancer as well. This anti-invasive and anti-angiogenic properties of TIPE2 was mediated through inhibition of Rac1, F-actin polymerization, and VEGF expression (Li et al., 2016, Padmavathi et al., 2018). In addition, recent studies have also reported the involvement of TIPE2 in breast cancer where once again the downregulation was witnessed and induction of TIPE2 overexpression led to decreased proliferation, tumor growth, invasion, migration, EMT and metastasis by modulating the levels of β-catenin, cyclin D1, c-Myc, p-p38 and p-Akt and stimulating immune response in the tumor microenvironment and spleen via increased secretion of IFN-γ and TNF-α (Wang et al., 2017, Zhang et al., 2016, 2017, Padmavathi et al., 2018).

In contrast to the aforementioned cancers, TIPE2 was found to be upregulated in colon cancer, ovarian adenocarcinoma, renal cell carcinoma (RCC), peripheral T lymphoma and diffuse large B-cell lymphoma (DLBCL) (Li et al., 2014, Zhang et al., 2013c, 2010b, Hao et al., 2016). And the expression of TIPE2 was positively correlated with TNM staging and lymph node metastasis of RCC and colon cancers respectively (Zhang et al., 2013c, Li et al., 2014). Additionally, silencing of TIPE2 in colon cancer cells was demonstrated to significantly increase caspase-8 activity suggesting it to act as a negative regulator of caspase-8 in colon cancer (Li et al., 2014). Moreover, skin squamous cell carcinoma patients having increased levels of TIPE2 in tumor-associated macrophages were found to be associated with poor

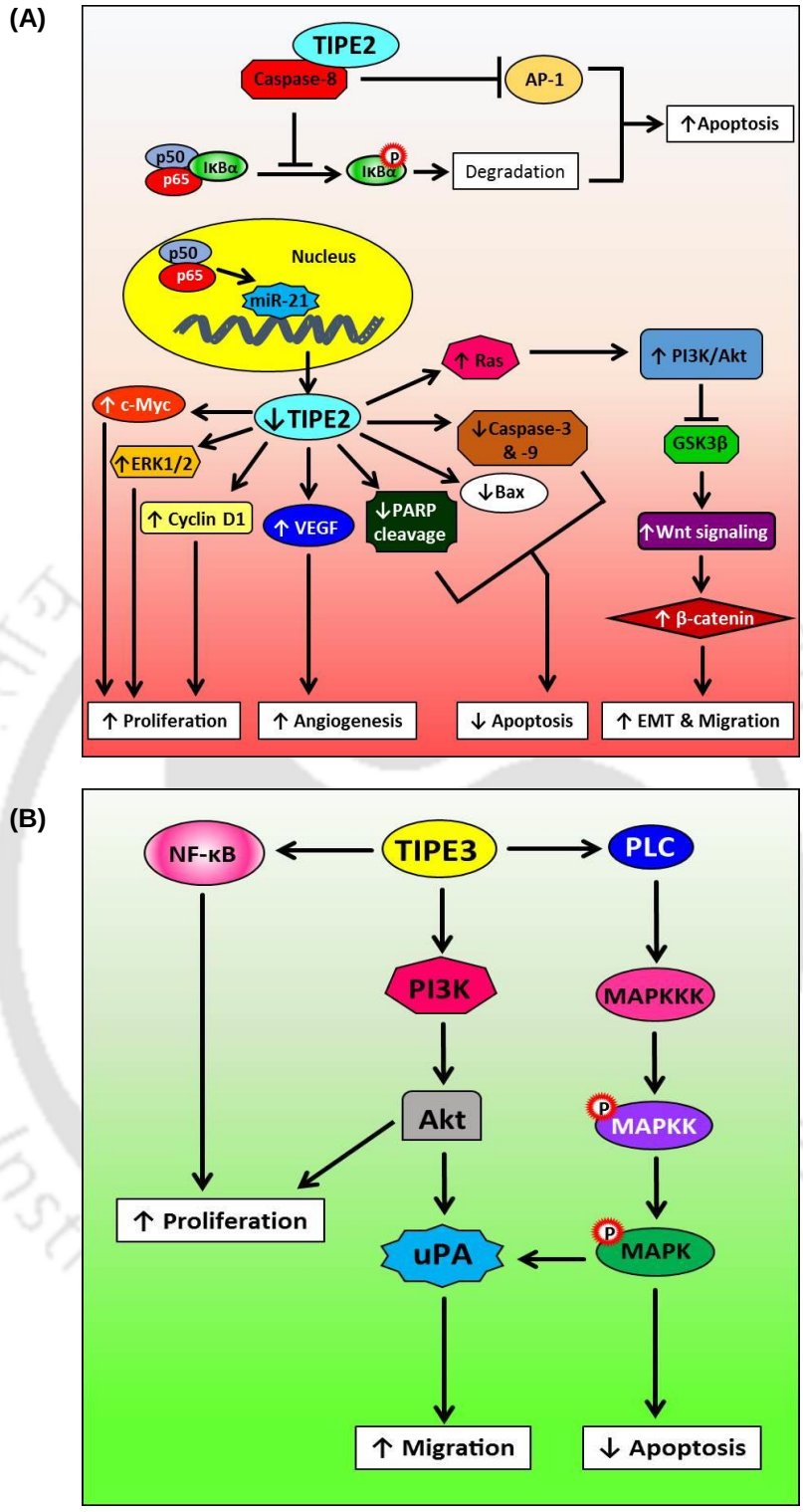


Figure 1.4: (A) Downregulation of TIPE2 leads to the activation of survival and proliferative signals; induction of angiogenesis, invasion, migration, and metastasis; and inhibition of pro-apoptotic signals, thus resulting in tumorigenesis; (B) TIPE3 acts as pro-tumorigenic molecule and induces proliferation and migration of cancer cells, and inhibits apoptotic cell death through the regulation of NF-κB, PI3K/Akt, and MAPK signaling pathways. ↑- upregulation; ↓- downregulation. (*Adapted from*: Padmavathi et al., 2018)

overall 5-year survival (Li, 2016, Padmavathi et al., 2018). Taken together, TIPE2 is known to have antitumor effect in most of the cancers and pro-tumorigenic effect in certain cancers suggesting a tissue specific role of TIPE2 in cancer (Figure 1.4A).

TIPE2 in other pathological conditions:

Similar to TIPE, elevated expression of TIPE2 was also found in glomeruli of streptozotocin (STZ)-induced diabetic rats and in renal biopsies of diabetic patients (Zhang et al., 2010a). Reduced mRNA expression of TIPE2 was observed in PBMC of patients with systemic lupus erythematosus (SLE), a multisystemic auto-immune disorder in which uncontrolled autoreactivity of B and T lymphocytes produces autoantibodies. Thus, it is believed to play a role in the pathogenesis of SLE (Li et al., 2009). Further, TIPE2 expression was observed to be deregulated in Hepatitis B virus (HBV)-induced hepatic inflammation. And reduced TIPE2 levels were associated with increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (Tbil) in blood as well as the HBV load of the patients. Interestingly, TIPE2 null mice were shown to develop more severe hepatitis than the wild type mice. These findings suggested that TIPE2 may hold an important role in HBV-induced hepatic inflammation (Xi et al., 2011, Zhang et al., 2015c). Moreover, elevated expression of TIPE2 was found in patients with acute-on-chronic hepatitis B liver failure (ACHBLF) which augmented the serum total bilirubin level. This upregulation induced disease progression through negative regulation of cell-mediated immunity (Wang et al., 2014a). Further, it is now well established that TIPE2 is a negative regulator of innate immunity downregulation of which induces hyperactivation of PI3K-Rac pathway and enhances Akt, Rac, P21-activated kinase, and IFN regulatory factor 3 activities. Consequently, TIPE2 null myeloid cells exhibited hyper reactivity to Polyinosinic-polycytidylic acid (Poly (I:C)) stimulation

and TIPE2 knockout mice displayed lethal hypersensitivity to Poly (I:C), a dsRNA receptor ligand that triggers Rac via its guanine nucleotide exchange factor Tiam which in turn activates cytokine genes. Therefore, it has been evidenced that TIPE2 regulates innate immunity to RNA by deregulating the PI3K-Rac pathway which implies that targeting TIPE2 can help in controlling the RNA viral infections (Sun et al., 2012). Furthermore, TIPE2 was also found to promote colitis by inhibiting mucosal immunity to commensal bacteria (Lou et al., 2014). Additionally, Jia et al., (2013) studied the relation between kidney allograft rejection and expression of TIPE2. They observed a significant increase in protein levels in the PBMC of chronic rejection group (CR) than that of normal control group suggesting TIPE2 to be a potential biomarker for the clinical monitoring of kidney chronic allograft rejection (Jia et al., 2013). Besides, TIPE2 was also shown to be capable of inhibiting atherosclerosis via inhibition of vascular smooth muscle cells (VSMCs) proliferation which depends on p38 and ERK1/2 kinase signals (Zhang et al., 2013b, Lou et al., 2013). Further, downregulation of TIPE2 was associated with childhood asthma and was correlated with the levels of immunoglobulin E (IgE), eosinophil, IL-4 that are higher in asthma children than the normal controls (Ma et al., 2013). Besides, downregulation of TIPE2 was also implicated in Hepatitis C virus (HCV) induced chronic hepatitis which increased TLR signalling (Kong et al., 2013). All these findings suggest, in addition to cancer, TIPE2 plays essential role in other chronic diseases as well (Bordoloi et al., 2018).

1.3.4 TNFAIP8L3 (TIPE3):

Tumor necrosis factor-α-induced protein 8-like 3 (TNFAIP8L3/TIPE3) is the recently discovered and the least explored member of TIPE family (Lou and Liu, 2011). Similar to other family members, TIPE3 was also found to share a significant

sequence and structural homology among the family counterparts (Lou and Liu, 2011, Luan et al., 2013). Recent reports have suggested that attributable to its unique structure, TIPE3 acts as transporter of lipid secondary messengers which are well known to promote tumorigenesis (Fayngerts et al., 2014). Expression of TIPE3 was detected in most of the tissues including cardiac muscle, pancreatic islets, neurons of central nervous system, epithelial-derived secretory cells, tissues of colon, kidney, lung, prostate, respiratory trachea, small intestine, stomach, testis and uterus but was absent in esophagus and skeletal muscles (Cui et al., 2015, Padmavathi et al., 2018).

Structural features of TIPE3:

Analysis of the crystal structure revealed that, TIPE3 also contains the TH domain with six α-helices (α1-6), similar to that of TIPE2 and an extra N-terminal α-helix (α0). Moreover, TIPE3 was also found to have the large central hydrophobic cavity which was proved to be the binding site of various lipid molecules (Fayngerts et al., 2014, Moniz and Vanhaesebroeck, 2014). In addition, the TIPE3 protein contains a unique N-terminal region (NT) of 19 amino acids which was found indispensable for the pro-tumorigenic activity of TIPE3 as removal of it significantly altered the protein function and exerted completely opposite effects from wild type (Fayngerts et al., 2014, Moniz and Vanhaesebroeck, 2014).

Functions of TIPE3:

Aforementioned, TIPE3 has been found to be a transporter of two major phospholipids namely, phosphatidyl insitol (4,5)-bisphosphate (PtdIns(4,5)P_2, PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3, PIP3) hence regarded as lipid sensor or lipid presenting protein. Supporting this, forced nuclear localization of this protein resulted in elevated levels of PIP2 in the nucleus (Fayngerts et al.,

2014, Moniz and Vanhaesebroeck, 2014). Besides, it was also found to have proliferative, anti-apoptotic and pro-tumorigenic functions (Padmavathi et al., 2018).

TIPE3 in cancer:

It is already well known that the phosphoinositide signals are significantly upregulated in various cancers and promote inflammation and tumorigenesis (Fayngerts et al., 2014, Moniz and Vanhaesebroeck, 2014, Padmavathi et al., 2018). Therefore, being a phospholipid carrier, TIPE3 is also considered to play essential role in the regulation of inflammation and cancer development. In line with this, TIPE3 was found to be overexpressed in cancers of cervix, colon, lung and esophagus (Fayngerts et al., 2014, Moniz and Vanhaesebroeck, 2014, Padmavathi et al., 2018). Further supporting its tumorigenic effect, overexpression of TIPE3 was shown to positively regulate the Akt and MAPK pathways and to accelerate tumor growth *in vivo* compared to that of TIPE3 null mice. Further, the highly conserved TH fold was found crucial for phospholipid binding as any alteration in this region completely abrogated the lipid binding capacity and Akt activation suggesting the importance of structural integrity of TIPE3 in maintaining its function in tumorigenesis (Fayngerts et al., 2014, Moniz and Vanhaesebroeck, 2014, Padmavathi et al., 2018). In addition to the structural integrity, localization of TIPE3 was also proved to contribute to its tumorigenic effects as elevated TIPE3 expression in plasma membrane was associated with increased proliferation and migration of NSCLC cells and forced cytoplasmic localization of the same had an opposite effect. And the proliferative and migratory effect of TIPE3 was again found to be mediated through activation of Akt and ERK pathways (Wang et al., 2018, Padmavathi et al., 2018). Similar to lung cancer, TIPE3 was also shown to be upregulated in breast cancers and correlated with disease progression from ductal carcinoma *in situ* to lymphatic metastasis. Akt and NF-κB

activation, MMP-2 and uPA overexpression were identified to be the downstream targets of TIPE3. Interestingly, silencing of TIPE3 resulted in decreased breast cancer cell proliferation, invasion and migration (Lian et al., 2017) (Figure 1.4B). Moreover, in contrast to the previous reports, very recently, downregulation of TIPE3 mRNA levels was reported in nasopharyngeal carcinoma (NPC) which was in association with hypermethylation of the CpG island of TIPE3 and poor prognosis. Further, induction of TIPE3 expression was found to suppress cancer cell proliferation, invasion and migration thus exhibiting an antitumor effect in NPC (Ren et al., 2018).

Taken together, last two decades of research explained the expression pattern and the importance of TIPE protein family in biological systems. Despite sharing structural and sequence homology, functions of the proteins varied significantly at least in the process of tumorigenesis. TIPE and TIPE3 were mostly pro-tumorigenic, anti-apoptotic, and overexpressed in cancers. TIPE1 and TIPE2 were antitumorigenic, pro-apoptotic, and downregulated in most cancers. Moreover, TIPE1 and TIPE3 proteins are highly unexplored highlighting the need for detailed studies on the role of these proteins in cancer development.

1.4 Importance of the study

Aforementioned, India has the highest incidence rate of oral cancer associated with poor survival. Supporting this fact, the 5-year survival rate of oral cancer is as low as 55% which is found to be further decreased every year. This sustained reduction in the survival rate is mainly due to the lack of effective therapy. Moreover, delayed diagnosis of the disease further added to the complications of disease management. In addition, chemoresistance and tumor recurrence also pose a great challenge and contribute to the poor survival and prognosis. Therefore, for the effective management of this disease, it is essential to decipher the complete molecular

mechanism of oral carcinogenesis. Development of oral cancer is a multi-stage process with several alterations in the genetic and epigenetic levels in each stage. Few such molecular alterations were well characterized to be involved in the disease pathogenesis. However, it is only the tip of the iceberg and therefore, more extensive studies are required to determine the other proteins involved in the development of oral cancer. TNFAIP8 or TIPE protein family contains four proteins with variety of functions. All the four members of this family- TIPE, TIPE1, TIPE2, and TIPE3 were found to have significant roles in the development and progression of different cancers. However, mechanism of regulation of tumorigenesis by these proteins remains ambiguous. Moreover, regardless of the high structural and sequence similarity, their functions in context of cancer differ for all four proteins. Further, despite the fact that the first member of the family, TIPE is initially discovered in head and neck squamous cell carcinoma cell lines, importance of these proteins in the etiopathogenesis of oral cancer is not yet completely understood. Therefore, unravelling the action of these four proteins would assist in understanding the role of TIPE family in the development and progression of oral cancer which would serve as early diagnostic, therapeutic and prognostic biomarkers. In addition, studying the effect of tobacco components, one of the major risk factors of oral cancer, on the expressions of TIPE, TIPE1, TIPE2, and TIPE3 would provide us new insights on oral cancer. Thus, decoding the role of TIPE family in oral cancer would help us to develop novel and highly efficacious therapies with fewer or no adverse effects against this vicious disease in order to increase the survival rate.

1.5 Objectives

Based on the extensive literature survey, the main objective of the current study are framed as,

1. To determine the expression of TNFAIP8 (TIPE), TNFAIP8L1 (TIPE1), TNFAIP8L2 (TIPE2) and TNFAIP8L3 (TIPE3) in normal oral epithelial and different developmental stages and grades of oral cancer (along with preneoplastic lesions) tissues samples.
2. To examine the effect of crude tobacco extract and various carcinogens (tobacco components and synthetic carcinogens) on the expression of TNFAIP8 (TIPE), TNFAIP8L1 (TIPE1), TNFAIP8L2 (TIPE2) and TNFAIP8L3 (TIPE3) in oral cancer cell lines.
3. To determine the role of TNFAIP8 (TIPE), TNFAIP8L1 (TIPE1), TNFAIP8L2 (TIPE2) and TNFAIP8L3 (TIPE3) in the regulation of different molecular mechanisms involved in the development and progression of oral cancer.

CHAPTER 2

Differential Expression of TNFAIP8 (TIPE) Proteins in Normal, Oral Precancerous and Cancer Tissues

Chapter 2

2.1 Introduction

As mentioned in the previous chapter, Melanesia, South-Central Asia, and Central and Eastern Europe were considered to have the highest incidence rate of oral cancer (Jemal et al., 2011). Particularly, among all other countries, India has the highest morbidity rate of oral cancer accounting for 30-40% of all cancers in India and the associated survival rate is as low as 55% owing to the late stage diagnosis and tumor recurrence (Byakodi et al., 2012, Joseph et al., 2018, Irani, 2016, Ribeiro et al., 2016). Therefore, it is essential to identify novel therapeutic targets which would assist in the better management of oral cancer by reducing tumor progression and recurrence thus improving the survival rate. Interestingly, a detailed review of literature suggested that the members of TNFAIP8 (TIPE) protein family play significant roles in the development of various cancers including the cancers of epithelial origin. In addition, they were also reported to critically regulate tumor invasion, migration, metastasis and angiogenesis processes further shouting their therapeutic and prognostic importance. Further, the differential expression of TIPE proteins were already established in various cancer tissues and cell lines including cancers of lung, breast, esophagus, colon, stomach, bone, bladder, liver, pancreas, endometrium, thyroid glands, kidney, prostate, cervix and ovary etc. (Padmavathi et al., 2018, Bordoloi et al., 2018). Moreover, the first member of the family, TIPE was initially identified to be highly overexpressed in metastatic HNSCC patient-derived cell lines showing a possible role of these proteins in head and neck cancers and oral cancer is the most prevalent cancer of head and neck. Therefore, we hypothesized that the TIPE proteins might play crucial role in the development and progression of oral cancer. In order to prove our hypothesis we initially analysed the differential expression of TIPE proteins

in oral cancer tissues and compared with normal tissues using immunohistochemical analysis of oral cancer tissue micro array.

2.2 Materials and methods

2.2.1 Tissue micro array (TMA):

Expression of TIPE proteins in normal and different stages of oral cancer tissues was determined by immunohistochemical analysis. For this purpose, tissue microarray (TMA) containing paraffin-embedded normal, preneoplastic and neoplastic oral cavity tissues (US Biomax, Inc., Cat. No. OR802) is used. The TMA slide contains total of 80 tissues from 79 different individuals and each tissue core is of 1.5 mm in diameter and 5 µm in thickness. It has, 28 tissues of squamous cell carcinoma, 4 adenocarcinoma, 8 mucoepidermoid carcinoma, 2 basal cell carcinoma, 4 metastatic carcinoma, 8 adamantinoma, 6 hyperplasia and 5 each of adjacent tissue, inflammation, adjacent normal tissue and normal tissue (Table 2.1). Further, the tissues were also classified based on tumor grades (grade 1, grade 2 and grade 3) and stages (stage I, stage II, stage III and stage IV).

Table 2.1: TMA details

Core	Sex	Age	Organ	Pathology	Grade	Stage	TNM	Type
A1	M	40	Gingiva	Squamous cell carcinoma	1	IV	T4N0 M0	Malignant
A2	F	47	Tongue	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
A3	M	81	Lip	Squamous cell carcinoma	1	II	T2N0 M0	Malignant
A4	M	57	Tongue	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
A5	F	52	Lip	Squamous cell carcinoma	2	I	T1N0 M0	Malignant
A6	M	53	Cheek	Squamous cell carcinoma	1	II	T2N0 M0	Malignant
A7	F	62	Cheek	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
A8	M	48	Base of tongue	Squamous cell carcinoma (salivary gland)	-	II	T2N0 M0	Malignant
A9	M	68	Right palate	Squamous cell carcinoma	1	II	T2N0 M0	Malignant

A10	F	56	Cheek	Squamous cell carcinoma	1	II	T2N0 M0	Malignant
B1	M	79	Cheek	Squamous cell carcinoma	1	II	T2N0 M0	Malignant
B2	M	60	Gingiva	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
B3	M	55	Cheek	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
B4	M	66	Tongue	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
B5	F	46	Tongue	Squamous cell carcinoma	1	IV	T1N0 M1	Malignant
B6	F	39	Tongue	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
B7	M	78	Tongue	Squamous cell carcinoma	1	II	T2N0 M0	Malignant
B8	F	78	Lip	Squamous cell carcinoma	1	IV	T1N0 M1	Malignant
B9	F	54	Lip	Squamous cell carcinoma	1	IV	T1N0 M1	Malignant
B10	F	75	Lip	Squamous cell carcinoma	1	IV	T1N0 M1	Malignant
C1	M	60	Tongue	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
C2	M	73	Lip	Squamous cell carcinoma	1	I	T1N0 M0	Malignant
C3	M	60	Gingiva	Squamous cell carcinoma	2	I	T1N0 M0	Malignant
C4	M	78	Lip	Squamous cell carcinoma	2	I	T1N0 M0	Malignant
C5	M	55	Gingiva	Squamous cell carcinoma	2--3	I	T1N0 M0	Malignant
C6	M	47	Lower mandible	Squamous cell carcinoma	3	II	T2N0 M0	Malignant
C7	M	41	Dental alveoli	Squamous cell carcinoma	3	I	T1N0 M0	Malignant
C8	M	60	Tongue	Squamous cell carcinoma	3	II	T2N0 M0	Malignant
C9	F	40	Palate	Adenoid cystic carcinoma	-	I	T1N0 M0	Malignant
C10	M	45	Left lower mandible	Adenoid cystic carcinoma (sparse)	-	I	T1N0 M0	Malignant
D1	M	64	Palate	Adenoid cystic carcinoma	-	II	T2N0 M0	Malignant
D2	M	66	Parotid gland	Acinic cell carcinoma	-	II	T2N0 M0	Malignant
D3	M	71	Mouth floor	Mucoepidermoid carcinoma	1	I	T1N0 M0	Malignant
D4	M	57	Palate	Mucoepidermoid carcinoma	1	II	T2N0 M0	Malignant
D5	F	50	Cheek	Mucoepidermoid carcinoma	1	II	T2N0 M0	Malignant
D6	M	57	Upper lip	Mucoepidermoid carcinoma (skeletal muscle and blood vessel)	-	I	T1N0 M0	Malignant
D7	F	48	Right lower	Mucoepidermoid	2	I	T1N0	Malignant

			mandible	carcinoma			M0	
D8	M	55	Gingiva	Mucoepidermoid carcinoma	3	I	T1N0 M0	Malignant
D9	M	60	Right lower mandible	Mucoepidermoid carcinoma	3	III	T3N0 M0	Malignant
D10	M	50	Root of tongue	Mucoepidermoid carcinoma (sparse)	-	I	T1N0 M0	Malignant
E1	F	79	Lip	Basal cell carcinoma (sparse)	-	II	T2N0 M0	Malignant
E2	F	48	Lip	Basal cell carcinoma	-	II	T2N0 M0	Malignant
E3	F	70	Lymph node	Metastatic squamous cell carcinoma of neck from cheek	2	-	-	Metastasis
E4	M	79	Lymph node	Metastatic squamous cell carcinoma of neck from tongue	1	-	-	Metastasis
E5	F	59	Lymph node	Metastatic squamous cell carcinoma of neck from mandible	2	-	-	Metastasis
E6	F	40	Lymph node	Metastatic mucoepidermoid carcinoma of neck from mandible	3	-	-	Metastasis
E7	M	11	Mandible	Adamantinoma	-	-	-	Benign
E8	M	28	Left mandible	Adamantinoma	-	-	-	Benign
E9	M	51	Right mandible	Adamantinoma	-	-	-	Benign
E10	M	64	Mandible	Adamantinoma (fibrous tissue and blood vessel)	-	-	-	Benign
F1	F	37	Mandible	Adamantinoma	-	-	-	Benign
F2	M	40	Lower mandible	Adamantinoma	-	-	-	Benign
F3	F	47	Mandible	Adamantinoma	-	-	-	Benign
F4	F	70	Right jaw bones	Adamantinoma	-	-	-	Benign
F5	M	67	Lip	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F6	M	40	Lip	Mild atypical hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F7	M	82	Lip	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F8	M	46	Tongue	Hyperplasia of squamous	-	-	-	Hyperplasia

				epithelium (skeletal muscle and blood vessel)				
F9	F	60	Tongue	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F10	F	3	Tongue	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
G1	M	68	Parotid gland	Cancer adjacent tissue (with squamous cell carcioma sparse)	-	-	-	AT
G2	F	53	Tongue	Cancer adjacent tissue	-	-	-	AT
G3	M	53	Tongue	Cancer adjacent tissue (hyperplasia of squamous epithelium)	-	-	-	AT
G4	M	70	Parotid gland	Cancer adjacent tissue (with mucoepidermoid carcinoma)	2	-	-	AT
G5	M	63	Lip	Cancer adjacent tissue (chronic inflammation of fibrous tissue and blood vessel)	-	-	-	AT
G6	M	43	Submaxillary gland	Chronic submaxillaritis	-	-	-	Inflammation
G7	F	23	Parotid gland	Chronic parotitis	-	-	-	Inflammation
G8	F	66	Parotid gland	Chronic parotitis	-	-	-	Inflammation
G9	F	40	Right cheek	Chronic inflammation of mucosa	-	-	-	Inflammation
G10	F	75	Lower lip	Chronic inflammation of mucosa of No. 20	-	-	-	Inflammation
H1	F	48	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H2	F	48	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H3	M	37	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H4	M	63	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H5	M	56	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H6	F	42	Tongue	Normal tongue tissue	-	-	-	Normal
H7	F	38	Salivary	Normal salivary	-	-	-	Normal

			gland	gland tissue				
H8	M	48	Tongue	Normal tongue tissue	-	-	-	Normal
H9	M	50	Salivary gland	Normal salivary gland tissue	-	-	-	Normal
H10	M	22	Salivary gland	Normal salivary gland tissue	-	-	-	Normal

2.2.2 Immunohistochemistry (IHC) and scoring:

Histostain-Plus IHC Kit, HRP, broad spectrum (Invitrogen, Cat. No. 859043) was used for immunostaining the tissue micro array. IHC was performed as per the manufacturer protocol: i.e.: first deparaffinization followed by rehydration, peroxidase quenching, antigen retrieval, blocking, primary antibody incubation, secondary antibody-peroxidase conjugate incubation, addition of DAB chromogen and counterstaining with hematoxylin. Finally, the slides were dehydrated and mounted with coverslip using D.P.X. mountant for microscopy. The primary antibodies namely anti-TNFAIP8 antibody (Cat. No. ab64988), anti-TNFAIP8L1 antibody (Cat. No. ab85409), anti-TNFAIP8L2 antibody (Cat. No. ab110389) and anti-TNFAIP8L3 antibody (Cat. No. ab111524) were obtained from abcam® and used in the dilutions of 1:25, 1:50, 1:50 and 1:100 respectively, for immunostaining. The immunostained microarrays were analysed under Olympus light microscope. Tissues that are stained brown are considered as positive for the presence of antigen of interest and given a score according to the staining intensity and percentage of positive cells. Score for percentage of positive cells is scaled from 0 to 4+ and for staining intensity is scaled from 1 to 3 (Table 2.2) (McDonald et al., 1999, Shiao et al., 2000, Charafe-Jauffret et al., 2004).

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 Staining	weak staining	moderate staining	strong staining		

2.2.3 Statistical analysis:

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

2.3 Results

As mentioned earlier, in the current chapter, for the first time we analysed the difference in the expression of TIPE proteins in normal, hyperplasic and neoplastic tissues from oral cancer patients by immunohistochemical analysis. Although the TMA slide contains tissues from different types of oral cancers, our prime focus was on oral squamous cell carcinoma (OSCC) as around 90% of oral cancers are squamous cell carcinomas (Joseph et al., 2018). Each tissue in the TMA slide was carefully analysed under the microscope and was given a total expression score (Q) based on the staining intensity and frequency as reported earlier (McDonald et al., 1999, Shiao et al., 2000, Charafe-Jauffret et al., 2004). The scorer was blinded for the details of TMA to avoid any manual bias. After scoring, in order to get a detailed idea of the involvement of TIPE proteins in OSCC development and progression, we analysed the IHC results, in context with preneoplastic to neoplastic transformation and progression of tumor grades and stages. Interestingly, the IHC results showed that the TIPE proteins are differentially expressed (either upregulated or downregulated) in oral cancer tissues compared to the normal tissues, which was in support to our hypothesis suggesting the deregulation of TIPE proteins to be associated with OSCC.

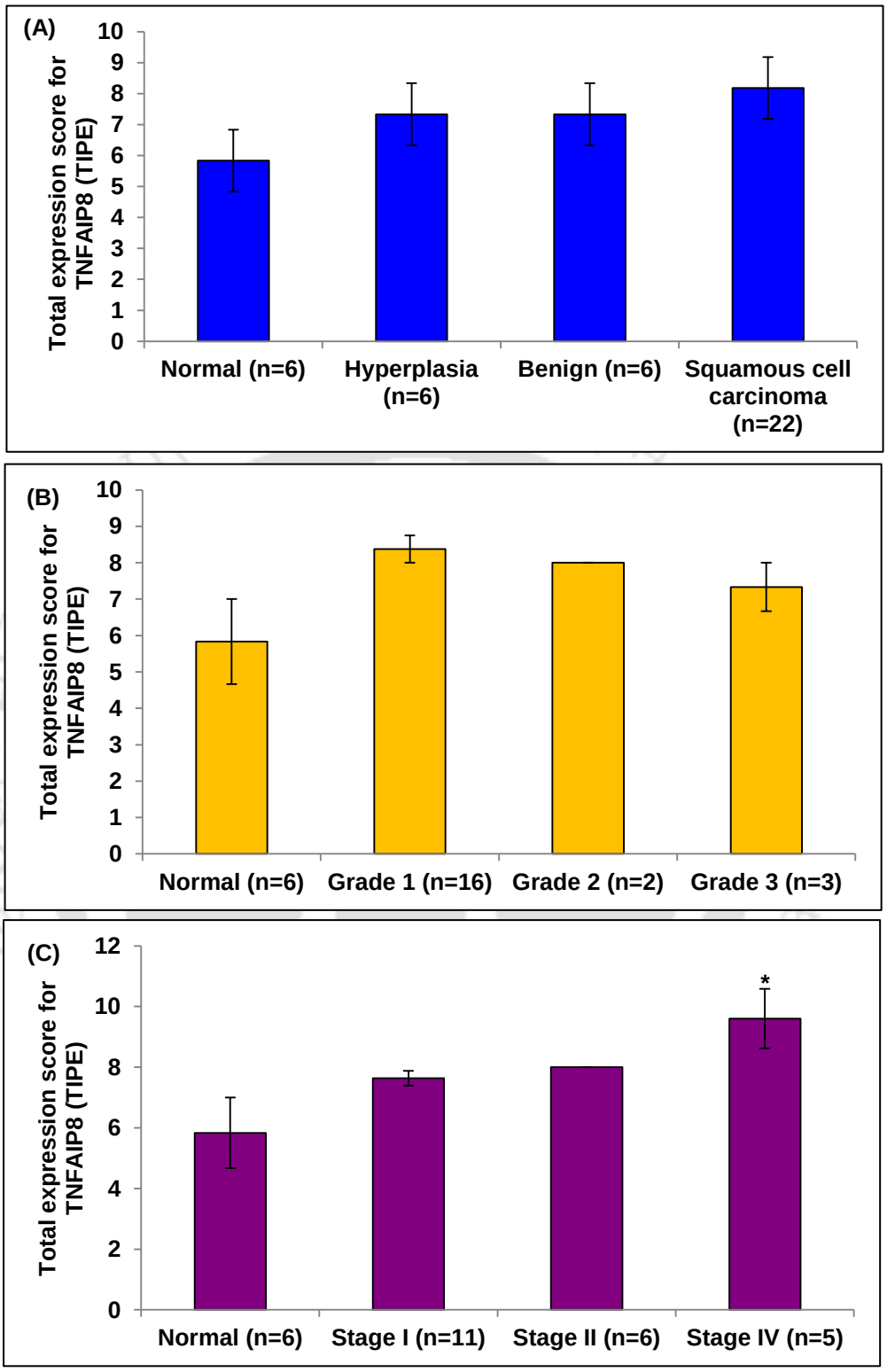


Figure 2.1: Differential expression of TNFAIP8 (TIPE) protein analysed by IHC. (A) Expression of TIPE in the developmental stages of OSCC; (B) Expression of TIPE in different grades of OSCC; (C) Expression of TIPE in different stages of OSCC. (*p-value ≤ 0.05 in comparison with normal tissues)

2.3.1 Upregulation of TIPE was observed in OSCC tissues and correlated with disease progression

Immunostaining of the oral cancer TMA revealed that levels of TIPE is marginally upregulated in cancer tissues compared to the normal. Further, the upregulation is considered to be in correlation with disease progression as TIPE expression progressively increased when moved from hyperplasia to benign and finally to malignant tumors with the average total expression scores (Q_{avg}) of 5.8, 7.3, 7.3, 8.2 for normal, hyperplasia, benign tumors and malignant tumors respectively (Figure 2.1A). Furthermore, TIPE level was also increased with increase in tumor stage. 7.6, 8 and 9.6 are the respective Q_{avg} for stage I, II and IV tumor tissues (Figure 2.1C). Additionally, grade wise analysis of TIPE expression also revealed that expression of TIPE is upregulated in grade 1 tumors whereas with increase in grades it tends to decrease. Nevertheless, compared to normal tissues, the TIPE level was high in all grade 1, 2 and 3 tissues with Q_{avg} of 8.4, 8 and 7.3 respectively (Figure 2.1B). However, the change in the expression was not statistically significant compared to normal tissues.

2.3.2 TIPE1 was downregulated in preneoplastic and OSCC tissues

Unlike TIPE, expression of TIPE1 was downregulated in oral cancer tissues compared to the normal tissues and the downregulation was in correlation with disease progression from normal (Q_{avg} = 8) to preneoplastic hyperplasia (Q_{avg} = 7.3) to benign (Q_{avg} = 7) and malignant carcinoma (Q_{avg} = 6.9) (Figure 2.2A) suggesting an inverse relation between TIPE1 expression and development of OSCC. Further assuring this, the decrease in TIPE1 expression was more with increase in tumor grade with the Q_{avg} of 7.1, 6 and 5.7 for grade 1, 2 and 3 tumors respectively (Figure 2.2B). Furthermore, downregulation of TIPE1 was also observed in the tissues of stage I, II and IV tumors.

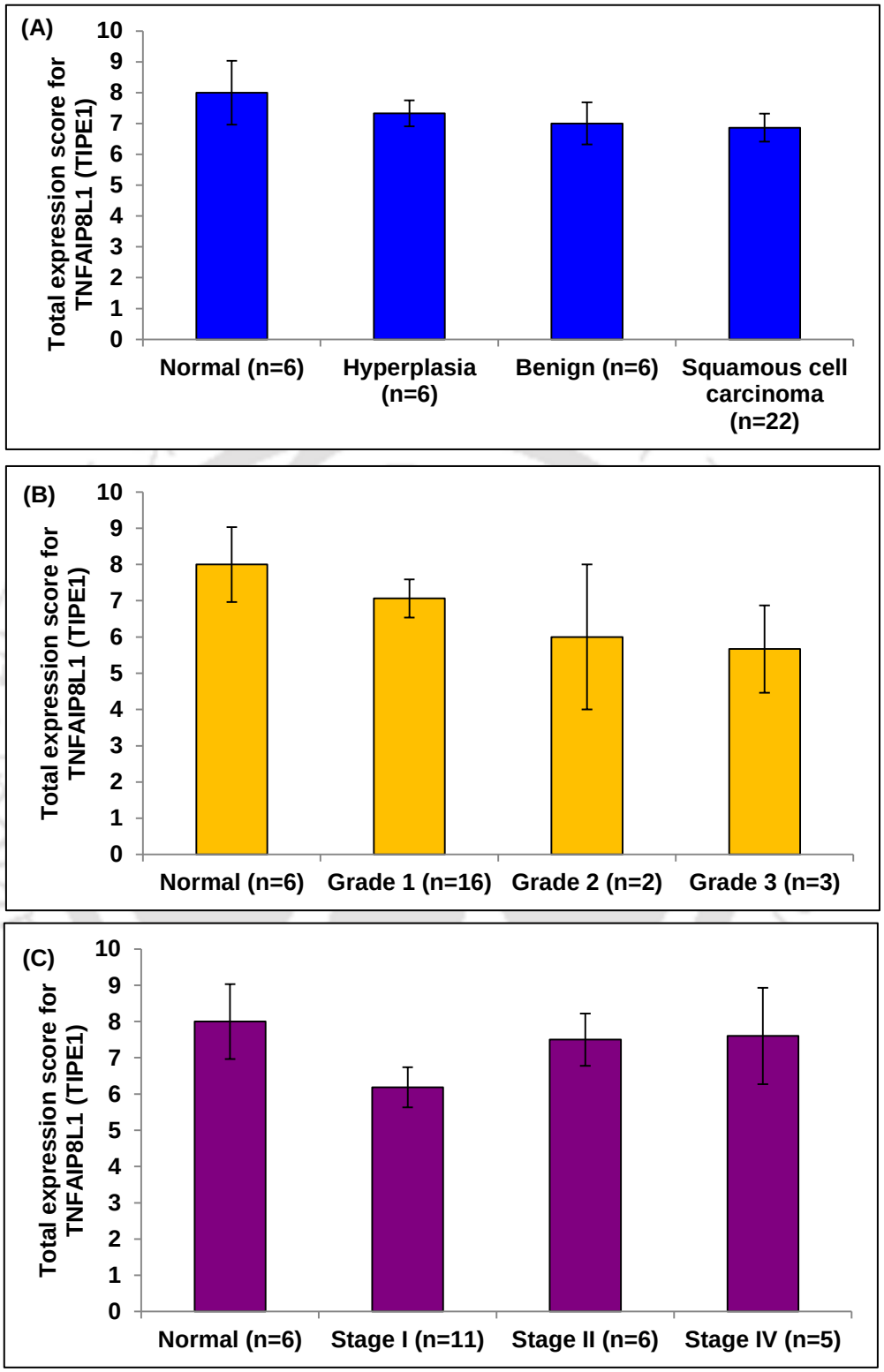


Figure 2.2: Differential expression of TNFAIP8L1 (TIPE1) protein analysed by IHC. (A) Expression of TIPE1 in the developmental stages of OSCC; (B) Expression of TIPE1 in different grades of OSCC; (C) Expression of TIPE1 in different stages of OSCC.

Nevertheless, stage I tissues had lesser TIPE1 expression (Q_{avg} = 6.2) compared to that of stage II (Q_{avg} = 7.5) and IV (Q_{avg} = 7.6) (Figure 2.2C). However, the decrease observed in the expression of TIPE1 was not statistically significant compared to normal tissues.

2.3.3 Upregulation of TIPE2 was observed in preneoplastic and OSCC tissues

Similar to TIPE, expression of TIPE2 was also upregulated in tumor tissues compared to the normal tissues. Further, the increased expression was significantly associated with the developmental stages of OSCC such as normal (Q_{avg} = 5.5), hyperplasia (Q_{avg} = 8.2), benign (Q_{avg} = 8.3) and malignant (Q_{avg} = 8.6) tissues (Figure 2.3A). Similarly, upregulation of TIPE2 was also correlated with increase in tumor stage from stage I to stage IV and the Q_{avg} values were 8.3, 8.7 and 9.4 for stage I, II and IV tissues respectively (Figure 2.3C). Further, TIPE2 overexpression was also observed in different grades of OSCC compared to normal tissues. However, with increase in grades of tumors change in the expression was very nominal with the Q_{avg} values of 8.8, 8 and 8.3 for grade 1, 2 and 3 tumors (Figure 2.3B). Moreover, the change observed in the expression of TIPE2 was statistically significant with respect to the normal tissues.

2.3.4 TIPE3 was overexpressed in preneoplastic and OSCC tissues

Expressional analysis of TIPE3 in normal, preneoplastic and neoplastic tissues revealed it to be highly overexpressed in both precancerous and cancerous tissues compared to normal tissues (Q_{avg} = 3.5) suggesting a strong correlation between TIPE3 upregulation and development of OSCC. Additionally, increase in the levels of TIPE3 was observed to be sustained in hyperplasia (Q_{avg} = 6.8), benign (Q_{avg} = 6.5) and malignant (Q_{avg} = 6.9) tissues (Figure 2.4A). Likewise, TIPE3 was also upregulated in grade 1 (Q_{avg} = 6.9), grade 2 (Q_{avg} = 6) and grade 3 (Q_{avg} = 7.3) tissues.

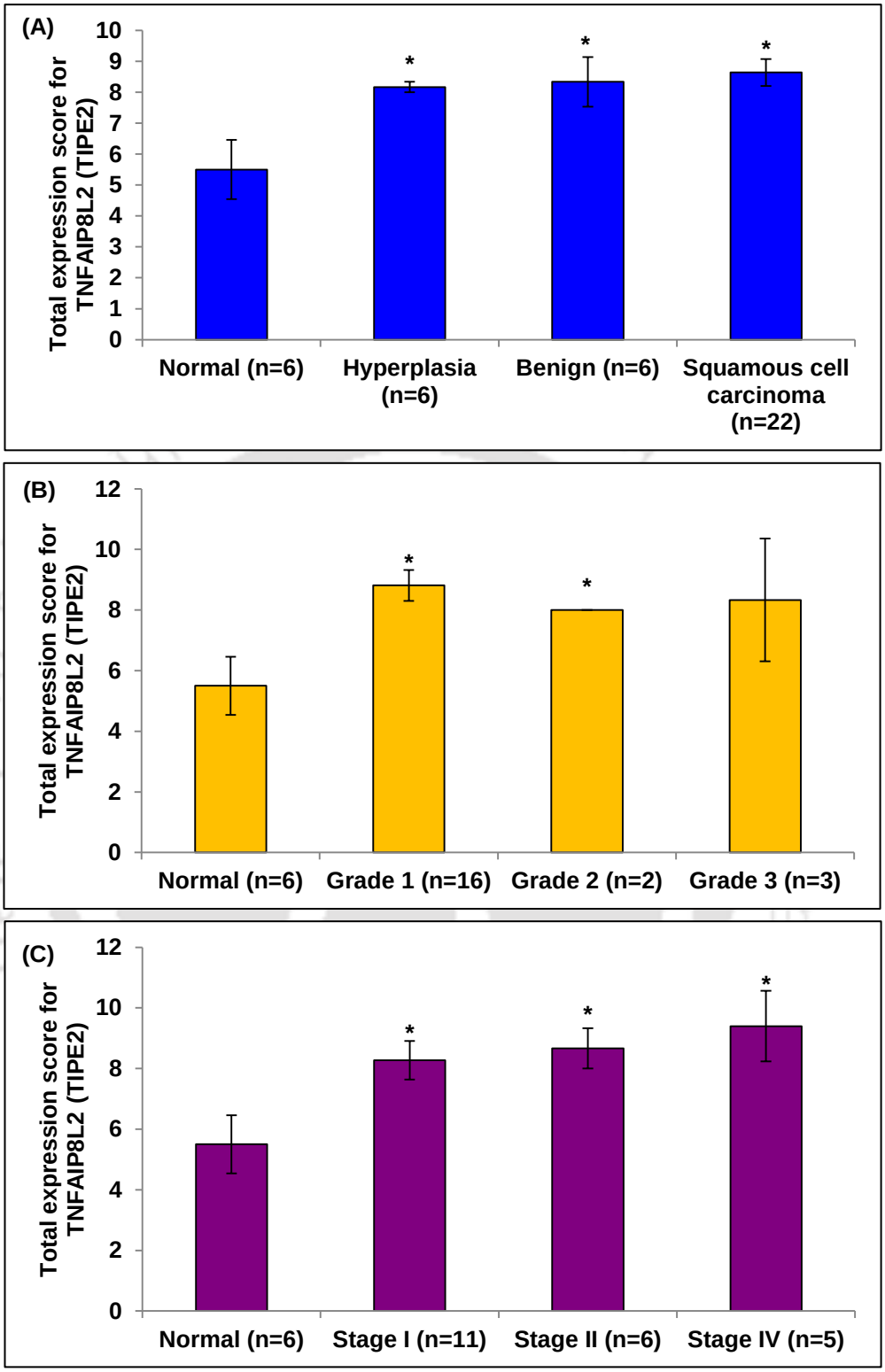


Figure 2.3: Differential expression of TNFAIP8L2 (TIPE2) protein analysed by IHC. (A) Expression of TIPE2 in the developmental stages of OSCC; (B) Expression of TIPE2 in different grades of OSCC; (C) Expression of TIPE2 in different stages of OSCC. (*p-value ≤ 0.05 in comparison with normal tissues)

Interestingly, the upregulation was significantly different from normal tissues however not between the grades. Further, analysis of the change in TIPE3 expression between different stages of oral cancer which includes tissues from stage I, II and IV showed a clear upregulation in all the three stages compared to normal. Moreover, a nominal increase was observed between stage I and II tumors and a slight decrease was observed in stage IV tumors (Figure 2.4C). Q_{avg} for TIPE3 in stage I, II and IV tissues were 6.8, 7.7 and 6.4 respectively. Furthermore, the changes witnessed in the TIPE3 levels were also statistically significant with respect to the normal tissues.

2.4 Discussion

2.4.1 TIPE proteins were differentially expressed in oral premalignant and malignant tissues

As mentioned in chapter 1, the TIPE family of proteins were reported to be differentially expressed in various cancer tissues and the difference in their expression was either positively or negatively correlated with disease progression, invasion, metastasis and prognosis. Moreover, despite the first member of the family being identified in HNSCC patient derived cell lines, expression of these proteins were not well established in HNSCC (Padmavathi et al., 2018). OSCC, the prime focus of this study, is one of the most prevalent cancers of head and neck. Therefore, in the current study we initially analysed the difference in the expression of these proteins in OSCC to get preliminary idea about their involvement in the development and disease progression. As expected, immunohistochemical analysis of the oral cancer TMA showed a differential expression of TIPE proteins in cancer tissues compared to the normal. For instance, levels of TIPE, TIPE2 and TIPE3 were upregulated and TIPE1 was downregulated in OSCC tissues and the change in the expression was also correlated with disease progression from hyperplasia to malignant. However, only the

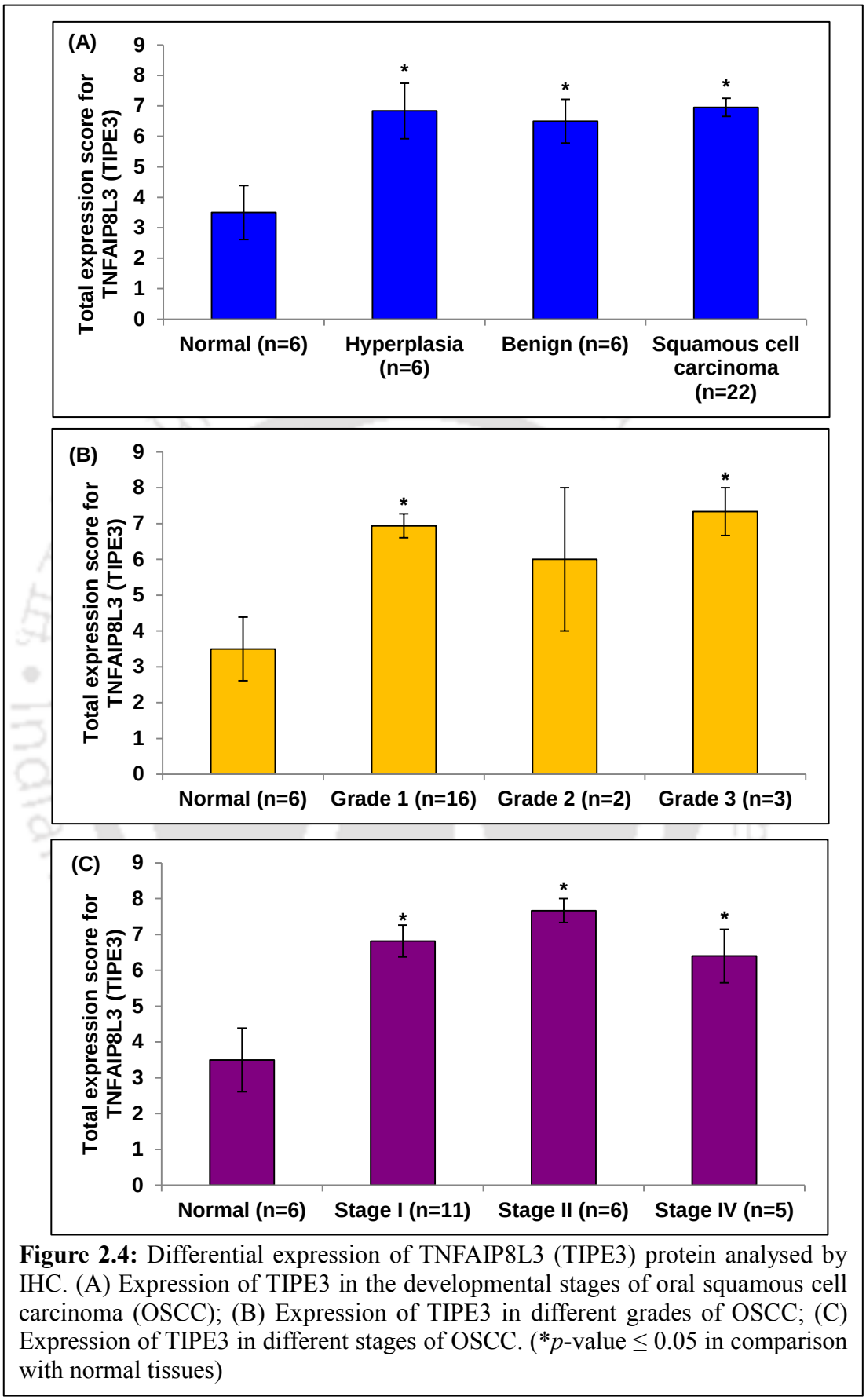


Figure 2.4: Differential expression of TNFAIP8L3 (TIPE3) protein analysed by IHC. (A) Expression of TIPE3 in the developmental stages of oral squamous cell carcinoma (OSCC); (B) Expression of TIPE3 in different grades of OSCC; (C) Expression of TIPE3 in different stages of OSCC. (*p-value ≤ 0.05 in comparison with normal tissues)

change in the levels of TIPE2 and TIPE3 were statistically significant whereas TIPE and TIPE1 levels were associated with minimal change in the expression. Overexpression of TIPE observed in OSCC tissues was in line with the previous reports showing elevated levels of TIPE in various cancers including breast cancer, chronic myelogenous leukemia, endometrial cancer, hepatocellular carcinoma (HCC), lung carcinoma, lymphoblastic leukemia, pancreatic cancer and renal cell carcinoma (Kumar et al., 2004, 2000, Liu et al., 2014b, Dong et al., 2017, 2010, Liu et al., 2012, Padmavathi et al., 2018). Moreover, the TIPE overexpression was also reported to be associated with tumor growth and progression in breast and liver cancers (Kumar et al., 2004, 2000, Dong et al., 2017). Similar results are observed in our study where we have found expression of TIPE to progressively increase with disease progression from hyperplasia to squamous cell carcinoma (Figure 2.1A). Further, expression of TIPE2 was found to be downregulated in many cancers including HCC, NSCLC, glioma, breast cancer, prostate cancer and gastric cancer, having low levels of TIPE2 in comparison to the normal cells/tissues and the reduced expression was associated with increased proliferation, EMT, invasion, migration and metastasis suggesting an anti-tumorigenic role (Cao et al., 2013, Zhang et al., 2015b, Li et al., 2016, Li et al., 2015b, Liu et al., 2016, Wang et al., 2017, Zhang et al., 2016, Lu et al., 2016, Wu et al., 2016, Padmavathi et al., 2018). However, in contrast to this, expression of TIPE2 was shown to be upregulated in colon cancer, non-Hodgkin's lymphoma (NHL) and renal cell carcinoma (RCC) and to have pro-tumorigenic effect (Li et al., 2014, Hao et al., 2016, Zhang et al., 2013c). All these reports suggest the expression and role of TIPE2 in cancer to be ambiguous and organ specific. In our study, we have observed a significant upregulation of TIPE2 in preneoplastic and neoplastic oral tissues compared to normal tissues. Further, the upregulation was also correlated with disease

progression from hyperplasia to benign and malignant tumors (Figure 2.3A). Therefore, our results support the pro-tumorigenic hypothesis of TIPE2 at least for OSCC. Similar to TIPE and TIPE2, another member of this family, TIPE3 was also found to be involved in tumorigenesis. Being recently identified, only very few reports are available on this protein and hence the difference in its expression in various cancers remains elusive. However, few studies have reported TIPE3 to be overexpressed in cancers of lung, cervix, colon, breast and esophagus (Fayngerts et al., 2014, Lian et al., 2017). Further, the upregulation of TIPE3 was also reported to increase tumor growth and aggressiveness (Padmavathi et al., 2018). For instance, it has been reported that increase in TIPE3 expression mediates transformation of ductal carcinoma *in situ* to invasive ductal breast carcinoma (IDC) and led to lymphatic metastasis (Lian et al., 2017). In line with these reports, in the current study, we have also witnessed a significant upregulation of TIPE3 in both oral precancerous and cancerous tissues compared to normal tissues. Even though, unlike breast cancer, the expression was not increased with disease progression from hyperplasia to malignant transformation of oral cancer. However, comparable upregulation was observed in all hyperplasia, benign and SCC tissues (Figure 2.4A). Interestingly, in contrast to all these three proteins, the second member of TIPE family, i.e. TIPE1 was downregulated in oral premalignant and malignant tissues. However, the change in expression was very minimal (Figure 2.2A). Supporting our results, reports on other cancers such as HCC and lung cancer have also denoted TIPE1 to be downregulated in cancer and restoring its expression was associated with reduced tumor burden, proliferation, invasion and migration and increased cell death (Zhang et al., 2015a, Wu et al., 2017a, Padmavathi et al., 2018). Thus, TIPE1 is a potential tumor suppressor that is downregulated in oral cancers.

2.4.2 Change in the expression of TIPE proteins was associated with increase in tumor stages and grades

Tumor staging and grading is the prime and crucial step in the management of cancer which gives information to the clinicians about the severity of disease which would help them to decide possible therapeutic strategies and to predict the prognosis of patient (Telloni, 2017). As discussed in previous chapter, staging is done on the basis of size and local growth of the tumor and the anatomic extent of the disease spread i.e. TNM staging. Considering the TNM status, the tumors are classified into different stages from stage I to IV. In general, stage I-III tumors regarded as curable with various treatment methods ranging from initial surgery to targeted therapies and stage IV tumors are highly advanced and mostly incurable (Telloni, 2017). Further, the tumors present only in the site of origin without spreading to proximal or distant sites and lymph nodes are grouped into stage I; stage II tumors include cancers that spread to the surrounding tissues and nearby lymph nodes; stage III includes tumors that invaded in deeper lymph nodes; finally the tumors spread to distant organs are grouped into stage IV disease (Telloni, 2017). Therefore, in order to find if the TIPE proteins involved in tumor growth, invasion and metastasis, we analysed the regulation of their expression with respect to tumor stage. We found that TIPE, TIPE2 and TIPE3 were upregulated with increase in tumor stage (Figures 2.1C, 2.3C and 2.4C). However, the change in the expression was minimal. Nevertheless, the minimal upregulation is hypothesised to contribute to tumor invasion, migration and metastasis, as all these proteins were previously reported to be strongly associated with invasion, migration and EMT of various cancers. Further assuring their importance in the regulation of cancer metastasis, downregulation of these proteins

resulted in significant reduction of cancer cell migration, invasion and angiogenesis and metastasis related protein markers in different cancers (Padmavathi et al., 2018).

Similar to tumor staging, grading is also equally important in the management of cancer. As mentioned in chapter 1, tumor grading is done based on architecture and histology of tumor tissues and denotes their differentiation status. Thus, the well differentiated, moderately differentiated and poorly differentiated tumors are categorized into grade 1, 2 and 3 cancers. Moreover, the increase in tumor grade is considered to be directly proportional to aggressiveness of the disease (Telloni, 2017). Therefore, in the current study we have also tested if there is any change in the expression of TIPE proteins with respect to grades of tumor, in order to establish a relation between TIPE proteins and aggressiveness of the disease. Interestingly, expression of TIPE, TIPE2 and TIPE3 proteins were upregulated in grade 1, 2 and 3 tumors compared to normal tissues whereas no remarkable change was observed between different grades of tumors (Figures 2.1B, 2.3B and 2.4B). In contrast to this, expression of TIPE1 was progressively downregulated with increase in tumor grades (Figure 2.2B) suggesting their association with degree of differentiation.

2.5 Conclusion

Taken together, in this chapter the differential expression of all four TIPE proteins was analysed in premalignant and malignant tissues from oral cancer patients and compared with normal tissues using immunostaining of TMA. Our results showed that TIPE, TIPE2 and TIPE3 are upregulated and TIPE1 is downregulated in oral precancerous lesion and oral squamous cell carcinoma. Moreover, the deregulation of TIPE proteins was correlated with advance in tumor stage and grade. Therefore, all these results combined with previous literature suggest that TIPE, TIPE2 and TIPE3 upregulation and TIPE1 downregulation might be crucial in the development and

progression of OSCC and also might play key role in the regulation of tumor migration, invasion and metastasis targeting which would give better treatment option for OSCC. However, more detailed investigation is required to confirm our hypothesis as the TMA results give only a preliminary idea about the change in the expression in comparison to normal tissues warranting a thorough study on the molecular alterations associated with TIPE mediated oral carcinogenesis and its upstream and downstream targets.

CHAPTER 3

Effect of Tobacco and Related Carcinogens on the Expression of TIPE Proteins

3.1 Introduction

From the results of our previous chapter, we have found that expression of TIPE proteins is deregulated in oral squamous cell carcinoma and the associated premalignant diseases. Therefore, we hypothesized a correlation of TIPE proteins with the development and progression of oral cancer. To further confirm our hypothesis, we attempted to analyse the effect of the risk factors of oral cancer on the levels of TIPE proteins. As discussed in chapter 1, tobacco and tobacco related products are the major risk factors for the development and progression of oral cancers (Gupta et al., 2013, Mignogna et al., 2004, Goel et al., 2014). Moreover, according to the Ministry of Health and Family Welfare, Govt. of India, reportedly 90% of oral cancer cases in India are associated with consumption of smokeless tobacco (SLT). Further, the SLT has been found to have numerous chemicals including more than 70 potential carcinogens (nicotine, benzo[a]pyrene (BaP) and tobacco specific N'-nitrosamines (TSNAs) such as N-Nitrosonornicotine (NNN) and 4 [methylnitrosoamino]-1-[3-pyridyl]-1-butanone (NNK) to name a few) which are well known to induce several genetic and epigenetic alterations in the genome of normal cells leading to the initiation and progression of at least 14 types of cancers (Hecht and Szabo, 2014, Hecht, 2003). Therefore, it is ideal to check if the deregulation of TIPE proteins observed in oral cancer tissues is mediated through these risk factors. In this chapter we have determined the effect of crude tobacco extracts, tobacco carcinogens (nicotine and BaP) and a synthetic carcinogen 4-nitroquinoline 1-oxide (4NQO) on the expression of all the four TIPE proteins in oral cancer cells *in vitro* by Western blot which would give a better knowledge on the involvement of TIPE protein family in oral cancer.

3.2 Materials and methods

3.2.1 Carcinogens and antibodies:

Nicotine (Cat. No. N3876), Benzo(a)pyrene (Cat. No. B1760) and 4NQO (Cat. No. N8141) were purchased from Sigma- Aldrich, Missouri, USA. Tobacco was procured from local markets of Guwahati, Assam, India and tuibur was procured from the local markets of Aizawl, Mizoram, India. The primary antibodies against TNFAIP8 (Cat. No. ab64988), TNFAIP8L1 (Cat. No. ab85409), TNFAIP8L2 (Cat. No. ab110389) and TNFAIP8L3 (Cat. No. ab111524) were purchased from abcam®, Cambridge, USA and used in the dilutions of 1: 2000, 1: 1000, 1: 8000 and 1: 4000 respectively. Antibodies against housekeeping genes α-Tubulin (Cat. No. 2144S) and β-Actin (Cat No. 4967S) were purchased from Cell Signaling Technology, Massachusetts, USA and used in the dilutions of 1: 4000 and 1: 2000 respectively. The anti-rabbit secondary antibody (Cat. No. ab97080) was obtained from abcam®, Cambridge, USA and used in the dilution of 1: 10000.

3.2.2 Tobacco extract (TE) preparation:

Dried tobacco leaves were mashed into fine powder and extracted with distilled water in the ratio of 100 mL of water for 4 g of tobacco powder for 24 h at room temperature (RT). After 24 h of constant stirring, the extract was filtered and the filtrate was lyophilized. A working stock concentration of 50 mg/mL of the lyophilized powder was prepared in sterile distilled water (tobacco extract (TE)) and stored in -20 °C for further use.

3.2.3 Tuibur:

Tuibur is a water-soluble tobacco smoke extract frequently consumed in the Indian state Mizoram. It was purchased from the local markets of Aizawl, Mizoram, India and filtered to remove any insoluble particles. The filtrate was then lyophilized into

powder and a working stock of 100 mg/mL was prepared by dissolving the lyophilized powder in sterile distilled water and stored in -20 °C deep freezer for further use.

3.2.4 Cell culture:

Two human oral cancer cell lines namely, SAS and KB were used for this study. SAS cells were obtained from the Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India. KB cells were purchased from National Centre for Cell Science (NCCS), Pune, India. Both the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco™; Life Technologies, NY, USA) enhanced with 10% fetal bovine serum (FBS; Gibco®, NY, USA) and 1X Pen-Strep (Invitrogen, CA, USA) and maintained at 37 °C in a humidified, CO_2-regulated (95% air/5% CO_2 atmosphere) incubator.

3.2.5 MTT assay:

MTT assay was performed to determine the effect of carcinogens on the proliferation and survival of oral cancer cells. All the experiments were carried out in sterile 96 well plates. In brief, SAS and KB cells were seeded at a density of 2000 cells/100 µL/ well and allowed overnight to adhere to the plate. Following the overnight incubation, the cells were treated with different concentrations of TE (0, 0.1, 0.5, 1, 5 and 10 µg/mL), tuibur (0, 1, 5, 10, 25 and 50 µg/mL), nicotine (0, 0.1 and 0.25 µM), BaP (0, 25 and 50 ng/mL) and 4NQO (0, 0.25, 0.5 and 0.75 ng/mL) for 0 and 24 h. 10 µL of 5 mg/mL MTT (Cat. No. M2128, Sigma-Aldrich, Missouri, USA) was added to the respective plates at the end of 0 and 24 h and incubated for 2 h in a CO_2-regulated 37 °C incubator. Following the 2 h incubation, the MTT containing media was replaced with 100 µL of dimethyl sulfoxide (DMSO) (Cat. No. 1.16743.0521, Merck, Darmstadt, Germany) to dissolve the formazan crystals and incubated in dark for 1 h

at RT. After the 1 h incubation, absorbance of the plate was measured at 570 nm using a microplate reader (TECAN Infinite 200 PRO multimode reader). % of proliferation was calculated by normalizing the absorbance of 24 h with 0 h absorbance and keeping the absorbance of untreated control as 100%.

3.2.6 Western blot:

Effect of tobacco carcinogens on the expression of TIPE proteins was determined using Western blot. Briefly, SAS and KB cells were treated with different concentrations of TE (0, 0.1, 0.5, 1, 5 and 10 µg/mL), tuibur (0, 1, 5, 10, 25 and 50 µg/mL), nicotine (0, 0.1 and 0.25 µM), BaP (0, 25 and 50 ng/mL) and 4NQO (0, 0.25, 0.5 and 0.75 ng/mL) for 24 h and the total protein was extracted 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 (250mM TrisHCl, 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 10% non-fat dry milk (Amulya) prepared in 1X TBST (0.2 M Tris base, 1.5 M NaCl and 1% Tween-20). Following the 2-3 h 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 secondary antibody for 2 h. Following the 2 h incubation, the membranes were again washed with 1X TBST and the blots were developed using Optiblot ECL Detection Kit (Cat. No. ab133406; Abcam, Cambridge, USA) and ChemiDoc™ XRS System (Bio-Rad, California, USA). The band intensity and the fold change in protein expression were calculated using ImageLab software considering the housekeeping genes β-actin and α-tubulin as loading controls.

3.2.7 Statistical analysis:

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

3.3 Results and discussion

3.3.1 Exposing oral cancer cells to crude tobacco extract altered the protein levels of TIPE family

As mentioned earlier, tobacco is one of the major risk factors of oral cancer and is proved to contain more than 70 potential carcinogens, consumption of which would induce chromosomal damage, mutagenesis and deregulation of various molecular pathways involved in cell death, inflammation, DNA repair, and cell cycle regulation etc. (Hecht and Szabo, 2014, Hecht, 2003, Gao et al., 2014, Schierl et al., 2014). Further, constitutive activation of STAT5-cyclin D1 pathway was found to be involved in tobacco mediated oral carcinogenesis (Mishra and Das, 2005). In addition to this, tobacco consumption is known to cause deregulation of several other signalling cascades responsible for the malignant transformation (Hecht and Szabo, 2014, Hecht, 2003, Xue et al., 2014). Moreover, reportedly 33% of Indian population regardless of age, were addicted to smokeless tobacco according to a Global Adult Tobacco Survey (GATS 2010) released in October 2010 suggesting the Indian

population to pose a major oral cancer risk. Additionally, in our previous chapter we have found TIPE proteins to be differentially expressed in oral cancer tissues compared to normal tissues. Therefore, it is important to study the effect of the crude smokeless tobacco extract on the regulation of these proteins in order to find the cause behind the deregulation of TIPE proteins in oral cancer. Further increasing our inquisitiveness, treatment of oral cancer cells (SAS and KB) for 24 h with non-toxic concentrations of the crude tobacco extract (TE) as obtained from MTT assay (Figure 3.1A) has given us interesting results (Figure 3.1). In case of SAS cells TE treatment induced minimal change in the expression of TIPE, TIPE1 and TIPE2 levels whereas expression of TIPE3 was several fold increased in response to TE (Figure 3.1B and C). In contrast to this, treatment of KB cells with TE significantly upregulated the expression of TIPE and TIPE2; downregulated TIPE1; and exerted very minimal effect on TIPE3 (Figure 3.1B and D) suggesting crude tobacco extract to target different proteins depending on the cell type. Further, it has been reported that chronic exposure of oral mucosa to tobacco induces genetic instability and activates various proliferative genes such as Cox-2, EGFR and Cyclin D1 etc. and inhibit the tumor suppressor genes such as p53 thus increasing the cell proliferation ultimately leading to the development of oral preneoplastic lesions and invasive oral carcinoma (Pérez-Sayáns et al., 2009). Moreover, nearly 90% of oral cancers were identified to possess genetic alterations that affect retinoblastoma or cyclin D1 or p16 genes (Brinkman and Wong, 2006). Interestingly, upregulation of TIPE was also reported to inhibit p53 and increase the expression of cyclin D1 and pRb genes in lung and colon cancers (Lowe et al., 2017, Miao et al., 2012, Padmavathi et al., 2018). Similarly, in pancreatic cancer, TIPE upregulation was observed to be correlated with increased EGFR levels (Liu et al., 2012). Therefore, TE induced upregulation of TIPE as

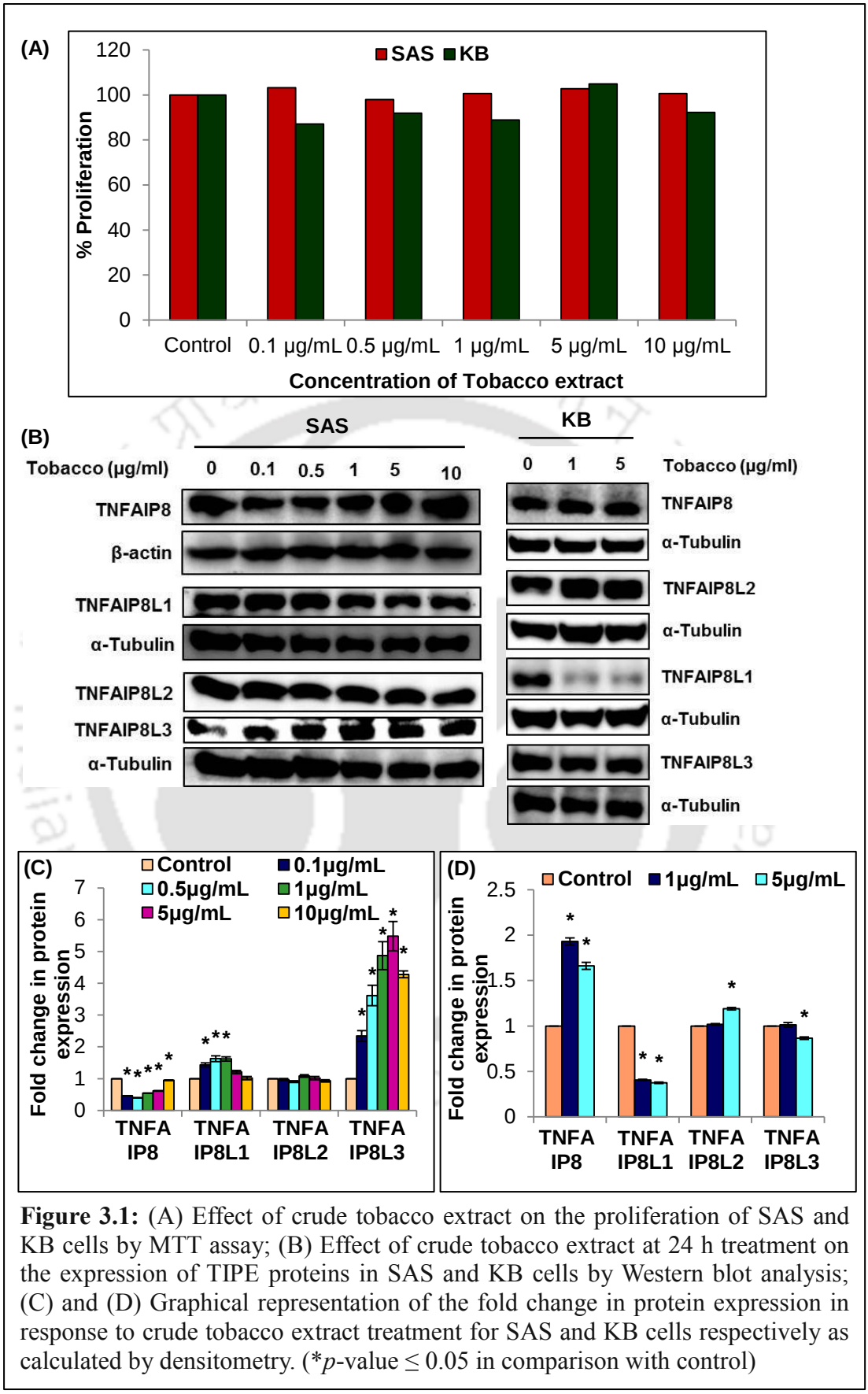


Figure 3.1: (A) Effect of crude tobacco extract on the proliferation of SAS and KB cells by MTT assay; (B) Effect of crude tobacco extract at 24 h treatment on the expression of TIPE proteins in SAS and KB cells by Western blot analysis; (C) and (D) Graphical representation of the fold change in protein expression in response to crude tobacco extract treatment for SAS and KB cells respectively as calculated by densitometry. (*p-value ≤ 0.05 in comparison with control)

observed in KB cells might regulate oral carcinogenesis through the upregulation of EGFR, cyclin D1 and pRb or inhibition of p53. In addition, the significant downregulation of TIPE1 observed in TE treated KB cells supported our IHC results where the expression of TIPE1 was downregulated with disease progression indicating the loss of TIPE1 to be an additional molecular event responsible for tumor growth and progression. In line with this, previous studies have also shown TIPE1 to be a potent anti-tumor molecule in cancers of lung and liver, downregulation of which increases tumor growth, proliferation, invasion and migration and reduces apoptosis in these cancers (Wu et al., 2017a, Zhang et al., 2015a). Similarly, upregulation of TIPE2 observed in KB cells was also in correlation with our IHC results. Moreover, overexpression of TIPE2 was earlier shown to be associated with progression of TNM staging and lymph node metastasis of renal cell carcinoma (RCC) and colon cancer (Li et al., 2014, Zhang et al., 2013c). Therefore, our finding in addition to the previous reports on colon and renal cancers suggest a possible involvement of TIPE2 in TE mediated oral carcinogenesis. Furthermore, a 5-fold increase in the expression of TIPE3 was observed in TE treated SAS cells (Figure 3.1C). As per the recent reports, TIPE3 is a carrier protein of phosphatidyl inositol (PIP) signals that have been known to activate multiple signalling cascades including PI3K/Akt pathway which in turn would result in the activation of cell survival, proliferative and anti-apoptotic molecules thus contributing to tumorigenesis (Fayngerts et al., 2014, Ye et al., 2013, Du et al., 2012). Therefore, we hypothesized that exposure to crude TE increases the levels of TIPE3 in oral cancer cells which would result in increased PIP signals ultimately leading to the activation of downstream pathways and contributing to tumor development and progression.

3.3.2 Exposing oral cancer cells to tuibur modulated the protein levels of TIPE family

Tuibur is a water-soluble tobacco smoke extract, consumed most frequently by the people of Mizoram, India. It is prepared by passing the tobacco smoke through water until it turns cognac-like in colour and gives pungent smell. It is commonly used by women of north-east region of India including Assam, Arunachal Pradesh, Mizoram, Meghalaya, Manipur, Sikkim, Nagaland and Tripura for gargling or sipping (Mahanta et al., 1998, Sinha et al., 2003). Since, it is produced from the tobacco smoke tuibur is also expected to contain the tobacco smoke related carcinogens. Further assuring its carcinogenic activity, reports have shown a correlation between regular use of tuibur and increased risk of stomach cancer (Phukan et al., 2005, Malakar et al., 2012). However, thus far only three reports are available on the effect of tuibur in tumorigenesis. As tuibur is used by keeping it in mouth for some time and spitting, we believe that it would have a carcinogenic effect on the oral mucosa similar to that of crude smokeless tobacco. Therefore, we have analysed the effect of tuibur on the TIPE protein levels in oral cancer. Initially, the non-toxic concentrations were estimated by MTT assay (Figure 3.2A) and the SAS and KB cells were treated with the selected concentrations of tuibur for 24 h before isolating total protein. Western blot analysis of tuibur treated oral cancer cells revealed that the expression of TIPE and TIPE3 were significantly upregulated and TIPE1 was downregulated in both the cell lines (Figure 3.2B-D). However, the TIPE2 levels were remarkably increased in tuibur treated SAS cells whereas the expression in KB cells was upregulated in lower concentration of tuibur and the effect on TIPE2 expression was seem to be lost in KB cells at higher concentrations of tuibur (Figure 3.2B and D). Effect of tuibur on TIPE proteins is in consistence with that of TE thus further confirming our hypothesis of

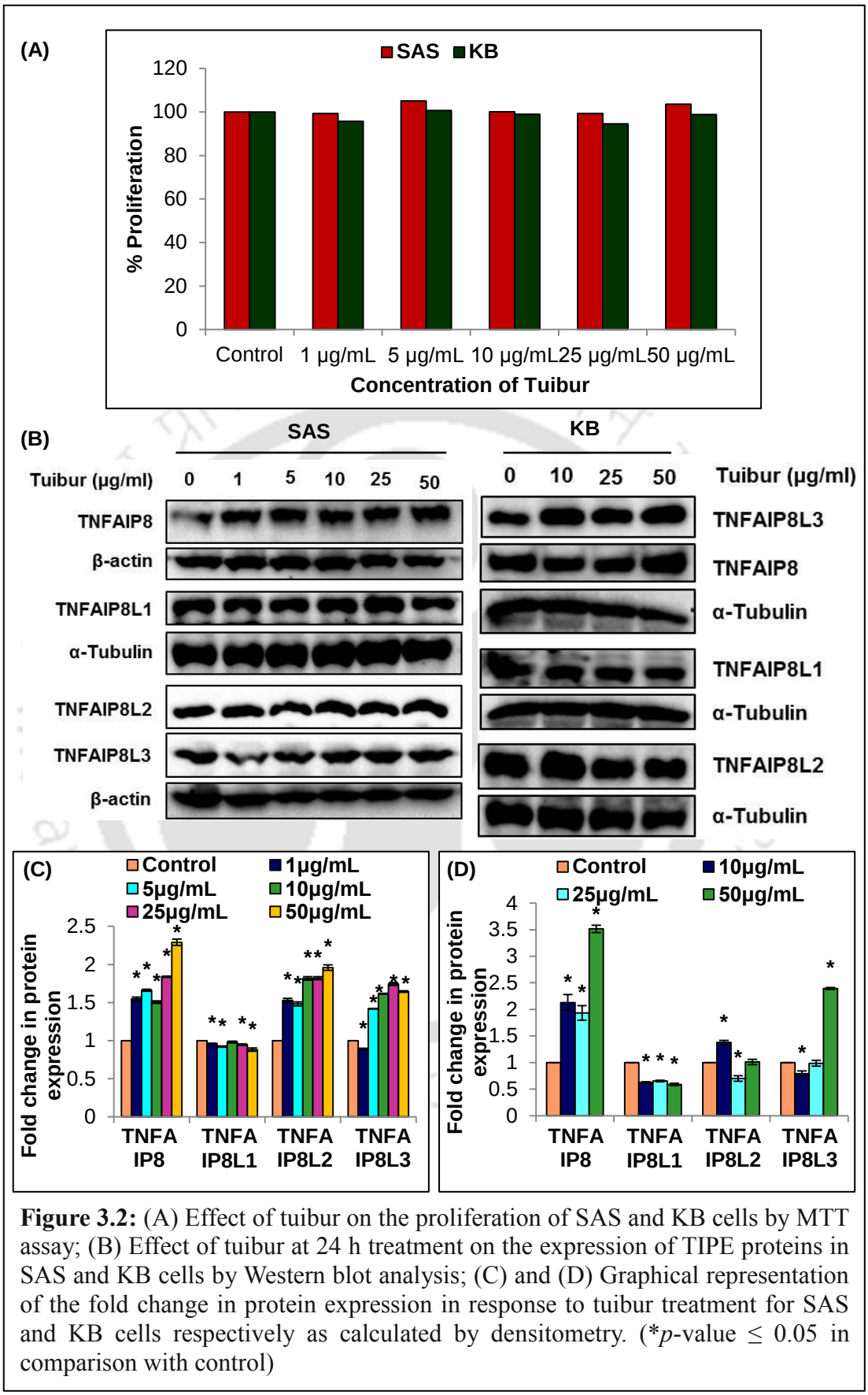


Figure 3.2: (A) Effect of tuibur on the proliferation of SAS and KB cells by MTT assay; (B) Effect of tuibur at 24 h treatment on the expression of TIPE proteins in SAS and KB cells by Western blot analysis; (C) and (D) Graphical representation of the fold change in protein expression in response to tuibur treatment for SAS and KB cells respectively as calculated by densitometry. (*p-value $\leq$ 0.05 in comparison with control)

TIPE, TIPE2 and TIPE3 having oncogenic and TIPE1 having anti-oncogenic role in oral carcinogenesis. Also, we presume that the downstream targets of TIPE proteins in response to tuibur would also be similar to that of TE. However, it has to be studied thoroughly as there is a severe lack of knowledge on the effect of tuibur in cancer related signaling molecules and its importance in the development and progression of oral cancer.

In the previous sections of the current chapter we have established the effect of crude tobacco extract and tuibur (tobacco smoke infused water) on the expression of TIPE proteins in oral cancer cells. However, aforementioned, both tobacco smoke and smokeless tobacco contain more than 70 identified carcinogens each having their own mode of action and molecular targets in the process of carcinogenesis. Moreover, as observed in the earlier sections, both TE and tuibur modulated the expression of TIPEs to a different extends in context to both concentration and cell type. Therefore, we believed that in addition to the crude tobacco, determining effect of the individual tobacco carcinogens on TIPE proteins is also mandatory. In the current study, we focussed on two tobacco carcinogens: nicotine and BaP.

3.3.3 Treatment of oral cancer cells with nicotine resulted in the deregulation of TIPE protein family

Nicotine present in tobacco is known to be the major compound responsible for addiction (Nishioka et al., 2018). Even though, there have been debates on the carcinogenic effect of nicotine, several reports have also proved the carcinogenicity of nicotine. For instance, at alkaline pH, it was found to exist in non-ionized form which can be easily absorbed across the epithelium of oral mucosa, lung, nose, and skin and known to induce several oncogenic signalling cascades (Sanner and Grimsrud, 2015). In addition, the nicotine induced carcinogenesis was found to be mediated through the

nicotinic acetylcholine receptors (nAChRs) as it exerts several fold more affinity to nAChRs than the neurotransmitter acetylcholine. Further, this competitive binding of nicotine to nAChRs was shown to result in the excessive secretion of epidermal growth factor (EGF) which in turn activates EGFR. In addition, it was also reported to activate several oncogenic, mitogenic and anti-apoptotic signaling pathways; increase the secretion of VEGF and arachidonic acid and promote cancer cell proliferation, invasion and angiogenesis (Sanner and Grimsrud, 2015, Nishioka et al., 2018). In line with this, nicotine treatment was also proved to activate PI3K/Akt and ERK pathways through increased phosphorylation of EGFR in human oral squamous cell carcinoma cell line (HSC-2) leading to increased cell proliferation (Nishioka et al., 2018). Further adding to its oncogenic potential once absorbed, nicotine is metabolized into its derivative nitrosamines such as NNN and NNK. Besides being nicotine derivatives NNN and NNK are also direct tobacco specific nitrosamines (TSNAs) present mainly in tobacco smoke. These nitrosamines were earlier reported to be strongly associated with the development of various cancers including lung cancer, oral cancer and esophageal cancer (Hecht and Hoffmann, 1988, Minna, 2003, Nishioka et al., 2010). Furthermore, nicotine was also reported to induce tumor growth, progression, invasion, metastasis and inhibit apoptosis through activation of JAK/STAT, PI3K/Akt, Ras/Raf/MEK/ERK and deregulation of calcium homeostasis (Grando, 2014). Further interestingly, the TIPE proteins were also found to be involved in the regulation of cell proliferation, apoptosis, invasion and migration denoting the significance of analysing the effect of nicotine on the expression of these proteins (Padmavathi et al., 2018). Therefore, we treated the oral cancer cells with non-toxic doses of nicotine as observed from the MTT assay (Figure 3.3B) and analysed the expression of TIPE proteins using Western blot. Interestingly, our results showed that

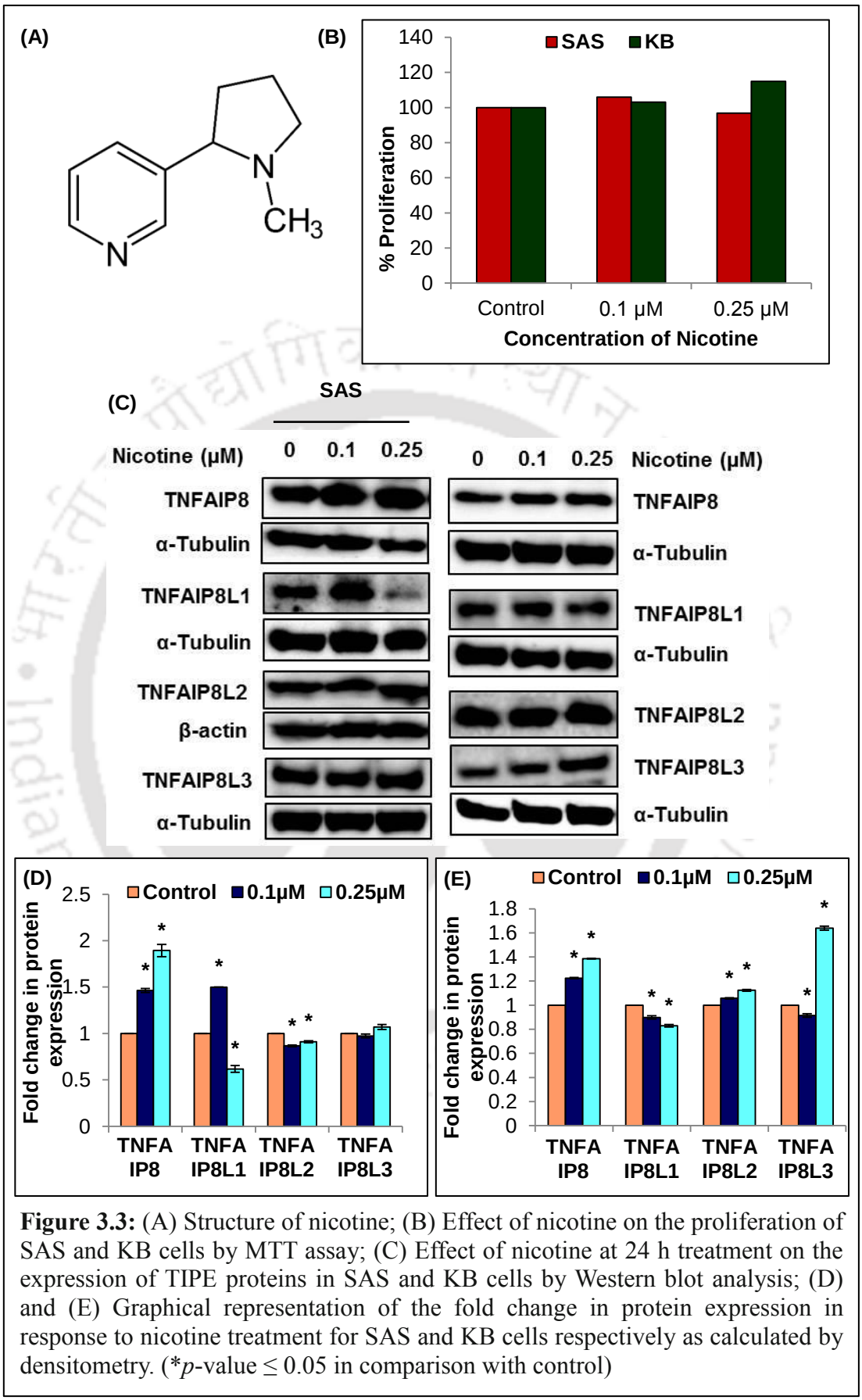


Figure 3.3: (A) Structure of nicotine; (B) Effect of nicotine on the proliferation of SAS and KB cells by MTT assay; (C) Effect of nicotine at 24 h treatment on the expression of TIPE proteins in SAS and KB cells by Western blot analysis; (D) and (E) Graphical representation of the fold change in protein expression in response to nicotine treatment for SAS and KB cells respectively as calculated by densitometry. (*p-value ≤ 0.05 in comparison with control)

nicotine treatment induces the overexpression of TIPE and downregulation of TIPE1 in both SAS and KB cells (Figure 3.3C-E). Further, it exerted a minimal or no effect on the expression of TIPE2 and TIPE3 in SAS cells whereas a significant upregulation of TIPE3 was observed in KB cells (Figure 3.3C-E). These results suggest that similar to crude tobacco, the individual compound nicotine also shows a cell type specific activation of TIPE, TIPE2 and TIPE3 and inhibition of TIPE1. Moreover, as we already discussed, nicotine exhibits anti-apoptotic and proliferative effect through activation of PI3K/Akt and NF-κB pathways and the TIPE and TIPE3 proteins were also reported to exhibit proliferative and anti-apoptotic effect through the regulation of similar signaling cascades (Grando, 2014, Padmavathi et al., 2018). Therefore, nicotine induced overexpression of these protein could be an earlier event in the nicotine-mediated activation of PI3K/Akt and MAPK signaling. Similarly, TIPE1 is a known pro-apoptotic protein downregulation of which results in the inhibition of several pro-apoptotic genes (Padmavathi et al., 2018). Thus, nicotine mediated downregulation of TIPE1 is expected to offer apoptosis resistance and proliferation to the cancer cells leading to increased progression. Furthermore, TIPE and TIPE3 were reported to be the activators of NF-κB and TIPE1 as inhibitor of NF-κB (Padmavathi et al., 2018). And nicotine is an NF-κB activator thus further assuring the involvement of TIPE proteins in nicotine-mediated oral carcinogenesis.

3.3.4 Treatment of oral cancer cells with BaP resulted in the deregulation of TIPE protein family

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) and a potential oral carcinogen present in tobacco smoke. In addition to tobacco smoke, BaP has also been found in coal combustion, motor-vehicle exhaust and industrial emissions etc. (Demetriou et al., 2018). Several preclinical studies have found that BaP is converted

to its reactive metabolites. Most of these BaP derived metabolites were found to be either carcinogenic or mutagenic that forms DNA adducts causing chromosomal aberrations, micronuclei or DNA damage and shown to induce different types of cancers *in vivo* (Demetriou et al., 2018). Further, BaP through its active metabolites was reported to contribute for different hallmarks of cancer. For example, BaP was shown to induce cancer cell proliferation through mutations and activation of Ras family of genes, EGFR and ERK pathways (Demetriou et al., 2018, Meng et al., 2010, Wei et al., 1999, Kometani et al., 2009). Similarly, BaP was also found to critically regulate apoptotic resistance and angiogenesis through modulation of apoptosis related genes and upregulation of hypoxia-inducible factor-1α. However, conflicting studies question the actual role of BaP in both apoptosis resistance and angiogenesis (Demetriou et al., 2018). Furthermore, BaP was also proved to induce inactivating p53 mutation in lung tumors (Liu et al., 2005). Additionally, it was also proved to induce invasion, migration and metastasis in lung, breast and liver cancers through elevated pro-inflammatory cytokines viz. IL8, CCL-2 and CCL-3 levels, MMP-2 and -9 and activation of NF-κB pathway (Demetriou et al., 2018). Moreover, BaP was also used to induce oral cancer in experimental animals (Janbaz et al., 2014, Nebert et al., 2013). Therefore, assessment of the effect of BaP on the expression of TIPE proteins would provide concrete evidence for the involvement of these proteins in BaP induced oral cancer. First the non-toxic concentrations of BaP were determined by MTT assay (Figure 3.4B) and both SAS and KB cells were exposed to the selected doses of BaP for 24 h. Western blot analysis revealed that BaP treatment of SAS cells significantly upregulates the levels of TIPE2 and TIPE3 and downregulated TIPE1 however, does not show any remarkable effect on TIPE expression (Figure 3.4C and D). In contrast to this, BaP treated KB cells had increased levels of TIPE and TIPE2

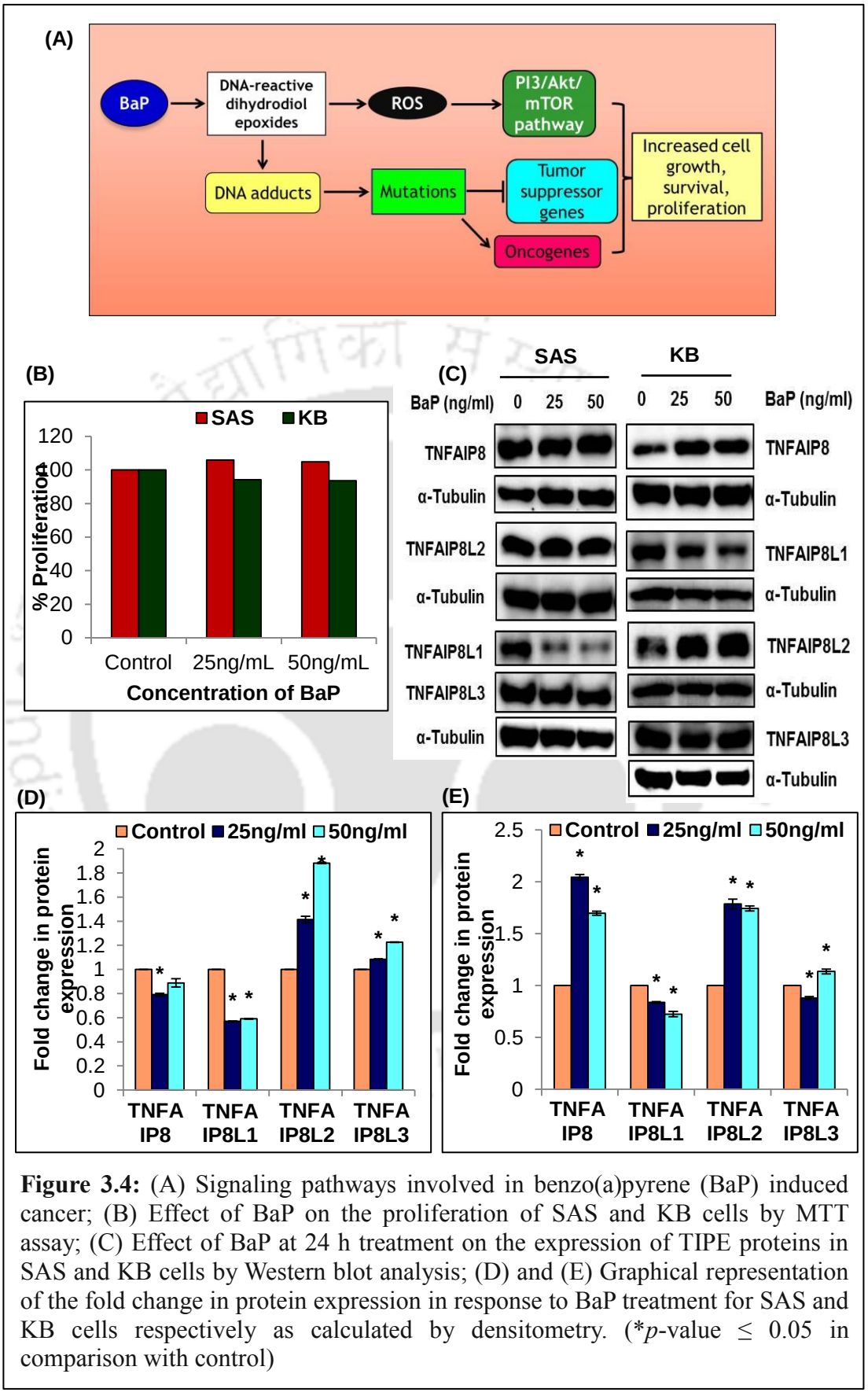


Figure 3.4: (A) Signaling pathways involved in benzo(a)pyrene (BaP) induced cancer; (B) Effect of BaP on the proliferation of SAS and KB cells by MTT assay; (C) Effect of BaP at 24 h treatment on the expression of TIPE proteins in SAS and KB cells by Western blot analysis; (D) and (E) Graphical representation of the fold change in protein expression in response to BaP treatment for SAS and KB cells respectively as calculated by densitometry. (*p-value $\leq$ 0.05 in comparison with control)

and decreased levels of TIPE1 whereas the expression of TIPE3 was minimally affected (Figure 3.4C and E). Therefore, yet again like other tobacco and related carcinogens, effect of BaP is also seem to be cell type specific. Supporting this, earlier reports have also shown that effect of BaP depends on the growth kinetics of a cell population, type of tissue and genetic differences (Hamouchene et al., 2011, Demetriou et al., 2018). Together, our results supported the IHC results showing upregulation of TIPE, TIPE2 and TIPE3 and downregulation of TIPE1 in response to BaP.

3.3.5 Exposure of oral cancer cells to 4NQO is also modulated the expression of TIPE proteins

4-Nitroquinoline 1-Oxide (4NQO) is a water soluble synthetic carcinogen widely known to induce oral carcinogenesis in rats and mice. It was reported to form DNA adducts and generate reactive oxygen species resulting in mutations and DNA strand breaks (Tang et al., 2004). Further, it was also shown to increase the mRNA levels of several inflammatory cytokines like TNF-α, IFN-γ, IL-1β, IL-6, IL-17, IL-8, iNOS and Cox-2 which mediate carcinogenesis (Tanaka et al., 2014). Moreover, 4NQO induced animals are the widely used models to study the molecular mechanisms involved in different stages of oral carcinogenesis (Nauta et al., 1996). Therefore, examining the effect of 4NQO on the expression of TIPE proteins would give new insights into the 4NQO-induced oral carcinogenesis and prove our hypothesis of TIPE proteins being involved in the development and progression of oral cancer. Firstly, we examined the effect of 4NQO on the proliferation of SAS and KB cells by MTT assay in order to determine the non-toxic doses (Figure 3.5B). Later, the cells were exposed to the selected concentrations of 4NQO for 24 h and the effect on protein levels of TIPE, TIPE1, TIPE2 and TIPE3 were examined. Our results showed that 4NQO

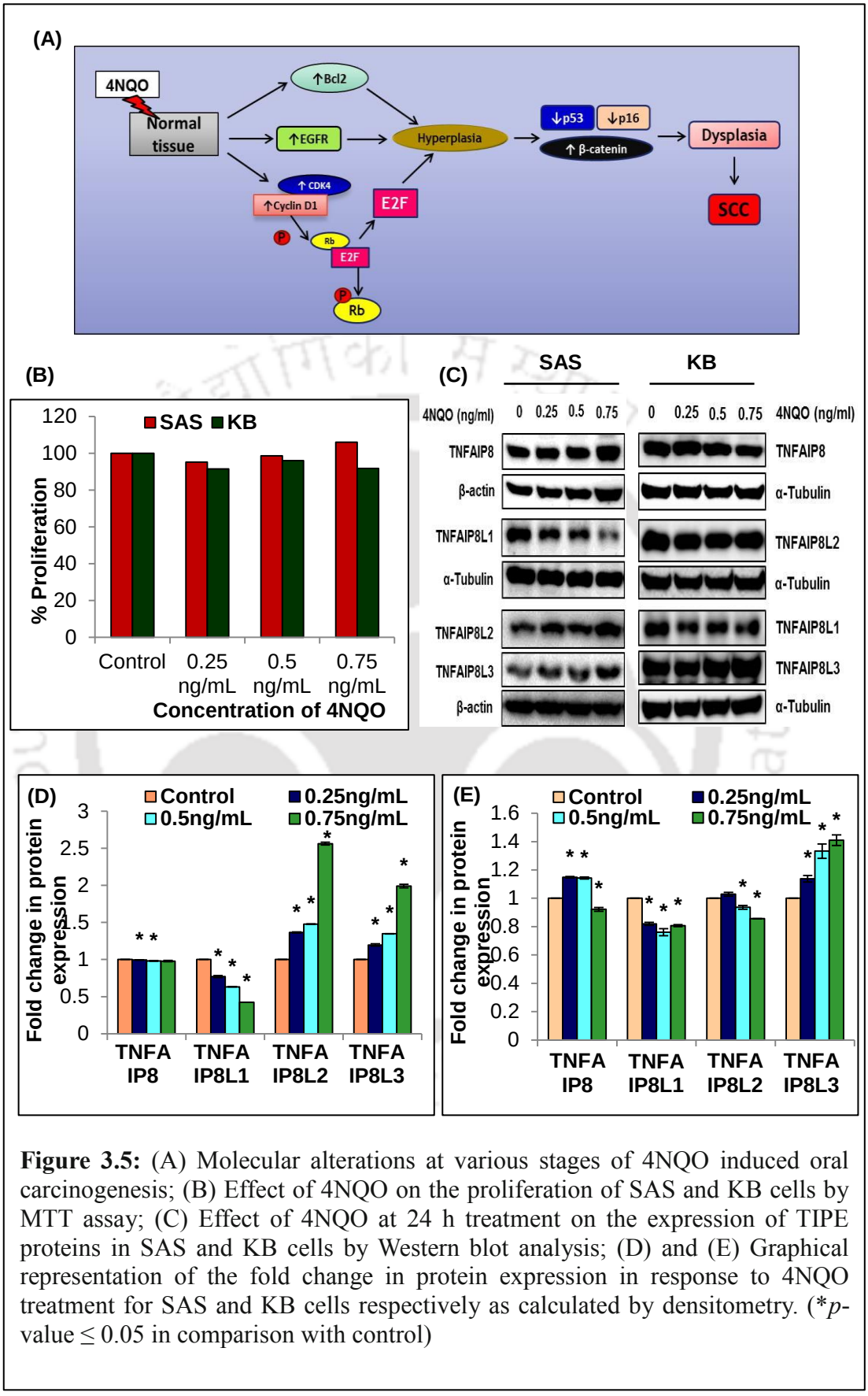


Figure 3.5: (A) Molecular alterations at various stages of 4NQO induced oral carcinogenesis; (B) Effect of 4NQO on the proliferation of SAS and KB cells by MTT assay; (C) Effect of 4NQO at 24 h treatment on the expression of TIPE proteins in SAS and KB cells by Western blot analysis; (D) and (E) Graphical representation of the fold change in protein expression in response to 4NQO treatment for SAS and KB cells respectively as calculated by densitometry. (*p-value ≤ 0.05 in comparison with control)

significantly increased the protein levels of TIPE2 and TIPE3; decreased TIPE1 level and exerted minimal effect on TIPE in case of SAS cells (Figure 3.5C and D). However, KB cells showed remarkable upregulation of TIPE3; downregulation of TIPE1 and minimal changes in TIPE and TIPE2 in response to 4NQO (Figure 3.5C and E). Interestingly, 4NQO has been shown to induce the development of oral hyperplasia through upregulation of EGFR, cyclin D1, cdk4 and Bcl-2 and phosphorylation of Rb. Further accumulation of β-catenin and downregulation of tumor suppressor genes p53 and p16 were reported to be associated with the development of dysplasia and SCC (Kanojia and Vaidya, 2006). Similarly, results of our previous chapter suggested deregulation of TIPE proteins to be associated with disease progression from hyperplasia to SCC. In addition, TIPE proteins were also found to regulate the expression of above mentioned genes (Padmavathi et al., 2018). Further adding to this, expression of TIPE1, TIPE2 and TIPE3 proteins were also modulated in response to 4NQO treatment. Therefore, our results combined with the previous studies suggest a strong correlation between TIPE proteins and 4NQO-induced oral cancer. Nevertheless, the very minimal effect of 4NQO on TIPE observed in both oral cancer cells questions the significance of this protein in 4NQO-mediated oral carcinogenesis.

3.4 Conclusion

Altogether, in the current chapter for the first time we have established a correlation between tobacco-related carcinogens and regulation of TIPE proteins in oral cancer. Interestingly, most of the tobacco carcinogens including crude tobacco extract, tobacco infused water, nicotine, BaP and synthetic carcinogen 4NQO, resulted in the upregulation of TIPE, TIPE2 and TIPE3 proteins and downregulation of TIPE1. This supports our IHC results showing downregulation of TIPE1 and upregulation of other

three family members in oral cancer tissues compared to normal. Therefore, we presumed that deregulation of TIPE proteins to be one of the key molecular events in the development and progression of oral cancer as tobacco, the major risk factor of oral cancer, modulated the levels of these proteins. Nevertheless, our findings has given only a preliminary confirmation for the involvement of TIPE proteins in tobacco induced oral cancer hence warranting detailed studies on the upstream regulators and downstream targets of TIPE proteins in tobacco mediated oral carcinogenesis.

CHAPTER 4

Role of TNFAIP8 (TIPE) Proteins in the Regulation of Oral Cancer

4.1 Introduction

In our preliminary studies we have observed a differential expression of TIPE proteins in oral premalignant and malignant tissues. In brief, expression of TIPE, TIPE2 and TIPE3 were upregulated and TIPE1 was downregulated in oral cancer tissues compared to normal tissues. Additionally, crude tobacco and tobacco carcinogens, the prime risk factor for oral cancer, also induced the expression of TIPE, TIPE2 and TIPE3 and decreased TIPE1 expression in oral cancer cells. Furthermore, the synthetic carcinogen 4NQO which has been successfully used to mimic the developmental stages of oral cancer in experimental animal models also resulted in the upregulation of TIPE2 and TIPE3 and downregulation of TIPE1 in oral cancer cells. Therefore, collectively, results of our preliminary studies proposed that deregulation of TIPE family of proteins to have vital role in the development and maintenance of oral cancers. Further, in order to prove our hypothesis and to identify the downstream targets of TIPE proteins involved in the regulation of oral cancer, it is mandatory to silence the expression of TIPE proteins. Earlier, several studies have shown the silencing of TIPE proteins to greatly impact cell growth, proliferation, invasion, migration and metastasis of various cancers both *in vitro* and *in vivo* (Padmavathi et al., 2018, Bordoloi et al., 2018). Therefore, in the current chapter, we knockout the expression of TIPE, TIPE1, TIPE2 and TIPE3 individually in oral tongue carcinoma cell line (SAS) using CRISPR/Cas9-mediated knockout and determined the effect of gene knockout on the proliferation, colony formation, migration and cell cycle progression of SAS cells *in vitro*. Also, we identified the downstream targets of TIPE proteins involved in the regulation of oral carcinogenesis.

4.2 Materials and methods

4.2.1 Cell culture:

The human tongue squamous cell carcinoma cell line, SAS was used for gene knockout study. SAS cells were obtained from the Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco™; Life Technologies, NY, USA) enhanced with 10% fetal bovine serum (FBS; Gibco®, NY, USA) and 1X Pen-Strep (Invitrogen, CA, USA) and maintained at 37 °C in a humidified, CO_2-regulated (95% air/5% CO_2 atmosphere) incubator.

4.2.2 CRISPR/Cas9-mediated gene knockout:

Expression of TIPE, TIPE1, TIPE2 and TIPE3 genes was impaired using the CRISPR/Cas9 gene editing. For this purpose, commercially available CRISPR/Cas9 All-in-One Lentivector sets (Human) expressing both human Cas9 and respective sgRNAs viz. TNFAIP8 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414505), TNFAIP8L1 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414605), TNFAIP8L2 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414705), TNFAIP8L3 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat. No. K2414805) and Scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (Cat. No. K010) were purchased from Applied Biological Materials, Richmond, BC, Canada. The sgRNA target sequences are provided in Table 4.1. In brief, SAS cells were plated in a 24 well plate at a density of 25000 cells/500 µL/well and allowed to grow until 70-80% confluency. After reaching the desired confluency, the cells were transfected with 1 µg of respective plasmids using Lentifectin™ transfection reagent (Cat. No. G074, Applied Biological Materials, Richmond, BC, Canada) in incomplete opti-MEM media and incubated in a 37 °C CO_2-regulated (95% air/5% CO_2 atmosphere) incubator for 5-8 h. Following the 5-8 h incubation, 10% FBS (Gibco®, NY, USA) was added to the wells and

incubated for 24 h. At the end of 24 h, the plasmid containing media was replaced with fresh complete DMEM and allowed to recover for 24 h. Post 24 h recovery, 2.5 µg/mL of puromycin (Cat. No. P8833, Sigma-Aldrich, Missouri, USA) was added to the cells for positive selection. As the CRISPR/Cas9 plasmids carry puromycin resistance gene only the transfected cells will be resistant to puromycin. Therefore, the puromycin resistant cells were allowed to form colonies before proceeding for single cell selection. Selected single cell clones were screened for gene knockout by Western blotting. Cells that carried complete inhibition of respective proteins were cloned further and used for further studies.

Table 4.1: sgRNA target sequences

Gene	Target	sgRNA target sequence
TNFAIP8 (TIPE)	Target 1	ACTTGTGTCGTCTATTAAGG
	Target 2	TCATCAGCTTGCTATGACCG
	Target 3	CAAAGGTATAATCCACCTGA
TNFAIP8L1 (TIPE1)	Target 1	TGAGCTGTACCGCGCCACCA
	Target 2	GTCCACCTGGTGGAAGCTGA
	Target 3	CACCTGACCGCCAAGTCCCA
TNFAIP8L2 (TIPE2)	Target 1	CCAAGGAGTACACGCACAGC
	Target 2	CAGGTCCTTGATCACGCGCT
	Target 3	CCCGCTTTCGCCAGAAGCTG
TNFAIP8L3 (TIPE3)	Target 1	AACGGATATGCAGGGACCCA
	Target 2	ACGTGGCATCCCTTGTGCCT
	Target 3	CGGAATCCGAATCCATGCTG
Scrambled	-	GCACTCACATCGCTACATCA

4.2.3 MTT assay:

MTT assay was performed to determine the proliferation of TIPE, TIPE1, TIPE2 and TIPE3 knockout SAS cells. In short, the scrambled sgRNA transfected cells (denoted as scrambled or SCR here after), TIPE knockout cells (denoted as Cr TNFAIP8 here after), TIPE1 knockout (denoted as Cr TNFAIP8L1 here after), TIPE2 knockout cells (denoted as Cr TNFAIP8L2 cells here after) and TIPE3 knockout cells (denoted as Cr TNFAIP8L3 cells here after) were seeded in 96 well plates at a density of 2000 cells/100 µL/well in and allowed to grow for 0 and 72 h. 10 µL of 5 mg/mL MTT (Cat. No. M2128, Sigma-Aldrich, Missouri, USA) was added to the respective plates at the end of 0 and 72 h and incubated for 2 h in a CO_2-regulated 37 °C incubator. Following the 2 h incubation, the MTT containing media was replaced with 100 µL of dimethyl sulfoxide (DMSO) (Cat. No. 1.16743.0521, Merck, Darmstadt, Germany) to dissolve the formazan crystals and incubated in dark for 1 h at RT. After the 1 h incubation, absorbance of the plate was measured at 570 nm using a microplate reader (TECAN Infinite 200 PRO multimode reader). % of proliferation was calculated by normalizing the absorbance of 72 h with 0 h absorbance and keeping the absorbance of scrambled as 100%.

4.2.4 Colony formation assay:

In colony formation assay, the clonogenic potential of a single is being tested which gives an estimate of the survival capacity of the cells. Colony formation assay was performed as reported earlier (Rafehi et al., 2011). In brief, scrambled, Cr TNFAIP8, Cr TNFAIP8L1, Cr TNFAIP8L2 and Cr TNFAIP8L3 cells were plated in six well plates at the density of 500 cells/2 mL/well and allowed to form colonies in a CO_2-regulated 37 °C incubator for at least two weeks with frequent replenishing of media. At the end of two weeks, the media was removed and colonies were washed gently

with 1X PBS and fixed with 70% ethanol for 20 min at -20 °C. After fixation, the colonies were again washed with 1X PBS and stained with 0.01% (w/v) crystal violet. Excessive staining was removed by washing with 1X PBS and the images of the colonies were captured. Later, the number of colonies was counted by analysing the images with the help of ImageJ software and survival fraction was calculated as per the following formula:

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

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

4.2.5 Cell cycle analysis:

Effect of gene knockout of TIPE family proteins on the cell cycle progression of SAS cells was determined by flow cytometry assisted cell cycle analysis. In short, the scrambled, Cr TNFAIP8, Cr TNFAIP8L1, Cr TNFAIP8L2 and Cr TNFAIP8L3 cells were plated in six well plates at a density of 200,000 cells/2 mL/well and allowed to divide for 24 h. At the end of 24 h, the cells were collected by trypsinization; washed with 1X PBS and fixed with 75% ethanol without forming clumps overnight at -20 °C. Just before the analysis, ethanol used for fixing was removed by centrifugation and the cells were washed with 1X PBS and stained with Propidium Iodide (PI)/RNase solution for 20 min in dark. Following the 20 mins incubation, the cells were analysed by flow cytometer (BD FACSCelesta™, Becton-Dickinson, New Jersey, USA) and the percentage of cells in each phase of the cell cycle was analysed using FCS express software.

4.2.6 *In vitro* wound healing assay:

Effect of TIPE genes' knockout on the migration of SAS cells was estimated using a simple *in vitro* wound healing assay. The scrambled, Cr TNFAIP8, Cr TNFAIP8L1, Cr TNFAIP8L2 and Cr TNFAIP8L3 cells were plated in six well plates at a density of

500,000 cells/2 mL/well and allowed to form monolayer. After monolayer formation, media was replaced with serum free DMEM in order to halt cell proliferation and allowed 6-8 h for adaptation. After the 6-8 h serum starvation, a wound was created across the monolayer with the help of a 100 µL micro tip. The detached cells were washed away with 1X PBS and fresh serum free DMEM was added to the cells. Finally, images of the wounds were captured using an inverted Nikon light microscope and a camera.

4.2.7 Western blot analysis:

The successful gene knockout and its downstream targets were determined by Western blotting. In brief, the total protein lysates were prepared from the scrambled, Cr TNFAIP8, Cr TNFAIP8L1, Cr TNFAIP8L2 and Cr TNFAIP8L3 cells 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% or 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel with 5X Laemmli Buffer (250 mM TrisHCl, 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 h 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 Optiblot ECL Detection Kit (Cat. No. ab133406; Abcam, Cambridge, USA) and ChemiDoc™ XRS System (Bio-Rad, California, USA). The housekeeping genes GAPDH, β-actin and α-Tubulin were used as loading controls. Table 4.2 summarizes the primary and secondary antibody details used for the analysis.

Table 4.2: Details of the primary and secondary antibodies used in Western blot analysis

Antibody	Cat. No.	Company	Dilution
Anti-TNFAIP8 antibody	ab64988	abcam®, Cambridge, USA	1: 2000
Anti-TNFAIP8L1 antibody	ab85409	abcam®, Cambridge, USA	1: 1000
Anti-TNFAIP8L2 antibody	ab110389	abcam®, Cambridge, USA	1: 8000
Anti-TNFAIP8L3 antibody	ab111524	abcam®, Cambridge, USA	1: 4000
Anti-α-Tubulin antibody	2144S	Cell Signaling Technology, Massachusetts, USA	1: 4000
Anti-β-actin antibody	4967S	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-GAPDH antibody	2118S	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- Akt (Ser473) antibody	4060S	Cell Signaling Technology, Massachusetts, USA	1: 4000
Anti- Akt1 antibody	2938S	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- mTOR protein (Ser2448) antibody	5536T	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-mTOR antibody	2983T	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- S6 Ribosomal protein (Ser235/236) antibody	4858T	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-S6 Ribosomal protein-antibody	2317S	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- STAT3	9134T	Cell Signaling Technology,	1: 2000

(Ser727) antibody		Massachusetts, USA	
Anti-STAT3 antibody	9139T	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-Phospho- NF-κB p65 (Ser536) antibody	3033P	Cell Signaling Technology, Massachusetts, USA	1: 5000
Anti- NF-κB p65 antibody	8242P	Cell Signaling Technology, Massachusetts, USA	1: 4000
Anti-p53 antibody	2524T	Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-PTEN antibody	11-7539	ABGENEX pvt. Ltd., Odisha , India	1: 1000
Anti-REDD1 antibody	2516S	Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-LC3B antibody	2775S	Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-Bcl-2 antibody	15071	Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-Cox-2 antibody	12282P	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-survivin antibody	2808BC	Cell Signaling Technology, Massachusetts, USA	1: 2000
Anti-XIAP antibody	20-1106	ABGENEX Pvt. Ltd., Odisha , India	1: 1000
Anti-cIAP-1/HIAP-2 antibody	20-1054	ABGENEX Pvt. Ltd., Odisha , India	1: 1000
Anti-CXCR4 antibody	ab124824	abcam®, Cambridge, USA	1: 2000
Anti-MMP-9 antibody	13667P	Cell Signaling Technology, Massachusetts, USA	1: 1000
Anti-VEGFA antibody	ab46154	abcam®, Cambridge, USA	1: 4000
Anti-mouse secondary antibody	ab97040	abcam®, Cambridge, USA	1: 4000
Anti-rabbit secondary antibody	ab97080	abcam®, Cambridge, USA	1: 10000

4.2.8 Statistical analysis:

Statistical analysis was performed using simple Student's *t*-test and p-value ≤ 0.05 was considered as statistically significant and all the data are represented in Mean±SE.

4.3 Results and discussion

It has been now well established that development of cancer is multifaceted and it requires several genetic and epigenetic anomalies for the normal cells to become cancerous. Such anomalies at the molecular level were reported to greatly influence the regulation of normal cellular processes such as survival, proliferation, cell cycle progression, cell death etc. Further assuring this, Hanahan and Weinberg, (2011) described certain hallmarks that are commonly associated with cancer such as non-stop proliferation, escape from growth inhibition, resistant to cell death, replicative immortality, angiogenesis, invasion and metastasis, escape from the immune system and reprogrammed energy metabolism that are acquired during the course of tumorigenesis (Hanahan and Weinberg, 2011). Interestingly, so far in our study we have established a correlation of TIPE family of proteins with the development and progression of oral cancer. However, to confirm our argument it is essential to prove the involvement of TIPE proteins in the regulation of cancer hallmarks. In order to achieve this, we used CRISPR/Cas9 based gene silencing technique to knockout TIPE proteins in SAS cells, a human tongue squamous cell carcinoma cell line.

4.3.1 Successful gene knockout was confirmed by Western blot

The TIPE proteins knockout SAS cell clones were generated using CRISPR/Cas9-mediated gene editing. For all the four proteins, transfection with target 2 sgRNA/Cas9 plasmids generated successful knockout clones and a scrambled sgRNA was used to generate SCR control cells. Further, knockout of TIPE proteins was confirmed by Western blot (Figure 4.1) in comparison with SCR and these clones were multiplied and used for further studies.

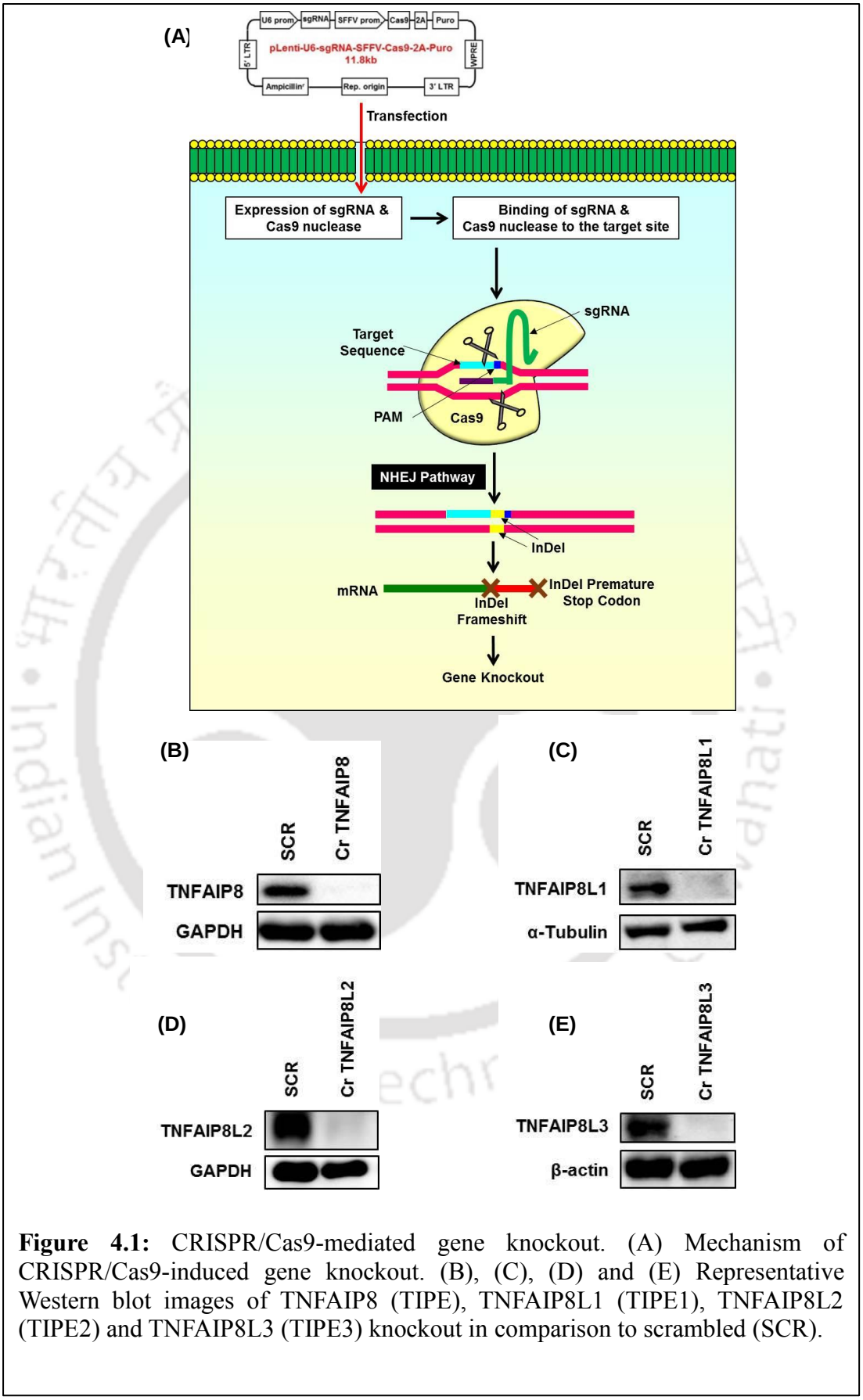


Figure 4.1: CRISPR/Cas9-mediated gene knockout. (A) Mechanism of CRISPR/Cas9-induced gene knockout. (B), (C), (D) and (E) Representative Western blot images of TNFAIP8 (TIPE), TNFAIP8L1 (TIPE1), TNFAIP8L2 (TIPE2) and TNFAIP8L3 (TIPE3) knockout in comparison to scrambled (SCR).

4.3.2 TIPE proteins play key role in the regulation of oral cancer cell proliferation

As defined by Hanahan and Weinberg, (2011), uncontrolled proliferation is the first and foremost hallmark of cancers. Therefore, initially we examined the effect of gene knockouts on the proliferation of SAS cells by MTT assay. As anticipated, the TIPE, TIPE2 and TIPE3 knockout clones exhibited significantly reduced proliferation compared to SCR control cells. However, the percentage of inhibition of proliferation was found to be different for all the three clones with TIPE3 knockout exhibiting highest inhibition (~75%) and TIPE2 knockout showing lowest inhibition (~30%). Further, the TIPE knockout cells were having an inhibition of 40% (Figure 4.2A, C and D). In contrast to this, silencing of TIPE1 protein was associated with a remarkable increase in the proliferation of SAS cells. The proliferation rate was almost doubled in TIPE1 knockout cells (Figure 4.2B). Therefore, these results supported our earlier results proving TIPE, TIPE2 and TIPE3 as oncogenic and TIPE1 as anti-oncogenic proteins. Further, our results of TIPE, TIPE1 and TIPE2 knockouts on oral cancer proliferation were in accordance to the previous reports showing the role of TIPE, TIPE1 and TIPE3 proteins in other cancers whereas, effect of TIPE2 in oral cancer was in contrast to the antitumor activity reported in cancers of breast, liver, lung and prostate, gastric cancer and glioma (Padmavathi et al., 2018). Nevertheless, increased expression of TIPE2 was also reported in certain cancers such as colon cancer and renal cell carcinoma and shown to downregulate the pro-apoptotic caspase proteins (Zhang et al., 2013c, Li et al., 2014). Together, it was proved that silencing of even one among the three oncogenic proteins TIPE, TIPE2 and TIPE3 is enough to significantly impair the proliferation of oral cancer cells. Further, our study

also revealed TIPE1 to hold a significant role in controlling the proliferation of oral cancer, loss of which can lead to uncontrolled cell proliferation.

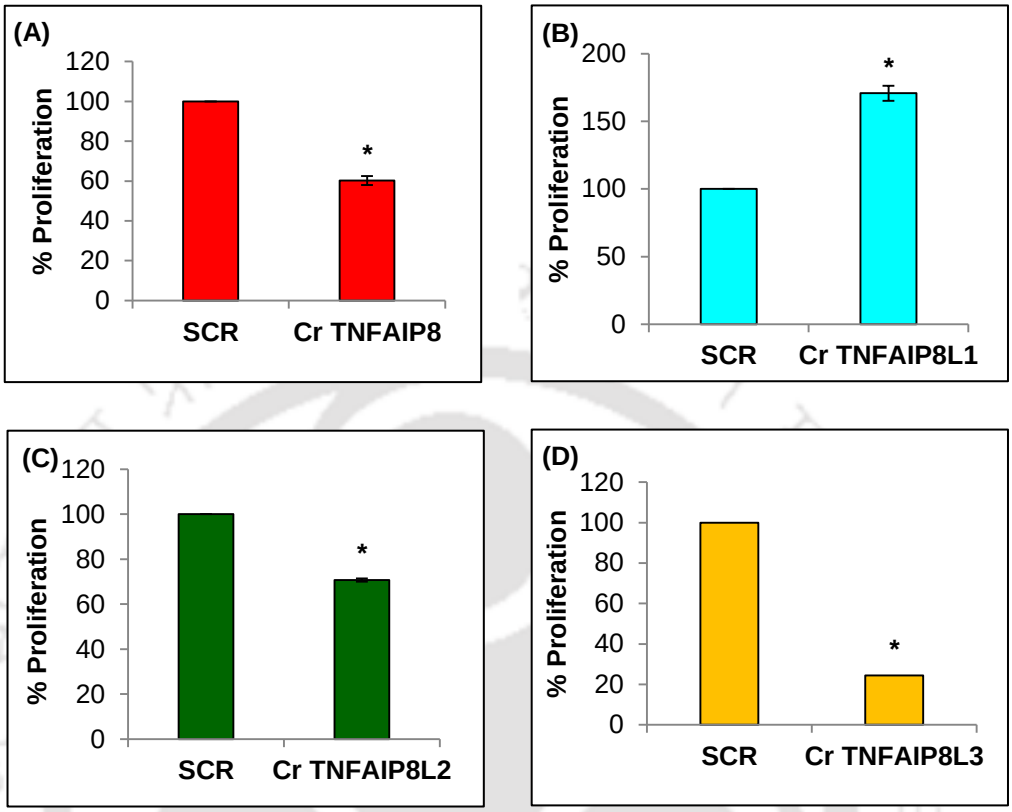


Figure 4.2: Effect of CRISPR/Cas9-mediated deletion of TIPE proteins on the proliferation of SAS cells was evaluated by MTT assay and the percentage proliferation was calculated in comparison to SCR control cells. (A)-(D) show the effect of TIPE, TIPE1, TIPE2 and TIPE3 knockout on the proliferation of SAS cells respectively. (*p-value ≤ 0.05 compared to SCR)

4.3.3 Effect of TIPE proteins on oral cancer cell proliferation was mediated through regulation of cell cycle progression

After proving TIPE proteins to critically regulate oral cancer cell proliferation, we further analysed if this regulation of proliferation is mediated through modulation of cell cycle. Therefore, we examined the effect of silencing of TIPEs on the cell cycle progression by analysing the percentage of cell distribution in each phase of the cell cycle using flow cytometry and compared the results with the cell cycle distribution in SCR cells. Analysis of the cell cycle data with FCS express software revealed that knockout of TIPE and TIPE3 results in accumulation of cells at S phase and a

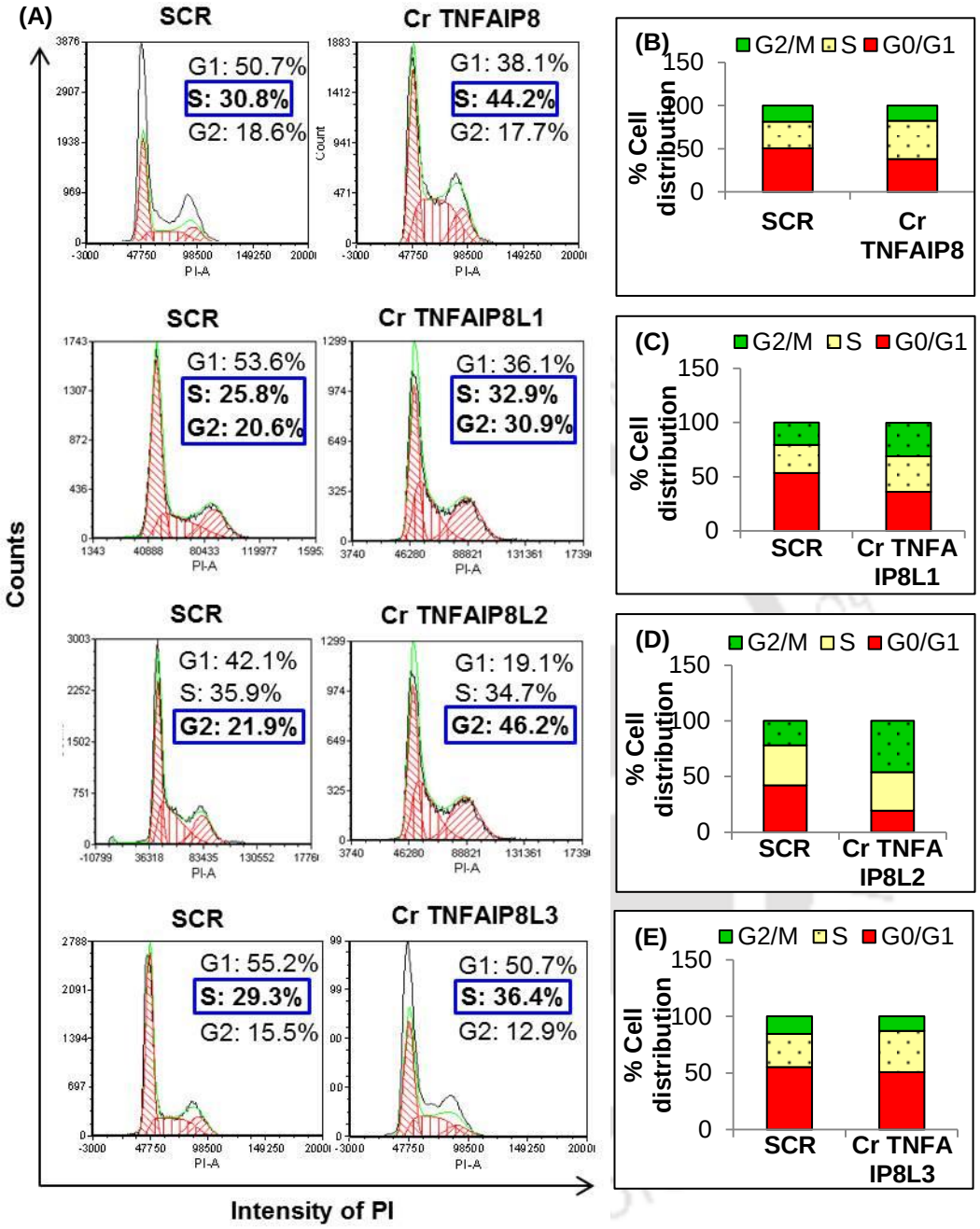


Figure 4.3: Effect of CRISPR/Cas9-mediated deletion of TIPE proteins on the cell cycle progression of SAS cells was analysed using flow cytometry. (A) Cell cycle data displaying the distribution of cells in different phases of the cells cycle as modeled with the help of FCS express data analysis software for SCR and knockout cells; (B)-(E) Percentage of cells in each phase of the cell cycle were plotted for TIPE, TIPE1, TIPE2 and TIPE3 knockout cells against the distribution in SCR cells.

subsequent reduction in G2/M and G0/G1 phases (Figure 4.3A, B and E). Further, TIPE2 knockout induced G2/M phase arrest unlike the other two oncogenic proteins (Figure 4.3A and D). Therefore, it has been proved that reduction in the proliferation of oral cancer cells acquired by silencing of oncogenic TIPE proteins is mediated through inhibition of cell cycle progression as accumulation of cells in S or G2/M phase and consequent decrease in other phases of cell cycle were known to inhibit cell proliferation (Haque et al., 2018, Banerjee et al., 2018, Wu et al., 2017b, Yang et al., 2006, Yu et al., 2016, Hu et al., 2017). Further, silencing of TIPE1 resulted in an increased percentage of cells in both S phase and subsequent G2/M phase suggesting an increased rate of cell division and increased proliferation as seen in our MTT results (Figure 4.3A and C). Hence, it is confirmed that TIPE proteins control oral cancer cell proliferation through regulation of cell cycle progression. Further interestingly, as these proteins were found to act on different phases of the cell cycle, a possible difference in the molecular targets of TIPE proteins is suggested in cell cycle regulation.

4.3.4 Silencing of TIPE proteins significantly altered the clonogenic potential of oral cancer cells

In addition to uncontrolled proliferation, increased survival, resistant to cell death and replicative immortality are some of the other basic characteristics of cancer cells. Therefore, in order to check the role of TIPE proteins in the regulation such processes we analysed the clonogenic potential of knockout cells in comparison to SCR control cells by colony forming assay. Clonogenic potential of a cell is defined by the ability of a single cell to multiply and form a colony which indirectly measures the survival and replicative cell death or immortality of the cells (Franken et al., 2006, Rafehi et al., 2011). In line with the previous results of the study, association of TIPE proteins

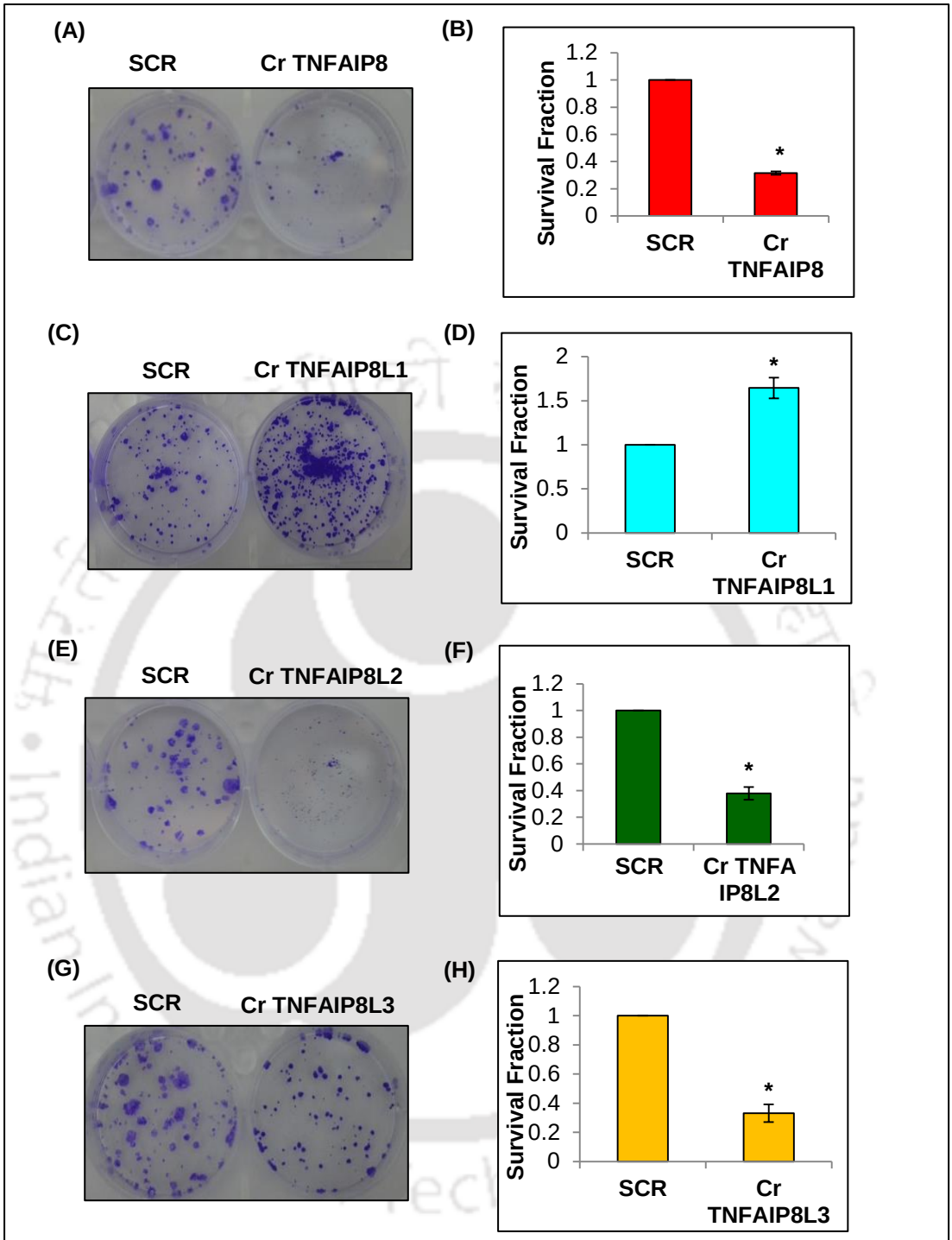


Figure 4.4: Effect of CRISPR/Cas9-mediated deletion of TIPE proteins on the survival and clonogenic potential of SAS cells was estimated by colony forming assay. (A), (C), (E) & (G) are the representing images showing the colonies of TIPE, TIPE1, TIPE2 & TIPE3 knockout cells in comparison to SCR respectively; (B), (D), (F) & (H) are the graphical representation of clonogenic potential of TIPE, TIPE1, TIPE2 & TIPE3 knockout cells measured in terms of survival fraction. (*p-value ≤ 0.05 compared to SCR)

with oral cancer is further confirmed by the colony forming assay results, measured in terms of survival fraction. i.e. the TIPE, TIPE2 and TIPE3 knockout cells produced colonies that are smaller in size and lesser in number than the SCR cells thus resulting in a significant reduction of survival fraction (Figure 4.4A, B, E-H). In contrast, TIPE1 knockout cells produced increased number of bigger colonies which was also reflected in the survival fraction (Figure 4.4C and D). Supporting our results, silencing of TIPE was also reported to reduce the colony formation of colon and lung cancer cells (Miao et al., 2012, Dong et al., 2010, Padmavathi et al., 2018). Similarly, retrieval of the lost expression of TIPE1 was shown to reduce the colony formation in lung cancer cells thus endorsing its antitumor activity (Wu et al., 2017a, Padmavathi et al., 2018). Further, in the current study we found TIPE2 knockout to decrease the colony formation of oral cancer cells. However, in case of lung cancer, overexpression of TIPE2 was proved to reduce colony formation (Padmavathi et al., 2018, Li et al., 2015). Together, TIPE, TIPE2 and TIPE3 proteins were found to positively regulate and TIPE1 to negatively regulate the survival of oral cancer cells.

4.3.5 TIPE proteins are also involved in the migration of oral cancer cells

As mentioned earlier, advanced stages of oral cancer often associated with poor prognosis and invasion, migration and metastasis of cancer cells is one of the major contributing factors for this disease progression and poor prognosis. Therefore, it is indispensable to determine the effect of TIPE proteins on the invasion and migratory potential of oral cancer cells in order to establish their involvement in disease progression. Hence, the knockout clones of TIPE proteins were checked for their migration potential compared to SCR control cells using *in vitro* wound healing assay. Interestingly, loss of TIPE, TIPE2 and TIPE3 significantly reduced the migration of oral cancer cells and the anti-migratory effect of TIPE2 knockout was remarkably

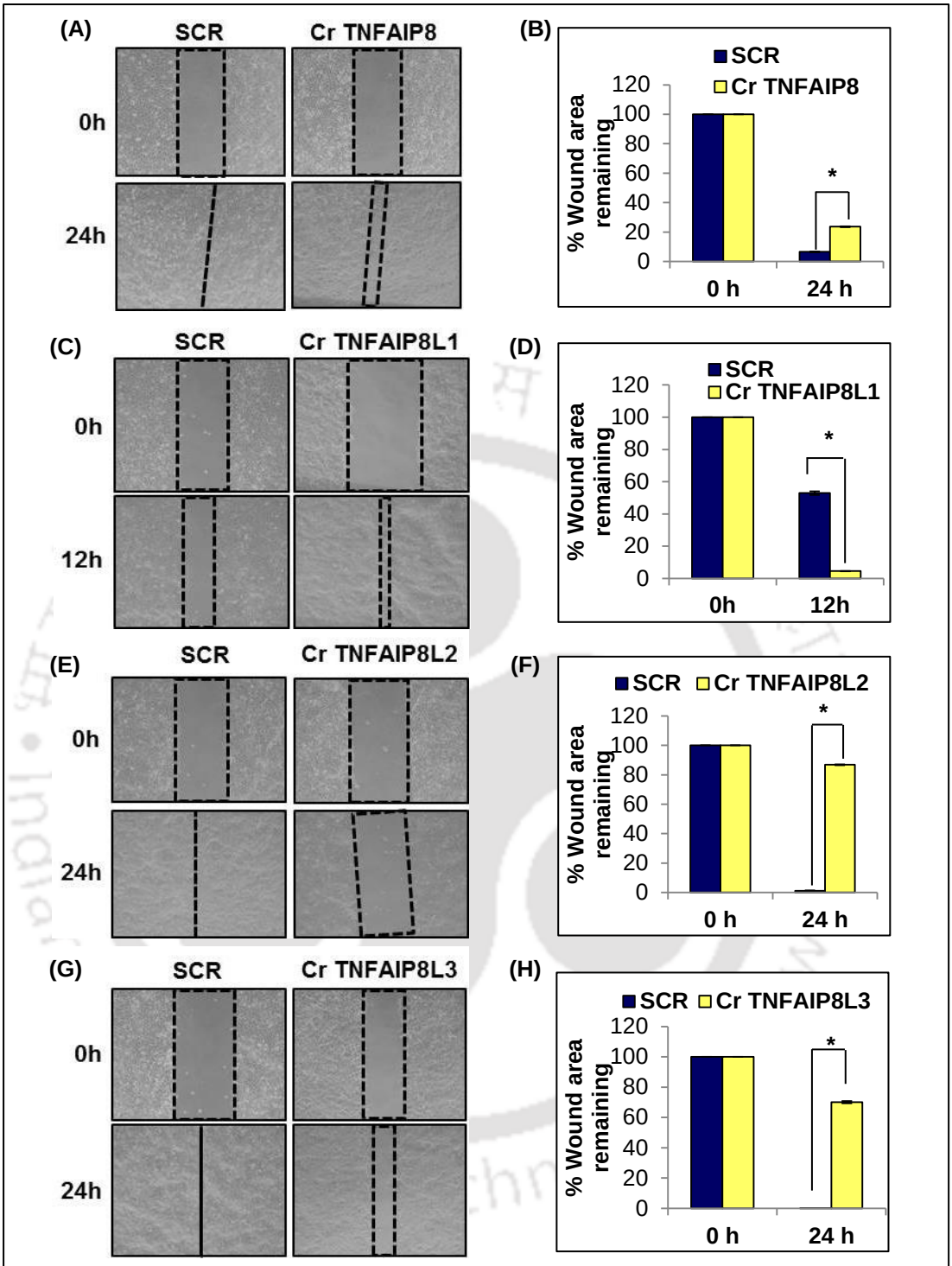


Figure 4.5: Effect of CRISPR/Cas9-mediated deletion of TIPE proteins on the migration of oral cancer cells was measured by *in vitro* scratch/ wound healing assay. (A), (C), (E) & (G) are the representative images of the effect of TIPE, TIPE1, TIPE2 & TIPE3 knockout on the migration of SAS cells; (B), (D), (F) & (H) are the graphical representation of % of wound area remaining at the end of 12 or 24 h in TIPE, TIPE1, TIPE2 & TIPE3 knockout cells as calculated by ImageJ software and plotted against SCR. (**p*-value ≤ 0.05 compared to SCR)

higher than (more than 85%) the other oncogenic counterparts of TIPE family, suggesting the importance of TIPE2 protein in the invasion and migration of oral cancer cells. Further, knockout of TIPE1 resulted in an increased migration of oral cancer cells with almost complete healing of the wound being observed at 12 h itself compared to the 24 h healing time of SCR cells (Figure 4.5A-H). These results were in accordance with the earlier reports on other cancers except for TIPE2 (Dong et al., 2010, 2017, Wang et al., 2018, Lian et al., 2017, Padmavathi et al., 2018). Collectively, in addition to proliferation and survival, deregulation of TIPE proteins was also found to be involved in the regulation of migration of oral cancer cells thus suggesting their importance in the disease progression and metastasis and their therapeutic values in preventing the tumor metastasis and in successful treatment of late stage oral cancers.

4.3.6 Effect of TIPE knockout on the oral cancer cell survival, proliferation and migration was mediated through downregulation of Akt/mTOR/S6 signaling axis

As evident from our results, deletion of TIPE significantly impaired the survival, proliferation and migration of oral cancer cells. Interestingly, these cancer hallmarks are known to be regulated by multiple signaling cascades that are abnormally activated or inhibited during the course of tumorigenesis. Therefore, we performed Western blot analysis of TIPE knockout cells to identify the molecular targets of TIPE protein involved in oral cancer progression. Our results revealed that knockout of TIPE is associated with the downregulation of proteins involved in survival, proliferation and inhibition of apoptosis such as Cox-2, survivin, Bcl-2 and cIAP1; invasion, migration and angiogenesis related proteins such as CXCR4, MMP-9 and VEGF-A; and upregulation of the tumor suppressor p53 (Figure 4.6.1A, B and D) (Hashemi Goradel et al., 2019, Hassan et al., 2014, Yu et al., 2017, Huang, 2018,

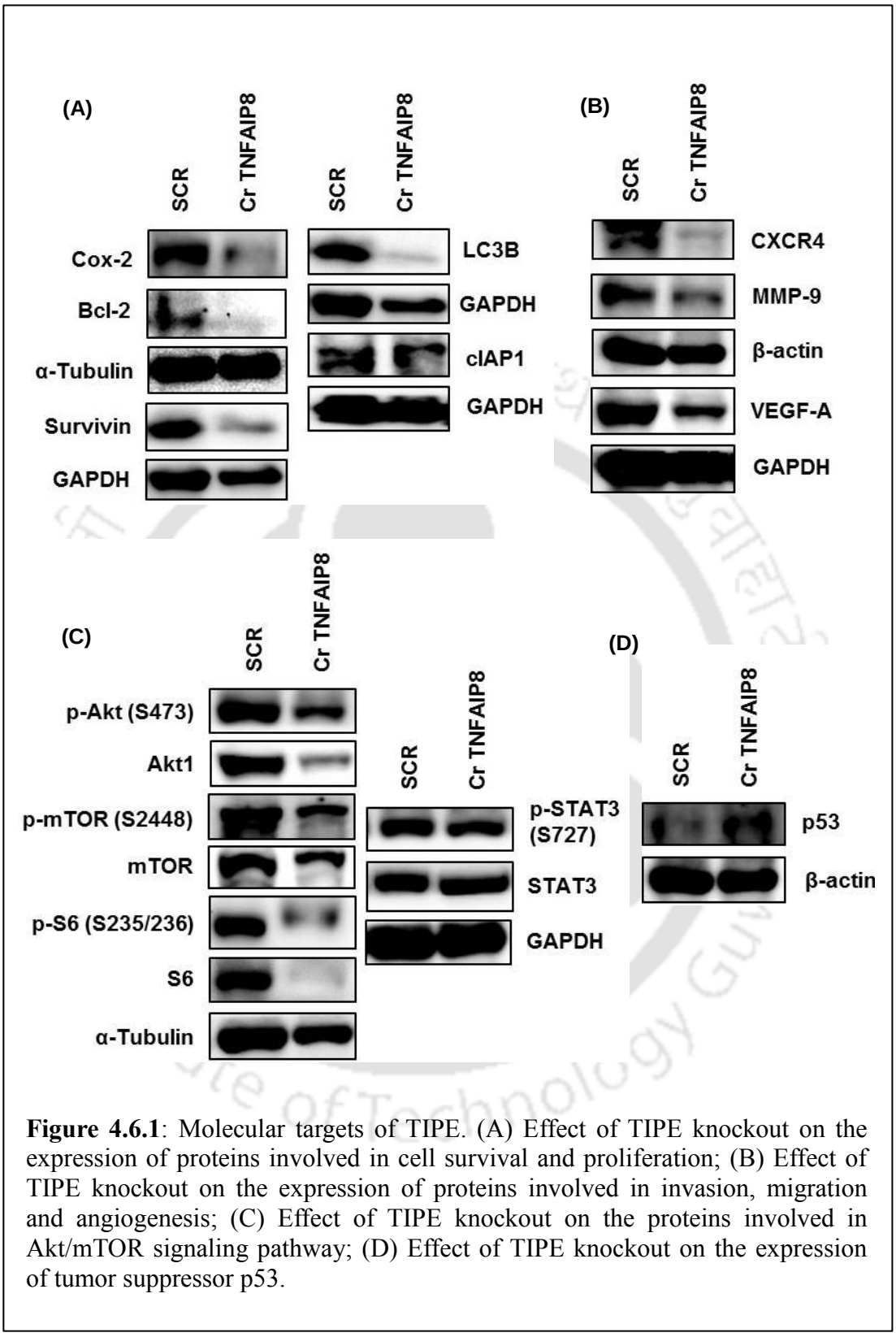


Figure 4.6.1: Molecular targets of TIPE. (A) Effect of TIPE knockout on the expression of proteins involved in cell survival and proliferation; (B) Effect of TIPE knockout on the expression of proteins involved in invasion, migration and angiogenesis; (C) Effect of TIPE knockout on the proteins involved in Akt/mTOR signaling pathway; (D) Effect of TIPE knockout on the expression of tumor suppressor p53.

Buijs et al., 2017, Moon et al., 2003). Further, loss of TIPE also inhibited the levels of LC3B, an autophagy related protein essential for the formation of autophagosomes (Figure 4.6.1A). Autophagy is a complex process known to be correlated both positively and negatively with tumorigenesis. Moreover, expression pattern of LC3 was also observed to vary significantly between cancer types. For instance, it was found to be downregulated in lung cancer and upregulated in colon cancer compared to respective normal tissues (Wu et al., 2015). Therefore, LC3B downregulation observed in TIPE knockout cells might contribute to the reduced cell survival and proliferation of oral cancer cells. Additionally, silencing of TIPE also resulted in the deregulation of various components of Akt/mTOR signaling pathway. For instance, TIPE knockout cells were found to have reduced levels of Akt, p-Akt (S473), mTOR, p-mTOR (S2448), S6 and p-S6 (S235/236) proteins (Figure 4.6.1C). Interestingly, other members of TIPE family such as TIPE2 and TIPE3 were also earlier shown to regulate Akt pathway in different cancers (Padmavathi et al., 2018). However, thus far no reports are available on the TIPE-mediated regulation of Akt/mTOR cascade in oral cancer. Therefore, for the first time in our study we have found TIPE to positively regulate the Akt/mTOR pathway in oral cancer and silencing of the same to result in the deactivation of Akt/mTOR components. Further, this Akt/mTOR signaling is a major growth regulator pathway, constitutive activation of which is well-known to be involved in the development and progression of various cancers including the cancers of head and neck through activation of survival and proliferative genes (Roy et al., 2017, Hennessy et al., 2017). Therefore, our findings together with the previous studies suggest that TIPE mediates survival, proliferation and migration of oral cancer cells by activating Akt/mTOR signaling and its downstream targets (Figure 4.6.2).

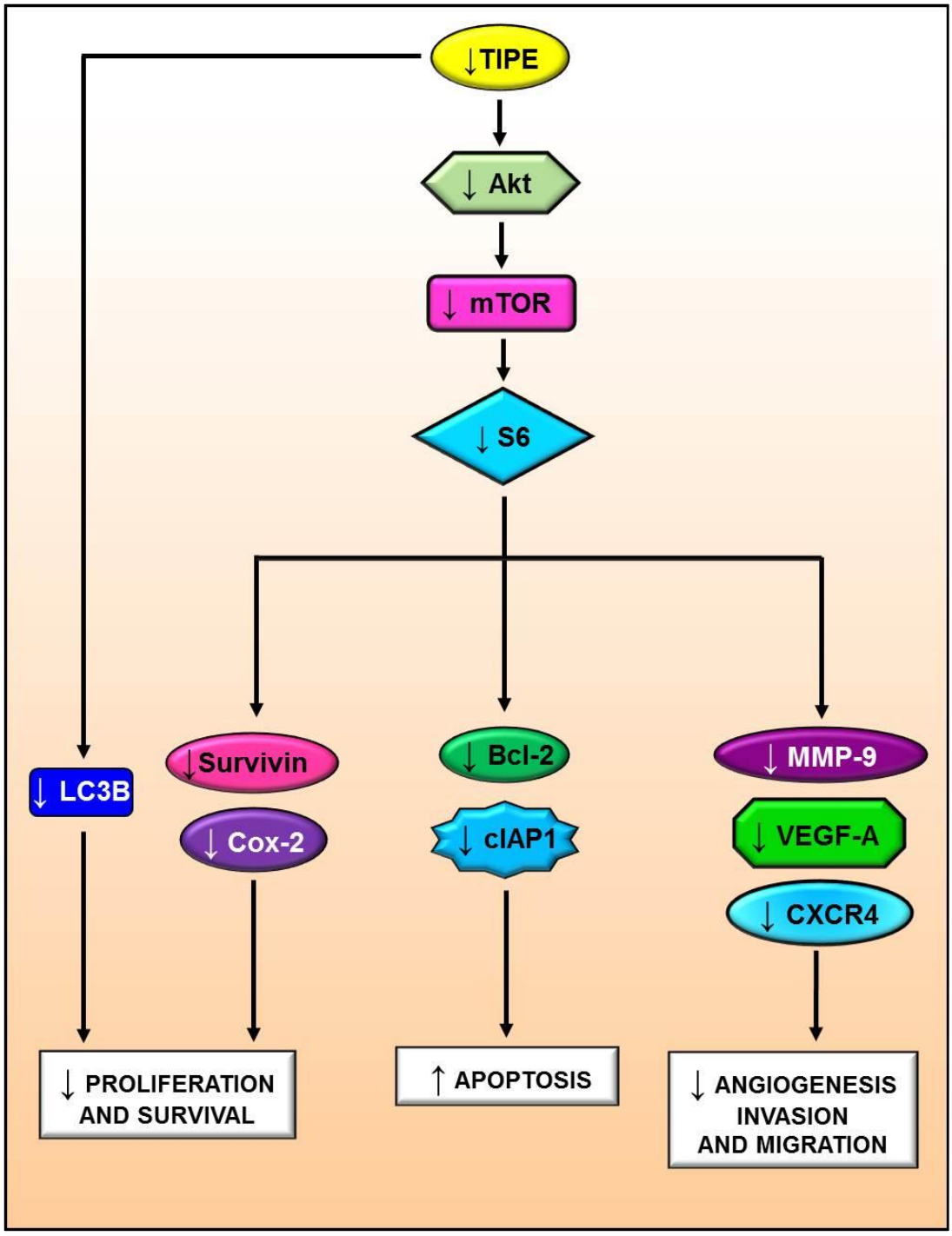


Figure 4.6.2: Suppression of TIPE inhibits oral cancer progression through downregulation of Akt/mTOR signaling and its downstream targets. ↑- upregulation; ↓- downregulation.

4.3.7 Loss of TIPE1 exhibits oncogenic activity through activation of Akt/mTOR pathway

In contrast to TIPE, loss of TIPE1 had an oncogenic effect on oral cancer cells. This was in line with the results of previous chapters showing TIPE1 expression to be downregulated in oral cancer tissues and in response to tobacco carcinogens. Further, the molecular analysis of the TIPE1 knockout cells revealed that loss of TIPE1 is associated with upregulation of anti-apoptotic, pro-survival and invasion related proteins such as Cox-2, cIAP1, Bcl-2 and MMP-9 (Figure 4.7.1A) (Hashemi Goradel et al., 2019, Hassan et al., 2014, Huang, 2018). Further, similar to TIPE, TIPE1 also modulated the expression of upstream and downstream targets of Akt/mTOR pathway. For example, TIPE1 increased the phosphorylation of Akt, mTOR and S6 proteins which in turn is responsible for the activation of survival and proliferative signals (Figure 4.7.1B) (Hennessy et al., 2005). In addition, TIPE1 knockout also inhibited the levels of PTEN and REDD1, inhibitors of p-Akt and mTOR (Figure 4.7.1B) (Al-Khouri et al., 2005, Kolesnichenko and Vogt, 2011, Katiyar et al., 2009). As learned from the review of literature, there are only very few studies are available on the role of TIPE1 and its association with cancer that have found TIPE1 to be downregulated in liver and lung cancers which in turn was associated with reduced apoptosis, increased tumor growth, proliferation, invasion and migration of cancer cells through deregulation of cyclins, caspases, MMP-9 and JNK and NF-κB pathways (Zhang et al., 2015a, Wu et al., 2017a). In line with these reports, our study for the first time established a negative correlation between TIPE1 expression and progression of oral cancer. Further, we also identified Akt/mTOR pathway and its upstream and downstream molecules to be the novel targets involved in the regulation of TIPE1-mediated oral cancer (Figure 4.7.2).

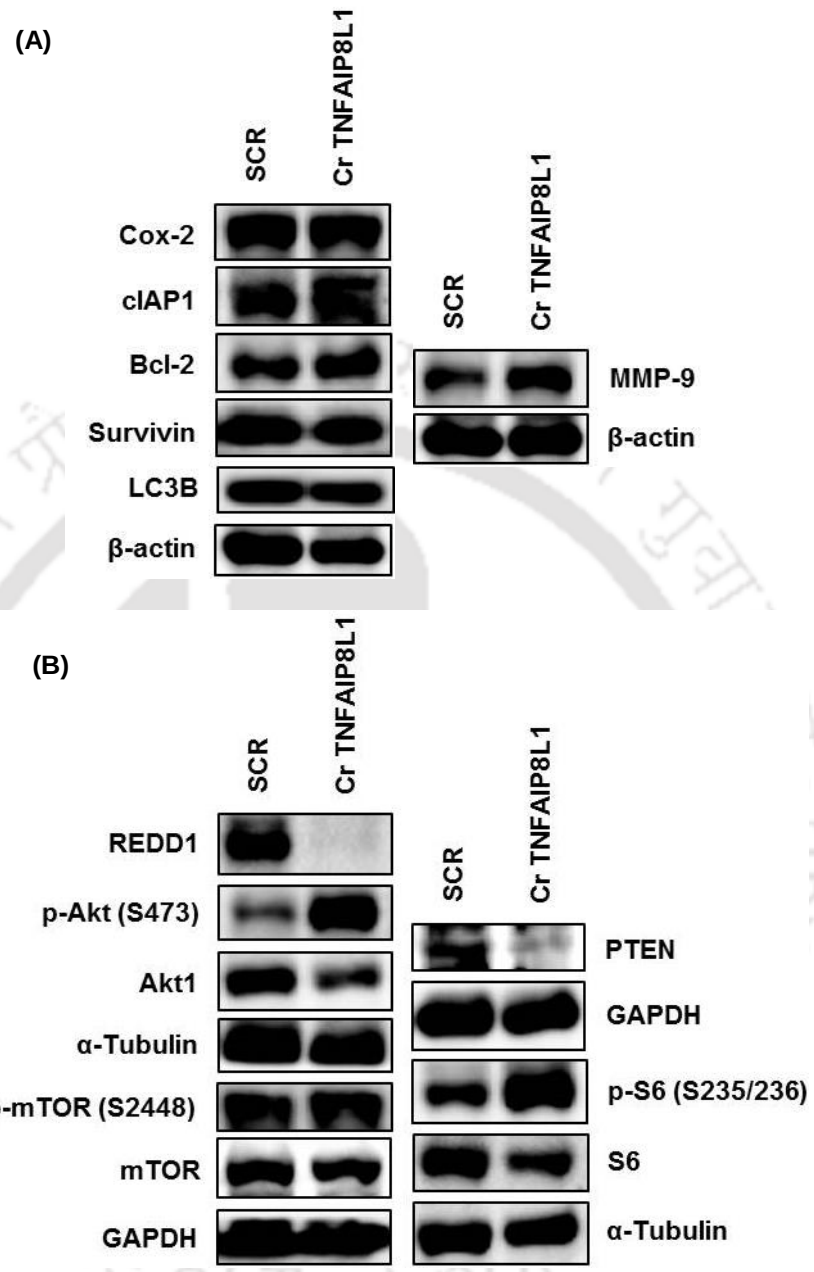


Figure 4.7.1: Molecular targets of TIPE1. (A) Effect of TIPE1 knockout on the expression of proteins involved in cell survival, proliferation and invasion; (B) Effect of TIPE1 knockout on the proteins involved in Akt/mTOR signaling pathway.

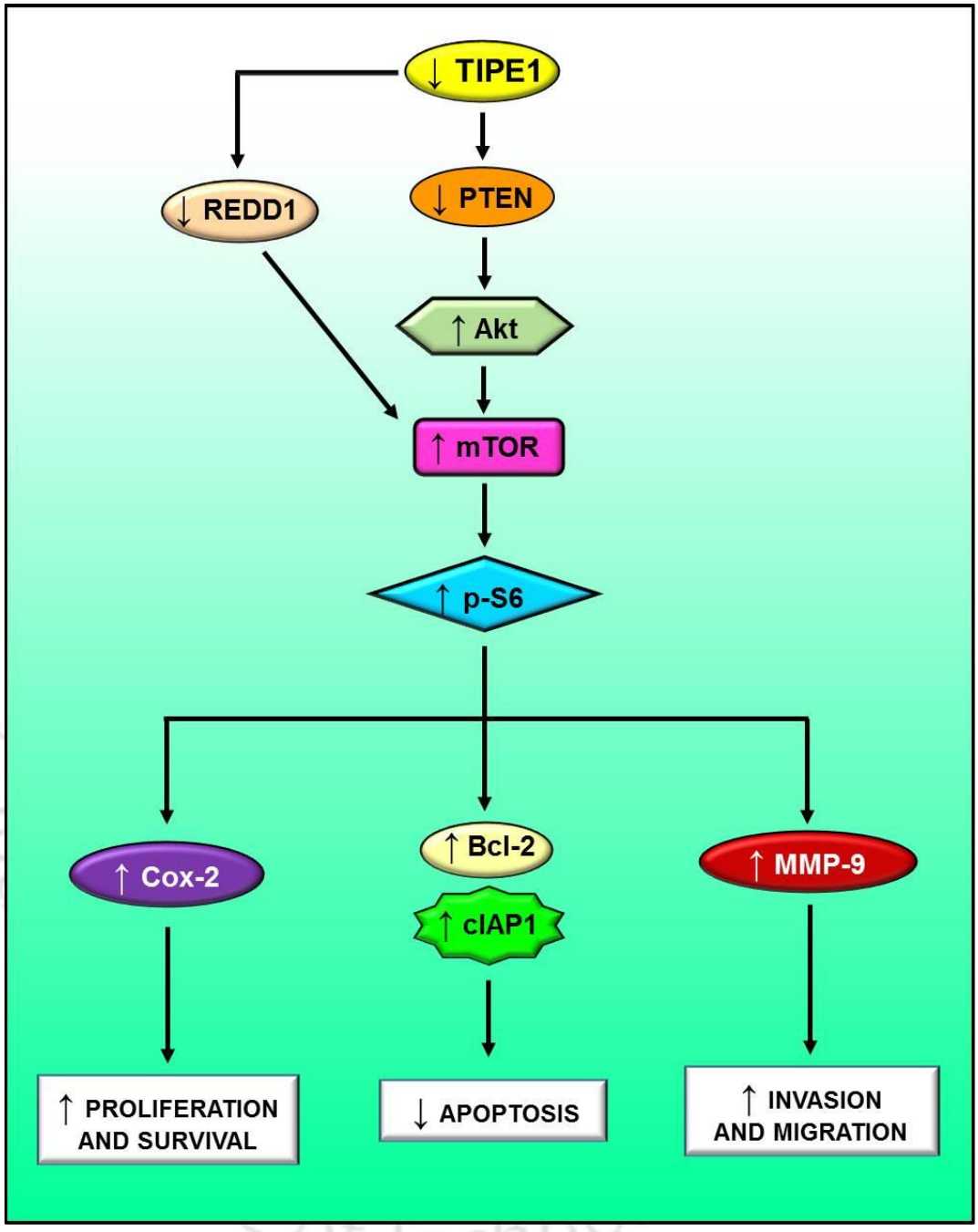


Figure 4.7.2: Deletion of TIPE1 augments oral cancer progression through activation of Akt/mTOR signaling and its downstream targets. ↑- upregulation; ↓- downregulation.

4.3.8 Suppression of TIPE2 expression reduced the survival, proliferation and migration of oral cancer cells through downregulation of Akt/mTOR/S6/NF-κB/STAT3 signaling axis

Similar to TIPE protein, in our preliminary studies we observed an upregulation of TIPE2 in oral premalignant and malignant tissues and in oral cancer cells in response to the tobacco components. Therefore we hypothesized TIPE2, another member of TIPE family, also to contribute to the development and progression of oral cancer. Supporting our hypothesis, post-knockout studies also revealed that loss of TIPE2 resulted in a significant reduction in the survival, proliferation, colony formation and migration of oral cancer cells *in vitro*. Further, in order to determine the molecular targets involved in this TIPE2-mediated oral carcinogenesis, we performed Western blot analysis of the TIPE2 knockout cells in comparison to SCR control cells. Interestingly, TIPE2 knockout was associated with the downregulation of various anti-apoptotic, proliferative and pro-survival proteins such as Cox-2, cIAP1, XIAP, Bcl-2 and survivin (Hashemi Goradel et al., 2019, Hassan et al., 2014). In addition, similar to TIPE silencing, TIPE2 knockout was also associated with reduced levels of autophagy related protein LC3B suggesting the inhibition of autophagy to contribute to its anti-survival and anti-proliferative activities in oral cancer (Figure 4.8.1A). Furthermore, TIPE2 knockout also resulted in reduced levels of invasion, migration and angiogenesis related proteins such as CXCR4, VEGF-A and MMP-9 (Figure 4.8.1B) thus inhibiting the migration of oral cancer cells as observed in the *in vitro* wound healing assay. Moreover, alike TIPE and TIPE1, knockout of TIPE2 also significantly modulated the levels of proteins involved in the Akt/mTOR pathway. For instance, TIPE2 knockout repressed the expression and activation of Akt and mTOR which resulted in the downregulation of S6 and p-S6, downstream target of

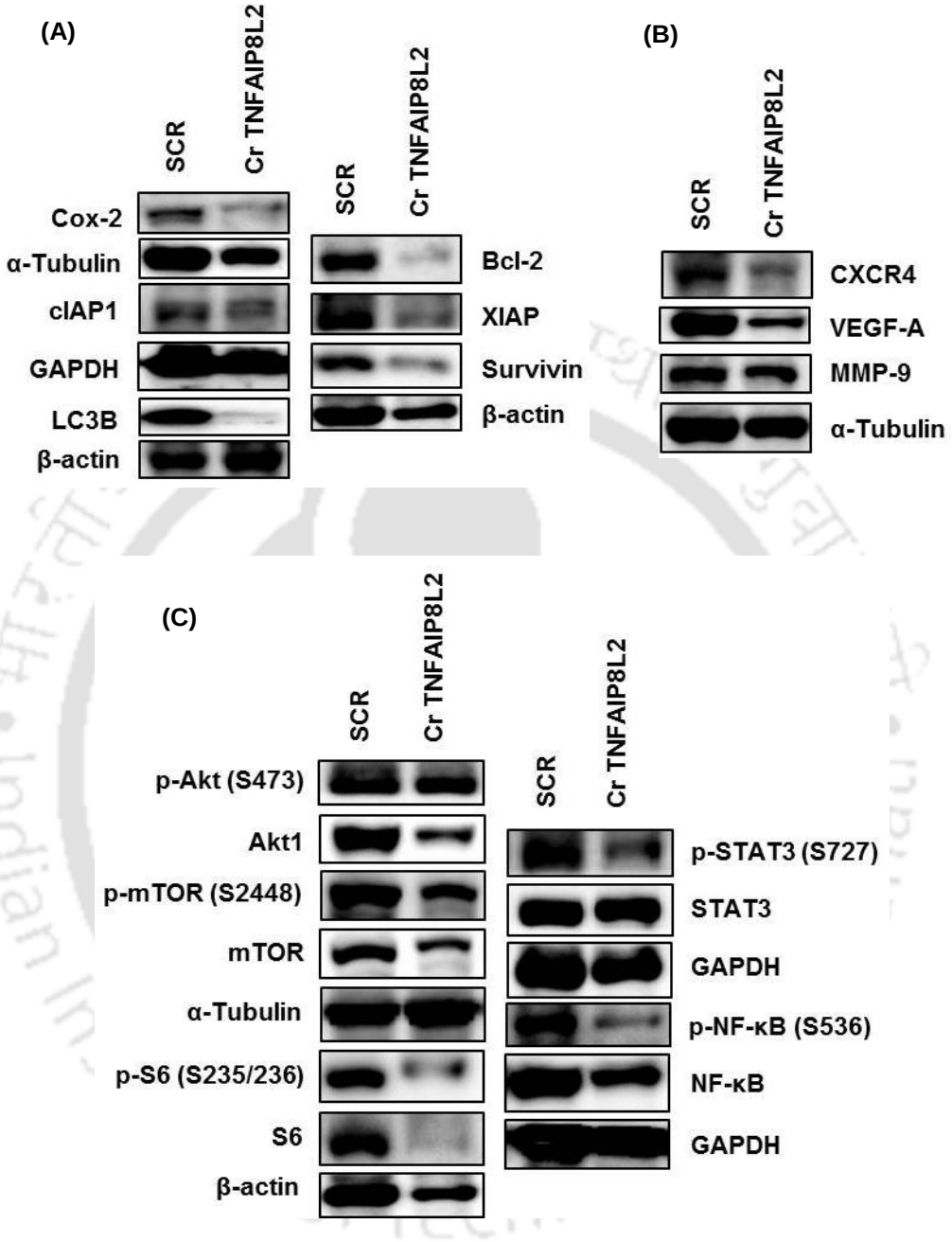


Figure 4.8.1: Molecular targets of TIPE2. (A) Effect of TIPE2 knockout on the expression of proteins involved in cell survival and proliferation; (B) Effect of TIPE2 knockout on the expression of proteins involved in invasion, migration and angiogenesis; (C) Effect of TIPE2 knockout on the proteins involved in Akt/mTOR signaling pathway.

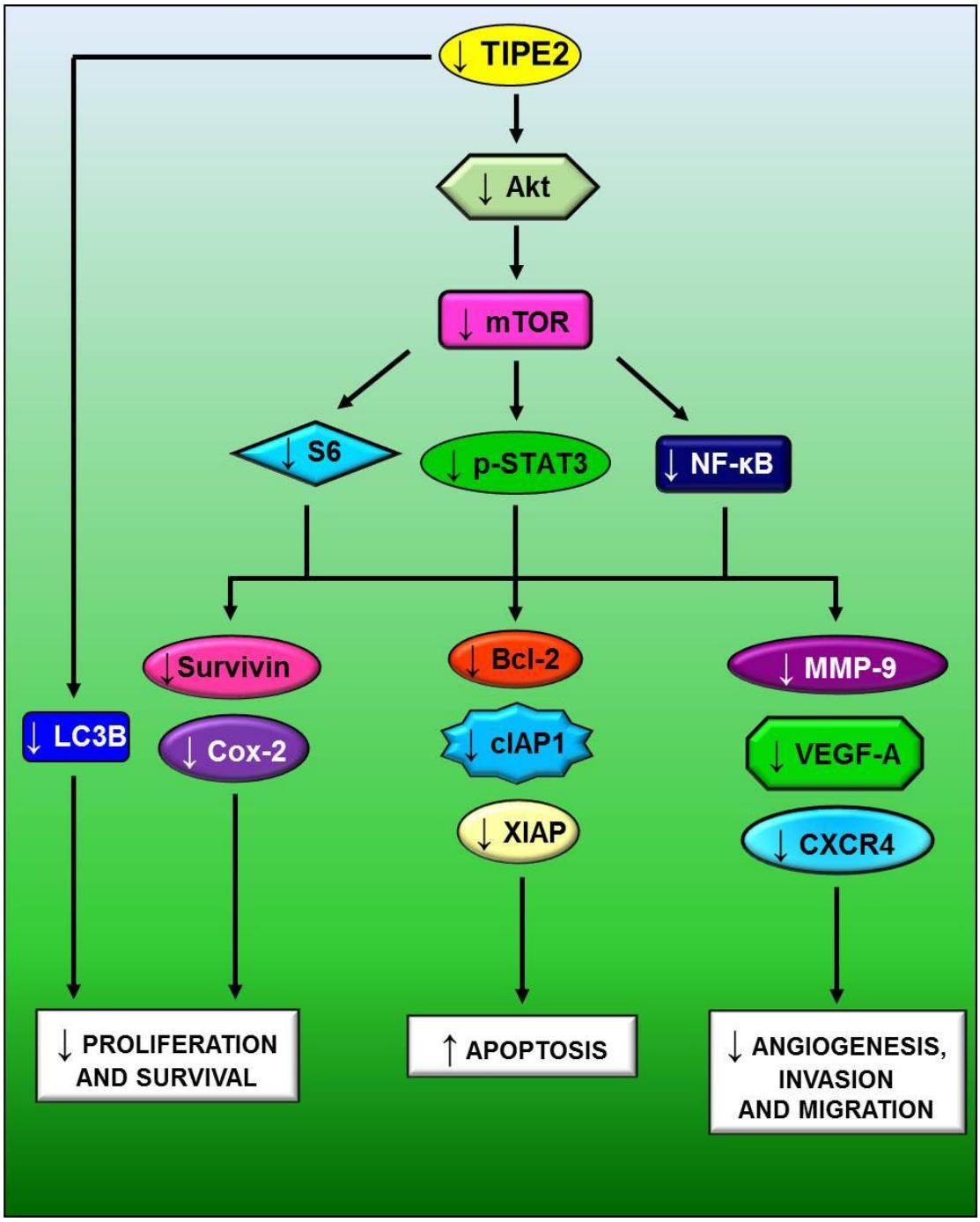


Figure 4.8.2: Downregulation of TIPE2 suppresses oral cancer progression through downregulation of Akt/mTOR signaling and its downstream targets. ↑- upregulation; ↓- downregulation.

mTOR (Figure 4.8.1C). Interestingly, the active form of Akt and mTOR were reported to activate NF-κB and STAT3 respectively (Zhou et al., 2007, Bai et al., 2009). In line with these reports, in our study, we observed that downregulation of TIPE2 resulted in inhibition of Akt and mTOR activation, which led to subsequent decrease in p-STAT3 and p-NF-κB levels ultimately resulting in reduced proliferation and migration of oral cancer cells (Figure 4.8.1C). However, our results were in contrast to the previous reports showing downregulation of TIPE2 to exert opposite effects in cancers of breast, liver, lung and prostate, gastric cancer and glioma with increased tumor growth, invasion and migration. Further, it was also reported to negatively regulate the PI3K/Akt pathway, NF-κB and VEGF (Padmavathi et al., 2018). Nevertheless, increased expression of TIPE2 was also reported to downregulate the pro-apoptotic caspase proteins in colon cancer and RCC (Zhang et al., 2013c, Li et al., 2014). Therefore, our results together with the previous studies suggested the cancer specific expression and role of TIPE2 protein and in oral cancer to exert oncogenic role (Figure 4.8.2).

4.3.9 The antitumor activity of TIPE3 gene silencing is governed through downregulation of Akt-independent mTOR/STAT3 signaling

TIPE3, the latest member of TIPE protein family has been implicated in the development and progression of breast and lung cancers (Lian, et al., 2017, Fayngerts et al., 2014, Wang et al., 2018). Further, increased expression of TIPE3 was also reported in different cancers including cervical cancer, colon cancer, esophageal cancer and liver cancer (Padmavathi et al., 2018). In line with these reports, we have also observed a remarkable increase in the expression of TIPE3 in oral premalignant and malignant tumor tissues. Also, treatment of oral cancer cells with tobacco carcinogens resulted in the upregulation of TIPE3 suggesting a strong correlation of

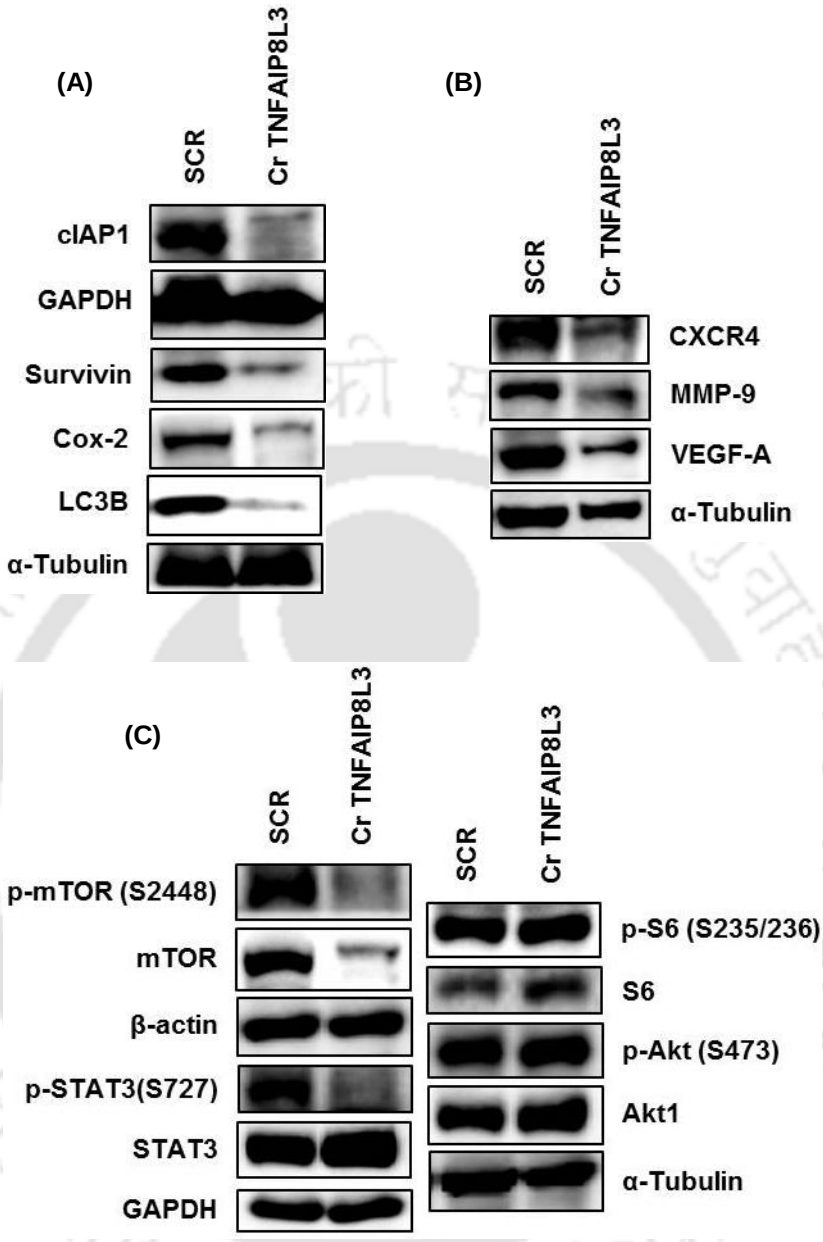


Figure 4.9.1: Downstream targets of TIPE3. (A) Effect of TIPE3 knockout on the expression of proteins involved in cell survival and proliferation; (B) Effect of TIPE3 knockout on the expression of proteins involved in invasion, migration and angiogenesis; (C) Effect of TIPE3 knockout on the proteins involved in Akt/mTOR signaling pathway.

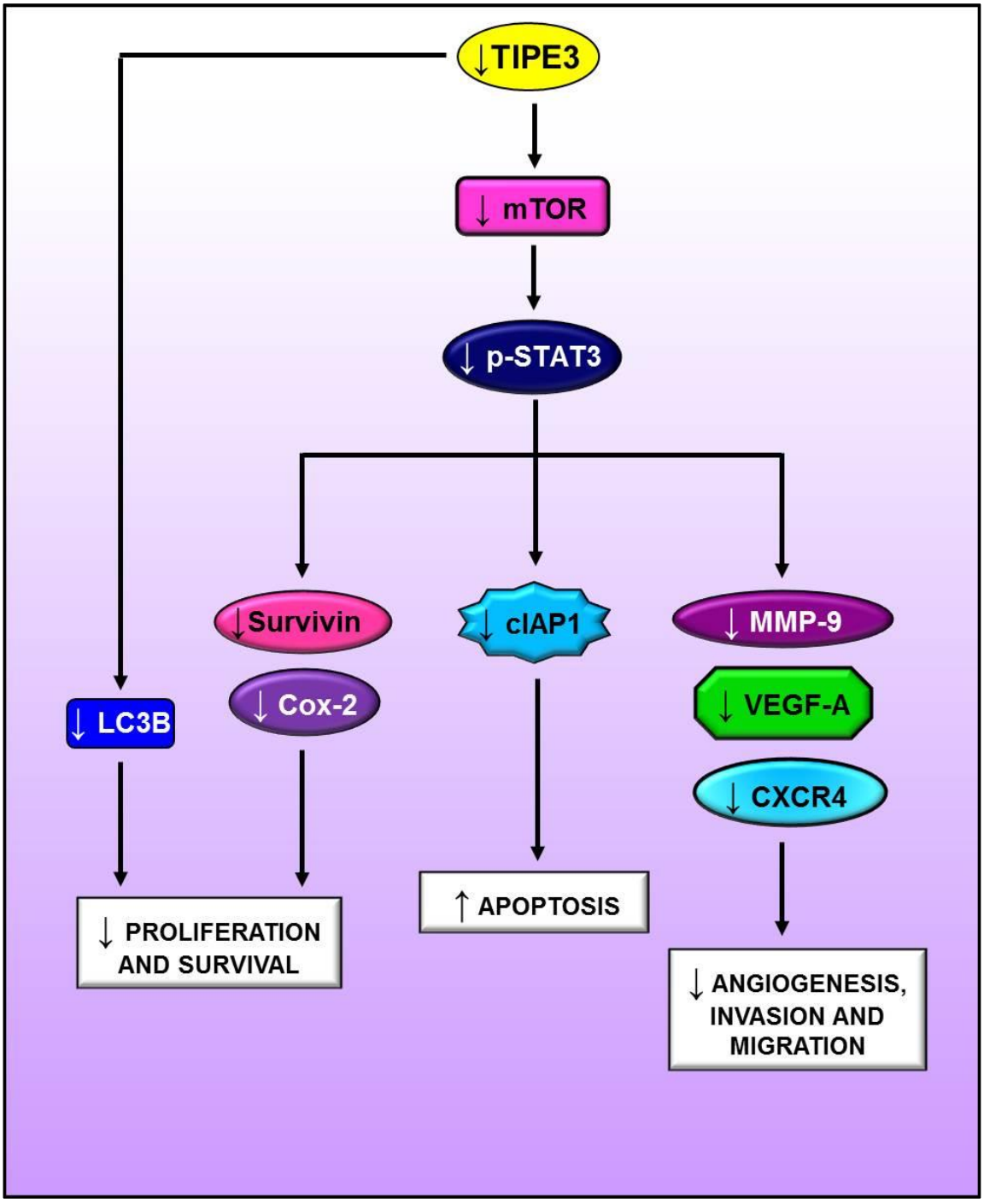


Figure 4.9.2: Suppression of TIPE3 inhibits oral cancer progression through downregulation of Akt-independent mTOR/STAT3 signaling and its downstream targets. ↑- upregulation; ↓- downregulation.

TIPE3 with the progression of oral cancer. Further assuring this, knockout of TIPE3 significantly reduced the survival, proliferation, cell cycle progression and migration of oral cancer cells. These results were in accordance with the earlier studies on breast and lung cancer showing downregulation of TIPE3 to inhibit tumor growth, proliferation, invasion and migration (Lian, et al., 2017, Fayngerts et al., 2014, Wang et al., 2018, Padmavathi et al., 2018). However, the underlying mechanism for oral cancer seems to be different as TIPE3 knockout failed to alter the levels of p-Akt in oral cancer cells unlike the breast and lung cancers. Interestingly, we observed a decrease in both mTOR and p-mTOR levels in TIPE3 null oral cancer cells. Likewise, levels of p-STAT3 also decreased in TIPE3 knockout cells however, expression of p-S6 remained unaltered in response to TIPE3 knockout (Figure 4.9.1C). Further, expression of survival, proliferative, anti-apoptotic, angiogenic, invasion and migration related proteins such as Cox-2, survivin, cIAP1, CXCR4, MMP-9 and VEGF-A were downregulated in TIPE3 null cells (Figure 4.9.1A and B) signifying a distinct Akt-independent activation of mTOR resulting in the activation of STAT3 ultimately leading to the overexpression of cellular proteins involved in proliferation, survival, invasion and migration thus contributing to the progression of oral cancer. Moreover, similar to TIPE and TIPE2 knockout, LC3B was downregulated in TIPE3 knockout cells as well denoting TIPE3 knockout to result in inhibition of autophagy thus contributing to its antitumor effects (Figure 4.9.2).

4.4 Conclusion

Collectively, this is the first report examining the role of TIPE proteins in oral cancer through CRISPR/Cas9-mediated gene knockout. In brief, results of the knockout studies supported our hypothesis where we postulated TIPE, TIPE2 and TIPE3 to play tumorigenic and TIPE1 to play antitumorigenic role in oral cancer. Further in detail,

all the four TIPE proteins were found to regulate the hallmarks of cancer such as survival, proliferation, invasion and migration as loss of these proteins greatly impacted these hallmarks in oral cancer. Moreover, while TIPE, TIPE1 and TIPE2 proteins exhibited their activity through regulation of Akt/mTOR signaling cascade, TIPE3 acted through an Akt-independent mTOR/STAT3 pathway. However, all the four proteins were found to commonly deregulate the levels of proteins involved in the maintenance of cell survival, proliferation, apoptosis, invasion, migration, metastasis and angiogenesis such as Cox-2, survivin, Bcl-2, cIAP1, XIAP, CXCR4, MMP-9 and VEGF-A etc. Furthermore, the oncogenic proteins TIPE, TIPE2 and TIPE3 were also found to regulate the autophagy marker LC3B, upregulation of which leads to autophagy which has been reported to be involved in tumorigenesis and development of resistance against cancer therapies endorsing TIPE proteins to exert their oncogenic activity through regulation of autophagy as well. Therefore, taken together, in our study, the TIPE proteins were found to play significant roles in the progression of oral cancer claiming the therapeutic values and the importance of specific targeting of TIPE proteins in the disease management. However, our results have to be validated *in vivo* and in clinical settings in order to further explore their diagnostic, prognostic and therapeutic importance in oral cancer which would help us to improve the survival rate.

CHAPTER 5

Discussion, Conclusion and Future Prospects

5.1 Discussion and conclusion

India is the global epicentre of oral cancer with highest incidence and mortality rates (Gupta et al., 2013). Further exemplifying the severity, oral cancer was reported to contribute for almost 30-40% of all cancer cases in India compared to the marginal 2–4% in the western world (Byakodi et al., 2012). This increased frequency of oral cancer in India is mainly attributable to the addictive habits such as consumption of smoke and smokeless tobacco, betel quid and alcohol. However, other factors including age, infection with human papillomavirus (HPV), poor oral hygiene, and immune deficiency syndromes etc. were also found to contribute to oral cancer risk (Gupta et al., 2013, Mignogna et al., 2004, Goel et al., 2014, Radhakrishnan et al., 2012, Macek, 2010, Oral cancer foundation). The currently available therapeutic options for oral cancer includes surgery, radiotherapy, chemotherapy with 5-fluorouracil, carboplatin, cisplatin, docetaxel and paclitaxel etc. or combination of these methods depending on the size, stage and severity of the disease and willingness of the patient (Rivera, 2015). Interestingly, the major advantage in this cancer is that the survival rate is significantly high (65-80%) compared to any other cancer if diagnosed at the early stages and treated accordingly. However, at the advanced stages, the survival rate decreases drastically despite using coadjutant therapies (Güneri and Epstein, 2014). Especially in India, survival rate of stage III and IV oral cancer patients decreased to 26.6 and 9.5% respectively from the 68.9% of Stage I and II patients according to a study involving around 15000 patients thus proving the importance of the stage of oral cancer in deciding the fate of therapy (Montero and Patel, 2015, Yeole et al., 2000). Unfortunately, almost 2/3 of the oral cancer cases are diagnosed only in the advanced stage of the disease making it difficult to treat and decreasing the chances of survival (Güneri and Epstein, 2014). Furthermore, side

effects associated with the conventional therapies, development of resistance and tumor recurrence were also known to contribute for poor prognosis (Su et al., 2010). Despite the recent development in molecular cancer therapeutics, survival rate of oral cancer does not seem to improve further. This is mainly due to the lack of biomarkers for effective targeting of the cancers in advanced stages. Recently, tumor necrosis factor (TNF)-α- induced protein 8 (TNFAIP8 or TIPE) family of proteins have been found to play significant roles in the development and maintenance of various cancers and the first member TNFAIP8/TIPE was first discovered to be differentially expressed in primary and metastatic head and neck cancer patients. Further, all the four identified members of TNFAIP8 family namely, TIPE, TIPE1, TIPE2 and TIPE3 were known to share significant sequence and structural homology. In addition, they were also reported to critically regulate tumor invasion, migration, metastasis and angiogenesis processes further shouting their therapeutic and prognostic importance (Padmavathi et al., 2018). Therefore, we hypothesized these proteins to be involved in the development and progression of oral cancer. To confirm our hypothesis, in the first place we analysed the difference in the expression of TIPE, TIPE1, TIPE2 and TIPE3 proteins in human tissue samples with the help of oral cancer tissue microarray which contains tissues from normal oral epithelia and different developmental stages and grades of oral cancer along with preneoplastic lesions. Our results have proved that the TIPE proteins are differentially expressed in OSCC tissues compared to normal tissues. In detail, TIPE, TIPE2 and TIPE3 proteins were upregulated in OSCC whereas TIPE1 was downregulated compared to the normal tissues. The upregulation of TIPE was observed to be correlated with disease progression from normal to premalignant to malignant disease and with increase in tumor stage from stage I to stage IV. Although, this is the first report showing the upregulation of TIPE in oral

premalignant and malignant tissues, there have been reports on other cancers such as cancers of blood (leukemia), breast, endometrium, liver, lung, pancreas and renal showing TIPE to be overexpressed (Kumar et al., 2004, 2000, Liu et al., 2014b, Dong et al., 2017, 2010, Liu et al., 2012, Padmavathi et al., 2018). Furthermore, earlier, the increased expression of TIPE was also shown to increase tumor growth and progression of breast cancer and HCC which is in line with the current results showing increased TIPE levels with increase in tumor progression (Kumar et al., 2004, 2000, Dong et al., 2017). Similar to TIPE, upregulation of TIPE2 was also found to be associated with oral tumor progression from hyperplasia to SCC and from stage I to IV disease suggesting TIPE2 overexpression to be a driving factor for oral cancer progression. However, this is in contrast to many other cancers such as breast, liver, lung, prostate and gastric cancers where TIPE2 was mostly shown to have antitumor effect. For example, downregulation of TIPE2 was found to increase breast cancer survival, growth, invasion, migration and metastasis through upregulation of β-catenin, cyclin D1 and c-Myc levels and phosphorylation of p38 and Akt (Wang et al., 2017, Zhang et al., 2016). Likewise, TIPE2 downregulation was also shown to decrease gastric cancer progression and metastasis through modulation of PI3K/Akt/GSK3β/β-catenin and ERK1/2 signaling pathways and to be associated with poor prognosis (Wu et al., 2016, Zhu et al., 2016, Padmavathi et al., 2018). Similarly, in glioma, liver cancer, lung cancer and prostate cancer as well the TIPE2 downregulation was proved to be positively correlated with cancer progression, invasion and metastasis by deregulating various signalling molecules (Liu et al., 2016, Cao et al., 2013, Zhang et al., 2015b, Li et al., 2016, 2015b, Lu et al., 2016). However, supporting our results, TIPE2 was also observed to be upregulated in colon cancer, NHL and RCC (Li et al., 2014, Hao et al., 2016, Zhang et al., 2013c).

Therefore, it is apparent that, role of TIPE2 in tumorigenesis varies depending upon the type of cancer and in oral cancer it acts as a pro-tumorigenic molecule with significant overexpression observed in SCC tissues compared to normal tissues. Unlike TIPE2, the recent family member TIPE3 has been reported to have majorly oncogenic role (Fayngerts et al., 2014, Lian et al., 2017, Padmavathi et al., 2018). However, being a recently identified protein, only very few studies are available to explain its role in cancer and in the present study, immunohistochemical analysis of oral TMA revealed TIPE3 to be highly overexpressed in hyperplasia, benign and OSCC compared to normal tissues. Also, the increase in the levels of TIPE3 was higher in the initial stages (stage I and II) than the stage IV tumors. Interestingly, the remaining member of TIPE family, TIPE1, which was found to be downregulated in OSCC in the current study, has also been reported earlier to exert antitumor effect in liver and lung cancers where the downregulation of TIPE1 led to decreased apoptosis and increased tumor growth, proliferation, invasion and migration (Zhang et al., 2015a, Wu et al., 2017a). Therefore, oral cancer TMA-IHC analysis proposed TIPE, TIPE2 and TIPE3 to paly oncogenic role and TIPE1 to play antitumorigenic role in oral cancer.

Further confirming this, treatment of oral cancer cells SAS and KB with the prime risk factors for the development of oral cancer i.e. crude tobacco extracts (TE and tuibur), tobacco carcinogens such as nicotine and BaP, and 4NQO (a synthetic carcinogen used to mimic oral cancer development *in vivo*) resulted in the upregulation of TIPE, TIPE2 and TIPE3 and downregulation of TIPE1. Interestingly, the extent of change in the expression of TIPE proteins was carcinogen and cell line specific. For instance, TE treatment resulted in a several fold increase of TIPE3 in SAS cells whereas exerted very minimal effect in KB cells. Similarly, expression of

TIPE and TIPE1 were significantly modulated in KB cells whereas effect in SAS cells was very nominal in response to TE treatment. Likewise, tuibur (tobacco smoke infused water extract), a commonly used tobacco product in Mizoram, India, amplified the expression of TIPE and TIPE3 regardless of the cell type however, exerted differential effect in regulating the levels of TIPE1 and TIPE2 depending on the cell line. Similar to the crude extracts, even the single purified carcinogens such as nicotine, BaP and 4NQO exhibited cell line specific impact on the regulation of TIPE proteins. i.e. Nicotine treatment led to a significant increase in TIPE expression, decrease in TIPE1 expression and minimal change in TIPE2 expression in both SAS and KB cells. However, TIPE3 expression was remarkably upregulated in KB cells whereas negligible change was observed in SAS cells in response to nicotine treatment. Likewise, treatment of SAS and KB cells with BaP significantly increased the levels of TIPE2, decreased the levels of TIPE1 and minimally altered TIPE3 levels in both cell lines. However, TIPE was upregulated in KB cells whereas minimally affected in SAS cells in response to BaP. Finally, treatment of oral cancer cells with 4NQO, a synthetic carcinogen, increased the expression levels of TIPE3, downregulated TIPE1 and had minimal effect on TIPE in both cell lines. Yet, it resulted in a profound upregulation of TIPE2 in case of SAS cells and not in case of KB cells. Moreover, the tobacco related carcinogens and TIPE proteins were found to share some common molecular targets that are known to be involved in the development and progression of oral cancer such as JAK/STAT, PI3K/Akt, Ras/Raf/MEK/ERK, EGFR, NF-κB, VEGF, MMPs, Cox-2, TNF-α (Sanner and Grimsrud, 2015, Nishioka et al., 2018, Grando, 2014, Demetriou et al., 2018, Meng et al., 2010, Wei et al., 1999, Kometani et al., 2009, Tanaka et al., 2014, Padmavathi et al., 2018). Together, our results confirmed that TIPE proteins are involved in the oral

cancer development and progression and supported our IHC results showing TIPE, TIPE2 and TIPE3 to be upregulated and TIPE1 to be downregulated upon exposure to oral cancer risk factors.

Although so far our results evidenced the involvement of TIPE proteins in oral cancer, they have given only preliminary idea requiring more confirmative results showing the signaling pathways involved in the TIPEs-mediated oral cancer and their importance in the regulation of various hallmarks of cancer. In order to achieve this, we generated four respective knockout clones for TIPE, TIPE1, TIPE2 and TIPE3 genes using CRISPR/Cas9-mediated genome editing of SAS cell line. In support to our earlier arguments, knockout of TIPE, TIPE2 or TIPE3 significantly impaired the survival, proliferation and migration of oral cancer cells and in contrast, TIPE1 knockout increased the survival, proliferation and migration of SAS cells. Our results were in line with the previous studies showing downregulation of TIPE and TIPE3 to significantly inhibit the survival, proliferation, invasion and migration of different tumor cells both *in vitro* and *in vivo* (Padmavathi et al., 2018). Likewise, the oncogenic role of TIPE1 knockout was also in accordance with other reports where ectopic expression of TIPE1 was proved to inhibit tumor growth, proliferation, invasion and induced cell death in HCC and lung cancer (Zhang et al., 2015a, Wu et al., 2017a, Padmavathi et al., 2018). However, effect of TIPE2 knockout observed in the current study was in contrast to the previous studies showing downregulation of TIPE2 to inhibit cell death and increase cancer cell survival, proliferation and migration in many cancers including cancers of breast, liver, lung and prostate, gastric cancer and glioma (Padmavathi et al., 2018). Nevertheless, in case of colon cancer, TIPE2 was shown to exhibit oncogenic role positively correlating with lymph node metastasis and downregulation of the same resulted in the activation of the pro-

apoptotic caspase-8 thus supporting our results in oral cancer (Li et al., 2014, Padmavathi et al., 2018).

Furthermore, the molecular targets of TIPE proteins in oral cancer were determined after successful knockout. Interestingly, loss of TIPE proteins modulated several proteins involved in the regulation and maintenance of cell survival, proliferation, migration, invasion and angiogenesis. For example, knockout of TIPE, TIPE2 and TIPE3 decreased the levels of Cox-2, survivin, Bcl-2, cIAP1, XIAP, CXCR4, MMP-9 and VEGF-A whereas knockout of TIPE1 increased Cox-2, cIAP1, Bcl-2 and MMP-9 expression and did not alter that of survivin. Further, Cox-2 has been reported to increase oral cancer progression and angiogenesis and its overexpression was found to be associated with poor prognosis of oral cancer patients (Wang et al., 2014b, Sharma et al., 2017). Similarly, survivin, Bcl-2, cIAP1 and XIAP are anti-apoptotic proteins overexpression of which was observed to be associated with tumor progression and resistance to therapy in oral cancers (Li et al., 2017, Mishra et al., 2015, Alam et al., 2017, Ning et al., 2014). Additionally, other downstream targets of TIPE proteins MMP-9, CXCR4 and VEGF-A were also known to regulate invasion, migration, metastasis and angiogenesis of oral cancer (Itoh and Nagase, 2002, Zhao et al., 2016, Uchida et al., 2007, Patel et al., 2015). Furthermore, TIPE and TIPE2 knockout resulted in the downregulation of Akt, p-Akt, mTOR, p-mTOR, S6 and p-S6, the key molecules of Akt/mTOR signaling cascade. In addition, TIPE2 knockout significantly inhibited the phosphorylation of STAT3 and NF-κB whereas TIPE knockout failed to alter the STAT3 phosphorylation status. Further, loss of TIPE3 was associated with decreased levels of mTOR, p-mTOR and p-STAT3. However, unlike the other oncogenic counterparts, TIPE3 knockout did not change the levels of p-Akt and p-S6. Furthermore, loss of the tumor suppressor member TIPE1 also resulted in the

modulation of several key components of Akt/mTOR pathway. i.e. TIPE1 knockout downregulated PTEN and REDD1 (inhibitors of p-Akt and mTOR) and activated Akt, mTOR and S6 through increased phosphorylation. Altogether, all the TIPE proteins except TIPE3 were found to regulate oral cancer progression through Akt/mTOR signaling and TIPE3 through Akt-independent mTOR/STAT3 activation.

Interestingly, the Akt/mTOR signaling cascade is a crucial regulator of cancer hallmarks such as cell cycle progression, survival, proliferation, invasion, migration, metastasis and genomic instability etc. and the most commonly deregulated signaling pathways in around 50% of solid cancers including breast cancer, colorectal cancer, prostate cancer, lung cancer, liver cancer, HNSCC, ovarian cancer, esophageal cancer, gastric cancer, pancreatic cancer, endometrial cancer and melanoma etc. Furthermore, it was also known to be abnormally activated in oral premalignant and malignant lesions. and numerous inhibitors have been developed and being examined for their anticancer potential in both preclinical and clinical settings (Zhang et al., 2012c, O'Donnell et al., 2018, Molinolo et al., 2009, Chaisuparat et al., 2016, Fruman and Rommel, 2014). In general, initially, cellular signals such as cytokines, chemokines or growth factors activate receptor tyrosine kinases (RTK) in the plasma membrane which in turn activates PI3K that generates PIP3 from PIP2 through phosphorylation. This lipid second messengers further results in the activation of Akt kinase and mTOR complex 1. This activation of TORC1 leads to the expression of gene products involved in several cellular processes such as survival, proliferation etc. through eukaryotic initiation factor-4E (eIF4E)-binding proteins (4E-BPs), S6 kinases, and autophagy regulators (O'Donnell et al., 2018, Fruman and Rommel, 2014). Therefore, our results clearly explain that the oncogenic potential of TIPE and TIPE2 and anti-tumor potential of TIPE1 are mediated through critical regulation of Akt/mTOR

signaling pathway. Further, explaining the Akt-independent mTOR regulation observed in TIPE3 knockout, mTOR has been reported to be regulated through AMPK, Ras-MAPK pathway and hypoxia-inducible REDD1 etc. (Zhang et al., 2012c, Memmott and Dennis, 2009). Therefore, collectively, from our results it is obvious that TIPE, TIPE2 and TIPE3 proteins positively regulate the progression of oral cancer and TIPE1 negatively regulates the same. Hence, targeting TIPE proteins holds great benefits in the treatment of oral cancer which would result in better prognosis.

5.2 Limitations and future prospects of the study

In the present study, we have established a strong correlation between TIPE proteins and oral cancer progression through various methods. However, there are few limitations that have to be clarified further. Firstly, the differential expression of TIPE proteins in oral cancer and normal oral tissues was determined by immunohistochemical analysis of TMA slides having only 80 tissue samples from different developmental stages of oral cancer. These tissue cores are very small in size i.e. each core is of 1.5 mm in diameter and 5 µm in thickness which confines our analysis to a smaller area. This could be a major concern as in cancer each and every cell might have numerous molecular alterations and differ from each other despite belonging to the same tissue. Further, the 80 tissues are categorized based on organ, disease pathology, grades and stages and the number of tissues in each category varies remarkably limiting our search of differential expression to only few categories leaving the knowledge on the expression of TIPE proteins in other categories such as different age groups, gender, organs, disease pathology and differentiation status highly unexplored. Furthermore, most importantly, it is crucial to include samples from Indian population as oral cancer is an Indian cancer and the underlying

molecular events may vary in accordance to ethnic and environmental differences. However, the TMA does not contain samples from Indian population. Therefore, keeping these limitations in mind more extensive analysis can be performed in future including more number of bigger tissue samples from Indian populace with a significant number of tissues in each aforementioned category. In addition, tissues from oral preneoplastic lesions such as leukoplakia, erythroplakia, OLP, OSF and oral dysplasia can also be included for the analysis in order to decipher the role of TIPE proteins in the development of oral cancer. Besides, expression of TIPE proteins can be determined in the tissues of different *in vivo* oral cancer models as well. Further, in the current study, factors like history of tobacco consumption, details of therapies given to the patients and their response to the treatment, chemoresistance and tumor recurrence status, and disease free and overall survival profiles etc. were not included. Hence, in future, analysing the change in the expression of TIPE proteins with respect to these factors will throw light on the clinical significance of TIPE proteins and their possible diagnostic, therapeutic and prognostic values.

Secondly, importance of oral cancer risk factors on the regulation of TIPE proteins was analysed by treating oral cancer cells with various oral carcinogens. In this case, as we examined the effect of carcinogens on established oral cancer cells which are already cancerous and having several molecular alterations, it only provided information about the involvement of TIPEs in the progression not the development of oral cancer. Hence, it is also important to analyse the effect of these carcinogens on the expression of TIPEs in normal oral epithelial cells adding more number of tobacco-related and other carcinogens and other risk factors such as viral infections which are known to induce oral cancer. Further, it is a well-known fact that oral cancer develops upon chronic exposure to tobacco and related carcinogens. Therefore,

a time-dependant study can be performed to examine the effect of such prolonged exposure to these carcinogens on the expression of TIPE proteins. Such analysis can also be performed on *in vivo* models which would give a precise knowledge on the involvement of the deregulation of TIPEs during the course of oral carcinogenesis. Furthermore, deciphering the other molecular events associated with carcinogen treatment would provide new insights into the crosstalk between different signaling cascades involved in the development and maintenance of oral cancer.

Thirdly, in the present study, we performed gene knockout analysis in order to check the effect of TIPE proteins on the regulation of cancer hallmarks and did not attempt to examine the result of retrieval of the expression which can be done in future using conditional knockout systems to further support our arguments regarding the importance of TIPE proteins in oral cancer. Further, we generated oral cancer cell lines baring only single gene knockout at a time for all four TIPE proteins. However, we haven't analysed the effect of co-deletion of these proteins. Hence, future studies can be directed towards generating co-deletion of TIPE genes and determining its effect on the different cancer hallmarks. Besides, it is also important to determine if there is a crosstalk between the TIPE proteins, as they were found to share common molecular targets at least in oral cancer. Further in the current study, we did not look into the chemoresistant and chemosensitizing potential of TIPEs. As development of resistance and tumor recurrence are the two major reasons for poor survival and recovery of oral cancer patients, effect of standard chemotherapeutic drugs on the expression of TIPE proteins and effect of silencing of TIPEs on the response to chemotherapy can also be evaluated in future to establish chemoresistant and chemosensitizing potential of these proteins in oral cancer. Furthermore, presently we established the correlation of TIPE proteins with oral cancer through *in vitro* assays

performed in only one cell line. Therefore, it is indispensable to validate our results in multiple cell lines and in *in vivo* and clinical settings before proceeding for the development of novel diagnostic, therapeutic and prognostic methods. Additionally, identifying the post translational modifications (PTMs) if any, methylation status of the promotor regions and the promotors responsible for TIPE genes' transcription is also necessary as these factors are well-established to regulate the expression and function of a protein and there are no data available on such factors of TIPEs till date which would give us more clarity on how the TIPEs deregulation occurs and its consequences in oral cancer. Above all, after the successful validation of our results, it is mandatory to identify non-toxic therapeutics that can specifically target TIPE proteins which would serve as better treatment option for earlier and advanced diseases and would greatly improve the overall survival of oral cancer patients.

BIBLIOGRAPHY

Alam M, Kashyap T, Pramanik KK, Singh AK, Nagini S, Mishra R. The elevated activation of NF-κB and AP-1 is correlated with differential regulation of Bcl-2 and associated with oral squamous cell carcinoma progression and resistance. Clin Oral Investig. 2017 Dec;21(9):2721-2731.

Al-Khouri AM, Ma Y, Togo SH, Williams S, Mustelin T. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. J Biol Chem. 2005 Oct 21;280(42):35195-202.

Alvi A, Myers EN, Johnson JT. Cancer of the oral cavity. In: Myers EN, Suen JY, editors. Cancer of the head and neck. 3rd edition. Philadelphia: Mosby. 1996; 321-360.

Awadallah M, Idle M, Patel K, Kademani D. Management update of potentially premalignant oral epithelial lesions. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018 Jun;125(6):628-636.

Awwad HK, Lotayef M, Shouman T, Begg AC, Wilson G, Bentzen SM, Abd El-Moneim H, Eissa S. Accelerated hyperfractionation (AHF) compared to conventional fractionation (CF) in the postoperative radiotherapy of locally advanced head and neck cancer: influence of proliferation. British Journal of Cancer. 2002; 86:517 – 523.

Bai D, Ueno L, Vogt PK. Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt. Int J Cancer. 2009 Dec 15;125(12):2863-70.

Banerjee K, Padmavathi G, Bhattacherjee D, Saha S, Kunnumakkara AB, Bhabak KP. Potent anti-proliferative activities of organochalcogenocyanates towards breast cancer. Org Biomol Chem. 2018 Nov 21;16(45):8769-8782.

Barik A, Rai RK, Gorain A, Majumdar S, Chowdhury A. Socio-economic disparities in tobacco consumption in rural India: evidence from a health and demographic surveillance system. Perspect Public Health. 2016 Sep;136(5):278-87.

Bartsch H, Rojas M, Nair U, Nair J, Alexandrov K. Genetic cancer susceptibility and DNA adducts: studies in smokers, tobacco chewers, and coke oven workers. Cancer Detect Prev. 1999;23(6):445-53.

Bordoloi D, Banik K, Shabnam B, Padmavathi G, Monisha J, Arfuso F, Dharmarajan A, Mao X, Lim LHK, Wang L, Fan L, Hui KM, Kumar AP, Sethi G, Kunnumakkara AB. TIPE Family of Proteins and Its Implications in Different Chronic Diseases. Int J Mol Sci. 2018 Sep 29;19(10). pii: E2974.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov;68(6):394-424.

Brinkman BM, Wong DT. Disease mechanism and biomarkers of oral squamous cell carcinoma. Curr Opin Oncol. 2006 May;18(3):228-33. Review.

Budach W, Hehr T, Budach V, Belka C and Dietz K. A meta-analysis of hyperfractionated and accelerated radiotherapy and combined chemotherapy and radiotherapy regimens in unresected locally advanced squamous cell carcinoma of the head and neck. BMC Cancer. 2006; 6:28.

Buijs N, Oosterink JE, Jessup M, Schierbeek H, Stolz DB, Houdijk AP, Geller DA, van Leeuwen PA. A new key player in VEGF-dependent angiogenesis in human hepatocellular carcinoma: dimethylarginine dimethylaminohydrolase 1. Angiogenesis. 2017 Nov;20(4):557-565.

Byakodi R, Byakodi S, Hiremath S, Byakodi J, Adaki S, Marathe K, Mahind P. Oral cancer in India: an epidemiologic and clinical review. J Community Health. 2012 Apr;37(2):316-9.

Cancer treatment centers of America; http://www.cancercenter.com/oral-cancer/risk-factors/

Cancer Treatment Centers of America® (CTCA): https://www.cancercenter.com/oral-cancer/types/

Cao X, Zhang L, Shi Y, Sun Y, Dai S, Guo C, Zhu F, Wang Q, Wang J, Wang X, Chen YH, Zhang L. Human tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 suppresses hepatocellular carcinoma metastasis through inhibiting Rac1. Mol Cancer. 2013 Nov 26;12(1):149.

Carmody RJ, Hilliard B, Maguschak K, Chodosh LA, Chen YH. Genomic scale profiling of autoimmune inflammation in the central nervous system: the nervous response to inflammation. J Neuroimmunol. 2002 Dec;133(1-2):95-107.

Chaisuparat R, Limpiwatana S, Kongpanitkul S, Yodsanga S, Jham BC. The Akt/mTOR pathway is activated in verrucous carcinoma of the oral cavity. J Oral Pathol Med. 2016 Sep;45(8):581-5.

Charafe-Jauffret E, Tarpin C, Bardou VJ, Bertucci F, Ginestier C, Braud AC, Puig B, Geneix J, Hassoun J, Birnbaum D, Jacquemier J, Viens P. Immunophenotypic analysis of inflammatory breast cancers: identification of an 'inflammatory signature'. J Pathol. 2004 Mar;202(3):265-73.

Chen L, Yang X, Yang X, Fan K, Xiao P, Zhang J, Wang X. Association between the expression levels of tumor necrosis factor-α-induced protein 8 and the prognosis of patients with gastric adenocarcinoma. Exp Ther Med. 2016 Jul;12(1):238-244.

Chen X, Zhao Y. Human papillomavirus infection in oral potentially malignant disorders and cancer. Arch Oral Biol. 2017 Nov;83:334-339.

Coelho KR. Challenges of the oral cancer burden in India. J Cancer Epidemiol. 2012;2012:701932.

Cui J, Hao C, Zhang W, Shao J, Zhang N, Zhang G, Liu S. Identical expression profiling of human and murine TIPE3 protein reveals links to its functions. J Histochem Cytochem. 2015 Mar;63(3):206-16.

Cui J, Zhang G, Hao C, Wang Y, Lou Y, Zhang W, Wang J, Liu S. The expression of TIPE1 in murine tissues and human cell lines. Mol Immunol. 2011Jul;48(12-13):1548-55.

Das BR, Nagpal JK. Understanding the biology of oral cancer. Med Sci Monit. 2002 Nov;8(11):RA258-67.

David G. Pfister, Kie-Kian Ang, David M. Brizel, Barbara A. Burtness, Anthony J. Cmelak, A. Dimitrios Colevas, Frank Dunphy, David W. Eisele, Jill Gilbert, Maura L. Gillison, Robert I. Haddad, Bruce H. Haughey, Wesley L. Hicks Jr., Ying J. Hitchcock, Merrill S. Kies, William M. Lydiatt, Ellie Maghami, Renato Martins, Thomas McCaffrey, Bharat B. Mittal, Harlan A. Pinto, John A. Ridge, Sandeep Samant, Giuseppe Sanguineti David E. Schuller, Jatin P. Shah,Sharon Spencer, Andy Trotti III, Randal S. Weber, Gregory T. Wolf Frank Worden. Head and Neck Cancers. J Natl Compr Canc Netw. 2011; 9:596-650.

Demetriou CA, Degli Esposti D, Pullen Fedinick K, Russo F, Robinson O, Vineis P. Filling the gap between chemical carcinogenesis and the hallmarks of cancer: A temporal perspective. Eur J Clin Invest. 2018 Jun;48(6):e12933.

Denoix PF. Tumor, Node and Metastasis (TNM). Bull Inst Nat Hyg (Paris) 1944;1:1–69.

Dong Q, Fu L, Zhao Y, Xie C, Li Q, Wang E. TNFAIP8 interacts with LATS1 and promotes aggressiveness through regulation of Hippo pathway in hepatocellular carcinoma. Oncotarget. 2017 Feb 28;8(9):15689-15703.

Dong QZ, Zhao Y, Liu Y, Wang Y, Zhang PX, Jiang GY, Dong XJ, Cui QZ, Wang EH. Overexpression of SCC-S2 correlates with lymph node metastasis and poor prognosis in patients with non-small-cell lung cancer. Cancer Sci. 2010 Jun;101(6):1562-9.

Du L, Shen J, Weems A, Lu SL. Role of phosphatidylinositol-3-kinase pathway in head and neck squamous cell carcinoma. J Oncol. 2012;2012:450179.

Duan D, Zhu YQ, Guan LL, Wang J. Upregulation of SCC-S2 in immune cells and tumor tissues of papillary thyroid carcinoma. Tumour Biol. 2014 May;35(5):4331-7.

Elango JK, Sundaram KR, Gangadharan P, Subhas P, Peter S, Pulayath C, Kuriakose MA. Factors affecting oral cancer awareness in a high-risk population in India. Asian Pac J Cancer Prev. 2009 Oct-Dec;10(4):627-30.

Fang M, Zhang W, Chen Y, He Z. Malignant transformation of oral lichen planus: a retrospective study of 23 cases. Quintessence Int. 2009 Mar;40(3):235-42.

Fayngerts SA, Wang Z, Zamani A, Sun H, Boggs AE, Porturas TP, Xie W, Lin M, Cathopoulis T, Goldsmith JR, Vourekas A, Chen YH. Direction of leukocyte

polarization and migration by the phosphoinositide-transfer protein TIPE2. Nat Immunol. 2017 Dec;18(12):1353-1360.

Fayngerts SA, Wu J, Oxley CL, Liu X, Vourekas A, Cathopoulis T, Wang Z, Cui J, Liu S, Sun H, Lemmon MA, Zhang L, Shi Y, Chen YH. TIPE3 Is the Transfer Protein of Lipid Second Messengers that Promote Cancer. Cancer Cell. 2014 Oct 13;26(4):465-78.

Feller L, Chandran R, Khammissa RA, Meyerov R, Lemmer J. Alcohol and oral squamous cell carcinoma. SADJ. 2013 May;68(4):176-80.

Fotedar V, Fotedar S, Seam RK, Gupta MK. Oral Cancer and Chemoprevention. International Journal of Pharmaceutical Science Invention. 2013; 2(2):16-20.

Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1(5):2315-9.

Freundt EC, Bidere N, Lenardo MJ. A different TIPE of immune homeostasis. Cell. 2008 May 2;133(3):401-2.

Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014 Feb;13(2):140-56.

Galluzzi L, Kroemer G. Necroptosis: a specialized pathway of programmed necrosis. Cell. 2008 Dec 26;135(7):1161-3.

Gao H, Prasad GL, Zacharias W. Combusted but not smokeless tobacco products cause DNA damage in oral cavity cells. Environ Toxicol Pharmacol. 2014 May;37(3):1079-89.

Goel A, Goel P, Mishra S, Saha R, Torwane N. Risk Factor Analysis for Oral Precancer among Slum Dwellers in Delhi, India. Ann Med Health Sci Res. 2014 Sep;4 (Suppl 3):S218-22.

Grando SA. Connections of nicotine to cancer. Nat Rev Cancer. 2014 Jun;14(6):419-29.

Güneri P, Epstein JB. Late stage diagnosis of oral cancer: components and possible solutions. Oral Oncol. 2014 Dec;50(12):1131-6.

Gupta B, Ariyawardana A, Johnson NW. Oral cancer in India continues in epidemic proportions: evidence base and policy initiatives. Int Dent J. 2013 Feb;63(1):12-25.

Gus-Brautbar Y, Johnson D, Zhang L, Sun H, Wang P, Zhang S, Zhang L, Chen YH. The anti-inflammatory TIPE2 is an inhibitor of the oncogenic Ras. Mol Cell. 2012 Mar 9;45(5):610-8.

Ha JY, Kim JS, Kang YH, Bok E, Kim YS, Son JH. Tnfaip8 l1/Oxi-β binds to FBXW5, increasing autophagy through activation of TSC2 in a Parkinson's disease model. J Neurochem. 2014 May;129(3):527-38.

Hadisaputri YE, Miyazaki T, Suzuki S, Yokobori T, Kobayashi T, Tanaka N, Inose T, Sohda M, Kuwano H. TNFAIP8 overexpression: clinical relevance to esophageal squamous cell carcinoma. Ann Surg Oncol. 2012 Jul;19 Suppl 3:S589-96.

Hamouchene H, Arlt VM, Giddings I, Phillips DH. Influence of cell cycle on responses of MCF-7 cells to benzo[a]pyrene. BMC Genomics. 2011 Jun 29;12:333.

Han Y, Tang Z, Zhao Y, Li Q, Wang E. TNFAIP8 regulates Hippo pathway through interacting with LATS1 to promote cell proliferation and invasion in lung cancer. Mol Carcinog. 2018 Feb;57(2):159-166.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

Hao C, Zhang N, Geng M, Ren Q, Li Y, Wang Y, Chen YH, Liu S. Clinical Significance of TIPE2 Protein Upregulation in Non-Hodgkin's Lymphoma. J Histochem Cytochem. 2016 Sep;64(9):556-64.

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.

Hashemi Goradel N, Najafi M, Salehi E, Farhood B, Mortezaee K. Cyclooxygenase-2 in cancer: A review. J Cell Physiol. 2019 May;234(5):5683-5699.

Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and molecular targeting therapy in cancer. Biomed Res Int. 2014;2014:150845.

Hecht SS, Hoffmann D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis. 1988 Jun;9(6):875-84.

Hecht SS, Szabo E. Fifty years of tobacco carcinogenesis research: from mechanisms to early detection and prevention of lung cancer. Cancer Prev Res (Phila). 2014 Jan;7(1):1-8.

Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer. 2003 Oct;3(10):733-44. Review. Erratum in: Nat Rev Cancer. 2004 Jan;4(1):84.

Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov. 2005 Dec;4(12):988-1004.

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell. 2008 Dec 26;135(7):1311-23.

Horrevoets AJ, Fontijn RD, van Zonneveld AJ, de Vries CJ, ten Cate JW, Pannekoek H. Vascular endothelial genes that are responsive to tumor necrosis factor-alpha in vitro are expressed in atherosclerotic lesions, including inhibitor of apoptosis protein-1, stannin, and two novel genes. Blood. 1999 May 15;93(10):3418-31.

Hu A, Huang JJ, Zhang JF, Dai WJ, Li RL, Lu ZY, Duan JL, Li JP, Chen XP, Fan JP, Xu WH, Zheng HL. Curcumin induces G2/M cell cycle arrest and apoptosis of head and neck squamous cell carcinoma in vitro and in vivo through ATM/Chk2/p53-dependent pathway. Oncotarget. 2017 Apr 13;8(31):50747-50760.

Hu R, Qiu X, Hong S, Meng L, Hong X, Qiu J, Yang J, Zhuang G, Liu Z. Clinical significance of TIPE expression in gastric carcinoma. Onco Targets Ther. 2016 Jul 25;9:4473-81.

Huang H. Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. Sensors (Basel). 2018 Sep 27;18(10). pii: E3249.

Irani S. Pre-Cancerous Lesions in the Oral and Maxillofacial Region: A Literature Review with Special Focus on Etiopathogenesis. Iran J Pathol. 2016 Fall;11(4):303-322.

Itoh Y, Nagase H. Matrix metalloproteinases in cancer. Essays Biochem. 2002;38:21-36.

Jain P, Kumar P, Pai VR, Parikh PM. Neoadjuvant chemotherapy or chemoradiotherapy in head and neck cancer. Indian J Cancer. 2008 Jul-Sep;45(3):83-9.

Jakymiw A, Patel RS, Deming N, Bhattacharyya I, Shah P, Lamont RJ, Stewart CM, Cohen DM, Chan EK. Overexpression of dicer as a result of reduced let-7 MicroRNA levels contributes to increased cell proliferation of oral cancer cells. Genes Chromosomes Cancer. 2010 Jun;49(6):549-59.

Jalouli J, Ibrahim SO, Mehrotra R, Jalouli MM, Sapkota D, Larsson PA, Hirsch JM. Prevalence of viral (HPV, EBV, HSV) infections in oral submucous fibrosis and oral cancer from India. Acta Otolaryngol. 2010 Nov;130(11):1306-11.

Janbaz KH, Qadir MI, Basser HT, Bokhari TH, Ahmad B. Risk for oral cancer from smokeless tobacco. Contemp Oncol (Pozn). 2014;18(3):160-4.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin. 2008 Mar-Apr;58(2):71-96.

Jia L, Gui B, Tian P, Yao G, Fu R, Wang L, Ge H, Ou Y. TIPE2, a novel biomarker for clinical chronic kidney allograft rejection. Artif Organs. 2013 Feb;37(2):221-5.

Johnson N. Tobacco use and oral cancer: a global perspective. J Dent Educ. 2001 Apr;65(4):328-39.

Joseph JP, Harishankar MK, Pillai AA, Devi A. Hypoxia induced EMT: A review on the mechanism of tumor progression and metastasis in OSCC. Oral Oncol. 2018 May;80:23-32.

Kanojia D, Vaidya MM. 4-nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol. 2006 Aug;42(7):655-67.

Katiyar S, Liu E, Knutzen CA, Lang ES, Lombardo CR, Sankar S, Toth JI, Petroski MD, Ronai Z, Chiang GG. REDD1, an inhibitor of mTOR signalling, is regulated by the CUL4A-DDB1 ubiquitin ligase. EMBO Rep. 2009 Aug;10(8):866-72.

Kerawala C, Roques T, Jeannon JP, Bisase B. Oral cavity and lip cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol. 2016 May;130(S2):S83-S89.

Kim JS, Park J, Kim MS, Ha JY, Jang YW, Shin DH, Son JH. The Tnfaip8-PE complex is a novel upstream effector in the anti-autophagic action of insulin. Sci Rep. 2017 Jul 24;7(1):6248.

Kim SK, Ioannidis JPA, Ahmed MA, Avins AL, Kleimeyer JP, Fredericson M, Dragoo JL. Two Genetic Variants Associated with Plantar Fascial Disorders. Int J Sports Med. 2018 Apr;39(4):314-321.

Kim Y, Shintani S, Kohno Y, Zhang R, Wong DT. Cyclin G2 dysregulation in human oral cancer. Cancer Res. 2004 Dec 15;64(24):8980-6.

Kolesnichenko M, Vogt PK. Understanding PLZF: two transcriptional targets, REDD1 and smooth muscle α-actin, define new questions in growth control, senescence, self-renewal and tumor suppression. Cell Cycle. 2011 Mar 1;10(5):771-5.

Kometani T, Yoshino I, Miura N, Okazaki H, Ohba T, Takenaka T, Shoji F, Yano T, Maehara Y. Benzo[a]pyrene promotes proliferation of human lung cancer cells by accelerating the epidermal growth factor receptor signaling pathway. Cancer Lett. 2009 Jun 8;278(1):27-33.

Kong L, Liu K, Zhang YZ, Jin M, Wu BR, Wang WZ, Li W, Nan YM, Chen YH. Downregulation of TIPE2 mRNA expression in peripheral blood mononuclear cells from patients with chronic hepatitis C. Hepatol Int. 2013 Jul;7(3):844-9.

Kramer IR, Lucas RB, Pindborg JJ, Sobin LH. Definition of leukoplakia and related lesions: an aid to studies on oral precancer. Oral Surg Oral Med Oral Pathol. 1978 Oct;46(4):518-39.

Kumar D, Gokhale P, Broustas C, Chakravarty D, Ahmad I, Kasid U. Expression of SCC-S2, an antiapoptotic molecule, correlates with enhanced proliferation and tumorigenicity of MDA-MB 435 cells. Oncogene. 2004 Jan 15;23(2):612-6.

Kumar D, Whiteside TL, Kasid U. Identification of a novel tumor necrosis factor-alpha-inducible gene, SCC-S2, containing the consensus sequence of a death effector domain of fas-associated death domain-like interleukin- 1beta-converting enzyme-inhibitory protein. J Biol Chem. 2000 Jan 28;275(4):2973-8.

Kushner J, Bradley G, Young B, Jordan RC. Aberrant expression of cyclin A and cyclin B1 proteins in oral carcinoma. J Oral Pathol Med. 1999 Feb;28(2):77-81.

Li D, Song L, Fan Y, Li X, Li Y, Chen J, Zhu F, Guo C, Shi Y, Zhang L. Down-regulation of TIPE2 mRNA expression in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Immunol. 2009 Dec;133(3):422-7.

Li S, Yang Y, Ding Y, Tang X, Sun Z. Impacts of survivin and caspase-3 on apoptosis and angiogenesis in oral cancer. Oncol Lett. 2017 Sep;14(3):3774-3779.

Li X. TIPE2 regulates tumor-associated macrophages in skin squamous cell carcinoma. Tumour Biol. 2016 Apr;37(4):5585-90.

Li XM, Su JR, Yan SP, Cheng ZL, Yang TT, Zhu Q. A novel inflammatory regulator TIPE2 inhibits TLR4-mediated development of colon cancer via caspase-8. Cancer Biomark. 2014;14(4):233-40.

Li Y, Jing C, Chen Y, Wang J, Zhou M, Liu X, Sun D, Mu L, Li L, Guo X. Expression of tumor necrosis factor α-induced protein 8 is upregulated in human gastric cancer and regulates cell proliferation, invasion and migration. Mol Med Rep. 2015a Aug;12(2):2636-42.

Li Y, Li X, Liu G, Sun R, Wang L, Wang J, Wang H. Downregulated TIPE2 is associated with poor prognosis and promotes cell proliferation in non-small cell lung cancer. Biochem Biophys Res Commun. 2015b Jan 30;457(1):43-9.

Li Y, Li X, Liu G, Sun R, Wang L, Wang J, Wang H. Downregulated TIPE2 is associated with poor prognosis and promotes cell proliferation in non-small cell lung cancer. Biochem Biophys Res Commun. 2015 Jan 30;457(1):43-9.

Li Z, Guo C, Liu X, Zhou C, Zhu F, Wang X, Wang Q, Shi Y, Wang J, Zhao W, Zhang L. TIPE2 suppresses angiogenesis and non-small cell lung cancer (NSCLC) invasiveness via inhibiting Rac1 activation and VEGF expression. Oncotarget. 2016 Sep 20;7(38):62224-62239.

Li Z, Jia W, Niu J, Zhang L. Understanding the roles of negative immune regulator TIPE2 in different diseases and tumourigenesis. Histol Histopathol. 2018 Sep;33(9):919-928.

Lian K, Ma C, Hao C, Li Y, Zhang N, Chen YH, Liu S. TIPE3 protein promotes breast cancer metastasis through activating AKT and NF-κB signaling pathways. Oncotarget. 2017 Jul 25;8(30):48889-48904.

Liu J, Huang XE, Feng JF. Further Study on Pemetrexed based chemotherapy in Treating Patients with Advanced Gastric Cancer (AGC). Asian Pac J Cancer Prev. 2014a; 15: 6587-90.

Liu K, Qin CK, Wang ZY, Liu SX, Cui XP, Zhang DY. Expression of tumor necrosis factor-alpha-induced protein 8 in pancreas tissues and its correlation with epithelial growth factor receptor levels. Asian Pac J Cancer Prev. 2012;13(3):847-50.

Liu T, Gao H, Chen X, Lou G, Gu L, Yang M, Xia B, Yin H. TNFAIP8 as a predictor of metastasis and a novel prognostic biomarker in patients with epithelial ovarian cancer. Br J Cancer. 2013 Sep 17;109(6):1685-92.

Liu T, Gao H, Yang M, Zhao T, Liu Y, Lou G. Correlation of TNFAIP8 overexpression with the proliferation, metastasis, and disease-free survival in endometrial cancer. Tumour Biol. 2014b Jun;35(6):5805-14.

Liu T, Xia B, Lu Y, Xu Y, Lou G. TNFAIP8 overexpression is associated with platinum resistance in epithelial ovarian cancers with optimal cytoreduction. Hum Pathol. 2014c Jun;45(6):1251-7.

Liu Z, Muehlbauer KR, Schmeiser HH, Hergenhahn M, Belharazem D, Hollstein MC. p53 mutations in benzo(a)pyrene-exposed human p53 knock-in murine fibroblasts correlate with p53 mutations in human lung tumors. Cancer Res. 2005 Apr 1;65(7):2583-7.

Liu ZJ, Liu HL, Zhou HC, Wang GC. TIPE2 Inhibits Hypoxia-Induced Wnt/β-Catenin Pathway Activation and EMT in Glioma Cells. Oncol Res. 2016;24(4):255-61.

Lou Y, Liu S, Zhang C, Zhang G, Li J, Ni M, An G, Dong M, Liu X, Zhu F, Zhang W, Gao F, Chen YH, Zhang Y. Enhanced atherosclerosis in TIPE2-deficient mice is associated with increased macrophage responses to oxidized low-density lipoprotein. J Immunol. 2013 Nov 1;191(9):4849-57.

Lou Y, Liu S. The TIPE (TNFAIP8) family in inflammation, immunity, and cancer. Mol Immunol. 2011 Oct;49(1-2):4-7.

Lou Y, Sun H, Morrissey S, Porturas T, Liu S, Hua X, Chen YH. Critical roles of TIPE2 protein in murine experimental colitis. J Immunol. 2014 Aug 1;193(3):1064-70.

Lowe JM, Nguyen TA, Grimm SA, Gabor KA, Peddada SD, Li L, Anderson CW, Resnick MA, Menendez D, Fessler MB. The novel p53 target TNFAIP8 variant 2 is increased in cancer and offsets p53-dependent tumor suppression. Cell Death Differ. 2017 Jan;24(1):181-191.

Lu Q, Liu Z, Li Z, Chen J, Liao Z, Wu WR, Li YW. TIPE2 Overexpression Suppresses the Proliferation, Migration, and Invasion in Prostate Cancer Cells by Inhibiting PI3K/Akt Signaling Pathway. Oncol Res. 2016;24(5):305-313.

Luan YY, Yao YM, Sheng ZY. The tumor necrosis factor-alpha-induced protein 8 family in immune homeostasis and inflammatory cancer diseases. J Biol Regul Homeost Agents. 2013 Jul-Sep;27(3):611-9.

Luan YY, Yao YM, Zhang L, Dong N, Zhang QH, Yu Y, Sheng ZY. Expression of tumor necrosis factor-α induced protein 8 like-2 contributes to the immunosuppressive property of CD4(+)CD25(+) regulatory T cells in mice. Mol Immunol. 2011 Oct;49(1-2):219-26.

Lyu J, Zhong Y, Li C, Song H, Ren G, Guo W. Prognostic value of residual node involvement after induction chemotherapy in operable oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014 Sep;118(3):295-9.

Ma Y, Liu X, Wei Z, Wang X, Wang Z, Zhong W, Li Y, Zhu F, Guo C, Zhang L, Wang X. The expression and significance of TIPE2 in peripheral blood mononuclear cells from asthmatic children. Scand J Immunol. 2013 Dec;78(6):523-8.

Macek MD. Younger adults with oral cavity squamous cell cancer have a significantly higher 5-year survival rate than do older adults in the united states, controlling for patient- and disease-specific characteristics. J Evid Based Dent Pract. 2010 Sep;10(3):158-9.

Mahanta J, Chetia M, Hazarika N, Narain K, Sharma S. Toxicity of tuibur, a unique form of tobacco smoke extract used in Mizoram, India. Current Science. 1998;75(4):381-4.

Malakar M, Devi KR, Phukan RK, Kaur T, Deka M, Puia L, Barua D, Mahanta J, Narain K. Genetic polymorphism of glutathione S-transferases M1 and T1, tobacco habits and risk of stomach cancer in Mizoram, India. Asian Pac J Cancer Prev. 2012;13(9):4725-32.

McDonald JW, Pilgram TK. Nuclear expression of p53, p21 and cyclin D1 is increased in bronchioloalveolar carcinoma. Histopathology. 1999 May;34(5):439-46.

Mehrotra R, Yadav S. Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations. Indian J Cancer. 2006 Apr-Jun;43(2):60-6.

Memmott RM, Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. Cell Signal. 2009 May;21(5):656-64.

Mendes RA., Oncogenic Pathways in the Development of Oral Cancer. J Carcinogene Mutagene 2012, 3: e110.

Meng F, Knapp GW, Green T, Ross JA, Parsons BL. K-Ras mutant fraction in A/J mouse lung increases as a function of benzo[a]pyrene dose. Environ Mol Mutagen. 2010 Mar;51(2):146-55.

Miao Z, Zhao T, Wang Z, Xu Y, Song Y, Wu J, Xu H. SCC-S2 is overexpressed in colon cancers and regulates cell proliferation. Tumour Biol. 2012 Dec;33(6):2099-106.

Mignogna MD, Fedele S, Lo Russo L. The World Cancer Report and the burden of oral cancer. Eur J Cancer Prev. 2004 Apr;13(2):139-42.

Minna JD. Nicotine exposure and bronchial epithelial cell nicotinic acetylcholine receptor expression in the pathogenesis of lung cancer. J Clin Invest. 2003 Jan;111(1):31-3.

Mishra R, Das BR. Activation of STAT 5-cyclin D1 pathway in chewing tobacco mediated oral squamous cell carcinoma. Mol Biol Rep. 2005 Sep;32(3):159-66.

Mishra R, Palve V, Kannan S, Pawar S, Teni T. High expression of survivin and its splice variants survivin ΔEx3 and survivin 2 B in oral cancers. Oral Surg Oral Med Oral Pathol Oral Radiol. 2015 Oct;120(4):497-507.

Moles MAG, Montoya JAG and Avila IR: Bases moleculares de la cancerización de cavidad oral. Av Odontoestomatol 2008, 24: 55-60.

Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol. 2009 Apr-May;45(4-5):324-34.

Moniz LS, Vanhaesebroeck B. A New TIPE of Phosphoinositide Regulator in Cancer. Cancer Cell. 2014 Oct 13;26(4):443-4.

Montero PH, Patel SG. Cancer of the oral cavity. Surg Oncol Clin N Am. 2015 Jul;24(3):491-508.

Moon WS, Rhyu KH, Kang MJ, Lee DG, Yu HC, Yeum JH, Koh GY, Tarnawski AS. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? Mod Pathol. 2003 Jun;16(6):552-7.

Mupparapu M, Shanti RM. Evaluation and Staging of Oral Cancer. Dent Clin North Am. 2018 Jan;62(1):47-58.

Nauta JM, Roodenburg JL, Nikkels PG, Witjes MJ, Vermey A. Epithelial dysplasia and squamous cell carcinoma of the Wistar rat palatal mucosa: 4NQO model. Head Neck. 1996 Sep-Oct;18(5):441-9.

Nebert DW, Shi Z, Gálvez-Peralta M, Uno S, Dragin N. Oral benzo[a]pyrene: understanding pharmacokinetics, detoxication, and consequences–Cyp1 knockout mouse lines as a paradigm. Mol Pharmacol. 2013 Sep;84(3):304-13.

Neville BW, Day TA. Oral cancer and precancerous lesions. CA Cancer J Clin. 2002 Jul-Aug;52(4):195-215.

Ning ZR, Li S, Guo YW, Fang DJ. [Expression and clinical significance of Cox-2 and XIAP in malignant tumors of the salivary gland]. Shanghai Kou Qiang Yi Xue. 2014 Jun;23(3):317-21.

Nishioka T, Guo J, Yamamoto D, Chen L, Huppi P, Chen CY. Nicotine, through upregulating pro-survival signaling, cooperates with NNK to promote transformation. J Cell Biochem. 2010 Jan 1;109(1):152-61.

Nishioka T, Tada H, Ibaragi S, Chen C, Sasano T. Nicotine exposure induces the proliferation of oral cancer cells through the α7 subunit of the nicotinic acetylcholine receptor. Biochem Biophys Res Commun. 2018 Dec 28. pii: S0006-291X(18)32823-7.

O'Donnell JS, Massi D, Teng MWL, Mandala M. PI3K-AKT-mTOR inhibition in cancer immunotherapy, redux. Semin Cancer Biol. 2018 Feb;48:91-103.

Ohta S, Uemura H, Matsui Y, Ishiguro H, Fujinami K, Kondo K, Miyamoto H, Yazawa T, Danenberg K, Danenberg PV, Tohnai I, Kubota Y. Alterations of p16 and p14ARF genes and their 9p21 locus in oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009 Jan;107(1):81-91.

Omura K. Current status of oral cancer treatment strategies: surgical treatments for oral squamous cell carcinoma. Int J Clin Oncol. 2014;19(3):423-30.

Oral cancer foundation; http://ocf.org.in/professional/IncidenceAndPrevalence.aspx

Ostwald C, Rutsatz K, Schweder J, Schmidt W, Gundlach K, Barten M. Human papillomavirus 6/11, 16 and 18 in oral carcinomas and benign oral lesions. Med Microbiol Immunol. 2003 Aug;192(3):145-8.

Padmavathi G, Banik K, Monisha J, Bordoloi D, Shabnam B, Arfuso F, Sethi G, Fan L, Kunnumakkara AB. Novel tumor necrosis factor-α induced protein eight (TNFAIP8/TIPE) family: Functions and downstream targets involved in cancer progression. Cancer Lett. 2018 Sep 28;432:260-271.

Patel KR, Vajaria BN, Begum R, Patel JB, Shah FD, Joshi GM, Patel PS. VEGFA isoforms play a vital role in oral cancer progression. Tumour Biol. 2015 Aug;36(8):6321-32.

Patel S, Wang FH, Whiteside TL, Kasid U. Identification of seven differentially displayed transcripts in human primary and matched metastatic head and neck squamous cell carcinoma cell lines: implications in metastasis and/or radiation response. Oral Oncol. 1997 May;33(3):197-203.

Peng Y, Zhao Q, Zhang H, Han B, Liu S, Han M, Liu S. TIPE2, a negative regulator of TLR signaling, regulates p27 through IRF4-induced signaling. Oncol Rep. 2016 Apr;35(4):2480-6.

Pereira MC, Oliveira DT, Landman G, Kowalski LP. Histologic subtypes of oral squamous cell carcinoma: prognostic relevance. J Can Dent Assoc. 2007 May;73(4):339-44.

Pérez-Sayáns M, Somoza-Martín JM, Barros-Angueira F, Reboiras-López MD, Gándara Rey JM, García-García A. Genetic and molecular alterations associated with oral squamous cell cancer (Review). Oncol Rep. 2009 Dec;22(6):1277-82.

Petersen PE. Strengthening the prevention of oral cancer: the WHO perspective. Community Dent Oral Epidemiol. 2005; 33: 397–9. Blackwell Munksgaard, 2005.

Petrelli F, Coinu A, Riboldi V, Borgonovo K, Ghilardi M, Cabiddu M, Lonati V, Sarti E, Barni S. Concomitant platinum-based chemotherapy or cetuximab with radiotherapy for locally advanced head and neck cancer: A systematic review and meta-analysis of published studies. Oral Oncol. 2014 Aug 27. pii: S1368-8375(14)00239-5.

Phukan RK, Zomawia E, Narain K, Hazarika NC, Mahanta J. Tobacco use and stomach cancer in Mizoram, India. Cancer Epidemiol Biomarkers Prev. 2005 Aug;14(8):1892-6.

Porturas TP, Sun H, Buchlis G, Lou Y, Liang X, Cathopoulis T, Fayngerts S, Johnson DS, Wang Z, Chen YH. Crucial roles of TNFAIP8 protein in regulating apoptosis and Listeria infection. J Immunol. 2015 Jun 15;194(12):5743-50.

Rafehi H, Orlowski C, Georgiadis GT, Ververis K, El-Osta A, Karagiannis TC. Clonogenic assay: adherent cells. J Vis Exp. 2011 Mar 13;(49). pii: 2573.

Raghu Radhakrishnan, Bijayata Shrestha and Dipshikha Bajracharya (2012). Oral Cancer - An Overview, Oral Cancer, Dr. Kalu U. E. Ogbureke (Ed.), ISBN: 978-953-51-0228-1, InTech.

Rajalalitha P, Vali S. Molecular pathogenesis of oral submucous fibrosis- a collagen metabolic disorder. J Oral Pathol Med. 2005 Jul;34(6):321-8.

Ren XY, Wen X, Li YQ, Zhang J, He QM, Yang XJ, Tang XR, Wang YQ, Zhang PP, Chen XZ, Cheng B, Ma J, Liu N. TIPE3 hypermethylation correlates with worse prognosis and promotes tumor progression in nasopharyngeal carcinoma. J Exp Clin Cancer Res. 2018 Sep 14;37(1):227.

Ribeiro IP, Barroso L, Marques F, Melo JB, Carreira IM. Early detection and personalized treatment in oral cancer: the impact of omics approaches. Mol Cytogenet. 2016 Nov 23;9:85.

Rivera C. Essentials of oral cancer. Int J Clin Exp Pathol. 2015 Sep 1;8(9):11884-94.

Roy NK, Bordoloi D, Monisha J, Padmavathi G, Kotoky J, Golla R, Kunnumakkara AB. Specific Targeting of Akt Kinase Isoforms: Taking the Precise Path for Prevention and Treatment of Cancer. Curr Drug Targets. 2017;18(4):421-435.

Ruan Q, Wang P, Wang T, Qi J, Wei M, Wang S, Fan T, Johnson D, Wan X, Shi W, Sun H, Chen YH. MicroRNA-21 regulates T-cell apoptosis by directly targeting the tumor suppressor gene Tipe2. Cell Death Dis. 2014 Feb 27;5:e1095.

Sanner T, Grimsrud TK. Nicotine: Carcinogenicity and Effects on Response to Cancer Treatment - A Review. Front Oncol. 2015 Aug 31;5:196.

Schierl M, Patel D, Ding W, Kochhar A, Adhami K, Zhou XK, Dannenberg AJ, Granstein RD. Tobacco smoke-induced immunologic changes may contribute to oral carcinogenesis. J Investig Med. 2014 Feb;62(2):316-23.

Schliephake H. Prognostic relevance of molecular markers of oral cancer- a review. Int J Oral Maxillofac Surg. 2003 Jun;32(3):233-45.

Scully C, Newman L, Bagan JV. The role of the dental team in preventing and diagnosing cancer: 2. Oral cancer risk factors. Dent Update. 2005 Jun;32(5):261-2, 264-6, 269-70 passim.

SEER Stat Fact Sheets: Oral Cavity and Pharynx Cancer 2003-2009 http://seer.cancer.gov/statfacts/html/oralcav.html

Sharma V, Nandan A, Sharma AK, Singh H, Bharadwaj M, Sinha DN, Mehrotra R. Signature of genetic associations in oral cancer. Tumour Biol. 2017 Oct;39(10):1010428317725923.

Shenoi R, Devrukhkar V; Chaudhuri, Sharma BK, Sapre SB, Chikhale A. Demographic and clinical profile of oral squamous cell carcinoma patients: a retrospective study. Indian J Cancer. 2012 Jan-Mar;49(1):21-6.

Shi TY, Cheng X, Yu KD, Sun MH, Shao ZM, Wang MY, Zhu ML, He J, Li QX, Chen XJ, Zhou XY, Wu X, Wei Q. Functional variants in TNFAIP8 associated with cervical cancer susceptibility and clinical outcomes. Carcinogenesis. 2013 Apr;34(4):770-8.

Shiao YH, Palli D, Caporaso NE, Alvord WG, Amorosi A, Nesi G, Saieva C, Masala G, Fraumeni JF Jr, Rice JM. Genetic and immunohistochemical analyses of p53 independently predict regional metastasis of gastric cancers. Cancer Epidemiol Biomarkers Prev. 2000 Jun;9(6):631-3.

Shirani S, Kargahi N, Razavi SM, Homayoni S. Epithelial dysplasia in oral cavity. Iran J Med Sci. 2014 Sep;39(5):406-17.

Simard EP, Torre LA, Jemal A. International trends in head and neck cancer incidence rates: differences by country, sex and anatomic site. Oral Oncol. 2014 May;50(5):387-403.

Sindhu SK, Bauman JE. Current Concepts in Chemotherapy for Head and Neck Cancer. Oral Maxillofac Surg Clin North Am. 2019 Feb;31(1):145-154.

Sinha DN, Gupta PC, Pednekar MS. Tobacco use among school personnel in eight North-eastern states of India. Indian J Cancer. 2003 Jan-Mar;40(1):3-14.

Su L, Wang Y, Xiao M, Lin Y, Yu L. Up-regulation of survivin in oral squamous cell carcinoma correlates with poor prognosis and chemoresistance. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010 Oct;110(4):484-91.

Sudbø J. Novel management of oral cancer: a paradigm of predictive oncology. Clin Med es. 2004 Nov;2(4):233-42.

Summerlin DJ. Precancerous and cancerous lesions of the oral cavity. Dermatol Clin. 1996 Apr;14(2):205-23.

Sun H, Gong S, Carmody RJ, Hilliard A, Li L, Sun J, Kong L, Xu L, Hilliard B, Hu S, Shen H, Yang X, Chen YH. TIPE2, a negative regulator of innate and adaptive immunity that maintains immune homeostasis. Cell. 2008 May 2;133(3):415-26.

Sun H, Zhuang G, Chai L, Wang Z, Johnson D, Ma Y, Chen YH. TIPE2 controls innate immunity to RNA by targeting the phosphatidylinositol 3-kinase-Rac pathway. J Immunol. 2012 Sep 15;189(6):2768-73.

Sun Z, Liu X, Song JH, Cheng Y, Liu Y, Jia Y, Meltzer SJ, Wang Z. TNFAIP8 overexpression: a potential predictor of lymphatic metastatic recurrence in pN0 esophageal squamous cell carcinoma after Ivor Lewis esophagectomy. Tumour Biol. 2016 Aug;37(8):10923-34.

Tahover E, Hubert A, Temper M, Salah A, Peretz T, Hamburger T, Uziely B. An observational cohort study of bevacizumab and chemotherapy in metastatic colorectal cancer patients: safety and efficacy with analysis by age group. Target Oncol. 2015 Mar;10(1):55-63.

Tanaka T, Ishigamori R. Understanding Carcinogenesis for Fighting Oral Cancer. Journal of Oncology. 2011; 603740.

Tanaka T, Shimizu M, Kochi T, Shirakami Y, Mori T, Watanabe N, Naiki T, Moriwaki H, Yoshimi K, Serikawa T, Kuramoto T. Apc-Mutant Kyoto Apc Delta (KAD) Rats Are Susceptible to 4-NQO-Induced Tongue Carcinogenesis. Cancers (Basel). 2014 Jul 21;6(3):1522-39.

Tang XH, Knudsen B, Bemis D, Tickoo S, Gudas LJ. Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. Clin Cancer Res. 2004 Jan 1;10(1 Pt 1):301-13.

Telloni SM. Tumor Staging and Grading: A Primer. Methods Mol Biol. 2017;1606:1-17.

Thomas GT, Lewis MP, Speight PM. Matrix metalloproteinases and oral cancer. Oral Oncol. 1999 May;35(3):227-33.

Tsantoulis PK, Kastrinakis NG, Tourvas AD, Laskaris G, Gorgoulis VG. Advances in the biology of oral cancer. Oral Oncol. 2007 Jul;43(6):523-34.

Uchida D, Onoue T, Tomizuka Y, Begum NM, Miwa Y, Yoshida H, Sato M. Involvement of an autocrine stromal cell derived factor-1/CXCR4 system on the distant metastasis of human oral squamous cell carcinoma. Mol Cancer Res. 2007 Jul;5(7):685-94.

Vermorken JB, Specenier P. Optimal treatment for recurrent/metastatic head and neck cancer. Annals of Oncology. 2010; 21 (7): 252–261.

Wakulich C, Jackson-Boeters L, Daley TD, Wysocki GP. Immunohistochemical localization of growth factors fibroblast growth factor-1 and fibroblast growth factor-2 and receptors fibroblast growth factor receptor-2 and fibroblast growth factor receptor-3 in normal oral epithelium, epithelial dysplasias, and squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002 May;93(5):573-9.

Wang G, Guo C, Zhao H, Pan Z, Zhu F, Zhang L, Wang Q. TIPE3 differentially modulates proliferation and migration of human non-small-cell lung cancer cells via distinct subcellular location. BMC Cancer. 2018 Mar 6;18(1):260.

Wang K, Ren Y, Liu Y, Zhang J, He JJ. Tumor Necrosis Factor (TNF)-α-Induced Protein 8-like-2 (TIPE2) Inhibits Proliferation and Tumorigenesis in Breast Cancer Cells. Oncol Res. 2017 Jan 2;25(1):55-63.

Wang L, Mosel AJ, Oakley GG, Peng A. Deficient DNA damage signaling leads to chemoresistance to cisplatin in oral cancer. Mol Cancer Ther. 2012a Nov;11(11):2401-9.

Wang LY, Fan YC, Zhao J, Gao S, Sun FK, Han J, Yang Y, Wang K. Elevated expression of tumour necrosis factor-α-induced protein 8 (TNFAIP8)-like 2 mRNA in peripheral blood mononuclear cells is associated with disease progression of acute-on-chronic hepatitis B liver failure. J Viral Hepat. 2014a Jan;21(1):64-73.

Wang Y, Jiang Y, Zhou J, Song W, Li J, Wang M, Chen J, Xu R, Zhang J, Ma F, Chen YH, Ma Y. Hepatitis C virus promotes hepatocellular carcinogenesis by targeting TIPE2, a new regulator of DNA damage response. Tumour Biol. 2016b Nov;37(11):15265-15274.

Wang Y, Liu Y, Hu C, Ni X, Huang X. Tumor necrosis factor α-induced protein 8-like 1 promotes apoptosis by regulating B-cell leukemia/lymphoma-2 family proteins in RAW264.7 cells. Oncol Lett. 2016a Nov;12(5):3506-3512.

Wang Z, Fayngerts S, Wang P, Sun H, Johnson DS, Ruan Q, Guo W, Chen YH. TIPE2 protein serves as a negative regulator of phagocytosis and oxidative burst during infection. Proc Natl Acad Sci U S A. 2012b Sep 18;109(38):15413-8.

Wang ZM, Liu J, Liu HB, Ye M, Zhang YF, Yang DS. Abnormal COX2 protein expression may be correlated with poor prognosis in oral cancer: a meta-analysis. Biomed Res Int. 2014b;2014:364207.

Wei SJ, Chang RL, Merkler KA, Gwynne M, Cui XX, Murthy B, Huang MT, Xie JG, Lu YP, Lou YR, Jerina DM, Conney AH. Dose-dependent mutation profile in the c-Ha-ras proto-oncogene of skin tumors in mice initiated with benzo[a]pyrene. Carcinogenesis. 1999 Sep;20(9):1689-96.

Woodward MJ, de Boer J, Heidorn S, Hubank M, Kioussis D, Williams O, Brady HJ. Tnfaip8 is an essential gene for the regulation of glucocorticoid-mediated apoptosis of thymocytes. Cell Death Differ. 2010 Feb;17(2):316-23.

Wu J, Cai Y, Li M, Zhang Y, Li H, Tan Z. Oxymatrine Promotes S-Phase Arrest and Inhibits Cell Proliferation of Human Breast Cancer Cells in Vitro through Mitochondria-Mediated Apoptosis. Biol Pharm Bull. 2017b;40(8):1232-1239.

Wu J, Zhang H, Xu C, Xu H, Zhou X, Xie Y, Tao M. TIPE2 functions as a metastasis suppressor via negatively regulating β-catenin through activating GSK3β in gastric cancer. Int J Oncol. 2016 Jan;48(1):199-206.

Wu S, Sun C, Tian D, Li Y, Gao X, He S, Li T. Expression and clinical significances of Beclin1, LC3 and mTOR in colorectal cancer. Int J Clin Exp Pathol. 2015 Apr 1;8(4):3882-91.

Wu X, Ma Y, Cheng J, Li X, Zheng H, Jiang L, Zhou R. TIPE1 function as a prognosis predictor and negative regulator of lung cancer. Oncotarget. 2017a Jul 28;8(45):78496-78506.

Xi W, Hu Y, Liu Y, Zhang J, Wang L, Lou Y, Qu Z, Cui J, Zhang G, Liang X, Ma C, Gao C, Chen Y, Liu S. Roles of TIPE2 in hepatitis B virus-induced hepatic inflammation in humans and mice. Mol Immunol. 2011 May;48(9-10):1203-8.

Xiao M, Xu Q, Lou C, Qin Y, Ning X, Liu T, Zhao X, Jia S, Huang Y. Overexpression of TNFAIP8 is associated with tumor aggressiveness and poor prognosis in patients with invasive ductal breast carcinoma. Hum Pathol. 2017 Apr;62:40-49.

Xing B, Ren C. Tumor-suppressive miR-99a inhibits cell proliferation via targeting of TNFAIP8 in osteosarcoma cells. Am J Transl Res. 2016 Feb 15;8(2):1082-90.

Xue J, Yang S, Seng S. Mechanisms of Cancer Induction by Tobacco-Specific NNK and NNN. Cancers (Basel). 2014 May 14;6(2):1138-56.

Yanamoto S, Kawasaki G, Yoshitomi I, Iwamoto T, Hirata K, Mizuno A. Clinicopathologic significance of EpCAM expression in squamous cell carcinoma of the tongue and its possibility as a potential target for tongue cancer gene therapy. Oral Oncol. 2007 Oct;43(9):869-77.

Yang M, Zhao Q, Wang X, Liu T, Yao G, Lou C, Zhang Y. TNFAIP8 overexpression is associated with lymph node metastasis and poor prognosis in intestinal-type gastric adenocarcinoma. Histopathology. 2014 Oct;65(4):517-26.

Yang XJ, Liu J, Ye L, Liao QJ, Wu JG, Gao JR, She YL, Wu ZH, Ye LB. HCV NS2 protein inhibits cell proliferation and induces cell cycle arrest in the S-phase in mammalian cells through down-regulation of cyclin A expression. Virus Res. 2006 Nov;121(2):134-43.

Ye Y, Jin L, Wilmott JS, Hu WL, Yosufi B, Thorne RF, Liu T, Rizos H, Yan XG, Dong L, Tay KH, Tseng HY, Guo ST, de Bock CE, Jiang CC, Wang CY, Wu M, Zhang LJ, Hersey P, Scolyer RA, Zhang XD. PI(4,5)P2 5-phosphatase A regulates PI3K/Akt signalling and has a tumour suppressive role in human melanoma. Nat Commun. 2013;4:1508.

Yeole BB, Sankaranarayanan R, Sunny M Sc L, Swaminathan R, Parkin DM. Survival from head and neck cancer in Mumbai (Bombay), India. Cancer. 2000 Jul 15;89(2):437-44.

Yin H, Huang X, Tao M, Hu Q, Qiu J, Chen W, Wu J, Xie Y. Adenovirus-mediated TIPE2 overexpression inhibits gastric cancer metastasis via reversal of epithelial-mesenchymal transition. Cancer Gene Ther. 2017 Apr;24(4):180-188.

You Z, Ouyang H, Lopatin D, Polver PJ, Wang CY. Nuclear factor-kappa B-inducible death effector domain-containing protein suppresses tumor necrosis factor-mediated apoptosis by inhibiting caspase-8 activity. J Biol Chem. 2001 Jul 13;276(28):26398-404.

Yu B, Xu L, Cai M, Zhang D, Li S. Effect of tumor necrosis factor-α-induced protein 8 on the immune response of CD4+ T lymphocytes in mice following acute insult. Mol Med Rep. 2018 May;17(5):6655-6660.

Yu X, Shi W, Zhang Y, Wang X, Sun S, Song Z, Liu M, Zeng Q, Cui S, Qu X. CXCL12/CXCR4 axis induced miR-125b promotes invasion and confers 5-fluorouracil resistance through enhancing autophagy in colorectal cancer. Sci Rep. 2017 Feb 8;7:42226.

Yu XD, Yang JL, Zhang WL, Liu DX. Resveratrol inhibits oral squamous cell carcinoma through induction of apoptosis and G2/M phase cell cycle arrest. Tumour Biol. 2016 Mar;37(3):2871-7.

Zhang C, Chakravarty D, Sakabe I, Mewani RR, Boudreau HE, Kumar D, Ahmad I, Kasid UN. Role of SCC-S2 in experimental metastasis and modulation of VEGFR-2, MMP-1, and MMP-9 expression. Mol Ther. 2006 May;13(5):947-55.

Zhang C, Kallakury BV, Ross JS, Mewani RR, Sheehan CE, Sakabe I, Luta G, Kumar D, Yadavalli S, Starr J, Sreenath TL, Srivastava S, Pollard HB, Eidelman O, Srivastava M, Kasid UN. The significance of TNFAIP8 in prostate cancer response to radiation and docetaxel and disease recurrence. Int J Cancer. 2013a Jul;133(1):31-42.

Zhang G, Hao C, Lou Y, Xi W, Wang X, Wang Y, Qu Z, Guo C, Chen Y, Zhang Y, Liu S. Tissue-specific expression of TIPE2 provides insights into its function. Mol Immunol. 2010b Sep;47(15):2435-42.

Zhang G, Zhang W, Lou Y, Xi W, Cui J, Geng M, Zhu F, Chen YH, Liu S. TIPE2 deficiency accelerates neointima formation by downregulating smooth muscle cell differentiation. Cell Cycle. 2013b Feb 1;12(3):501-10.

Zhang L, Shi Y, Wang Y, Zhu F, Wang Q, Ma C, Chen YH, Zhang L. The unique expression profile of human TIPE2 suggests new functions beyond its role in immune regulation. Mol Immunol. 2011 May;48(9-10):1209-15.

Zhang S, Zhang Y, Wei X, Zhen J, Wang Z, Li M, Miao W, Ding H, Du P, Zhang W, He M, Yi F. Expression and regulation of a novel identified TNFAIP8 family is associated with diabetic nephropathy. Biochim Biophys Acta. 2010a Nov;1802(11):1078-86.

Zhang W, Haines BB, Efferson C, Zhu J, Ware C, Kunii K, Tammam J, Angagaw M, Hinton MC, Keilhack H, Paweletz CP, Zhang T, Winter C, Sathyanarayanan S, Cheng J, Zawel L, Fawell S, Gilliland G, Majumder PK. Evidence of mTOR Activation by an AKT-Independent Mechanism Provides Support for the Combined Treatment of PTEN-Deficient Prostate Tumors with mTOR and AKT Inhibitors. Transl Oncol. 2012c Dec;5(6):422-9.

Zhang W, Zhang J, Zhao L, Shao J, Cui J, Guo C, Zhu F, Chen YH, Liu S. TIPE2 protein negatively regulates HBV-specific $CD8^+$ T lymphocyte functions in humans. Mol Immunol. 2015c Mar;64(1):204-9.

Zhang X, Wang J, Fan C, Li H, Sun H, Gong S, Chen YH, Shi Y. Crystal structure of TIPE2 provides insights into immune homeostasis. Nat Struct Mol Biol. 2009 Jan;16(1):89-90.

Zhang Y, Wang MY, He J, Wang JC, Yang YJ, Jin L, Chen ZY, Ma XJ, Sun MH, Xia KQ, Hong XN, Wei QY, Zhou XY. Tumor necrosis factor-α induced protein 8 polymorphism and risk of non-Hodgkin's lymphoma in a Chinese population: a case-control study. PLoS One. 2012a;7(5):e37846.

Zhang Y, Wei X, Liu L, Liu S, Wang Z, Zhang B, Fan B, Yang F, Huang S, Jiang F, Chen YH, Yi F. TIPE2, a novel regulator of immunity, protects against experimental stroke. J Biol Chem. 2012b Sep 21;287(39):32546-55.

Zhang YH, Yan HQ, Wang F, Wang YY, Jiang YN, Wang YN, Gao FG. TIPE2 inhibits TNF-α-induced hepatocellular carcinoma cell metastasis via Erk1/2 downregulation and NF-κB activation. Int J Oncol. 2015b Jan;46(1):254-64.

Zhang Z, Liang X, Gao L, Ma H, Liu X, Pan Y, Yan W, Shan H, Wang Z, Chen YH, Ma C. TIPE1 induces apoptosis by negatively regulating Rac1 activation in hepatocellular carcinoma cells. Oncogene. 2015a May 14;34(20):2566-74.

Zhang Z, Liu L, Cao S, Zhu Y, Mei Q. Gene delivery of TIPE2 inhibits breast cancer development and metastasis via CD8(+) T and NK cell-mediated antitumor responses. Mol Immunol. 2017 May;85:230-237.

Zhang Z, Liu L, Liu C, Cao S, Zhu Y, Mei Q. TIPE2 suppresses the tumorigenesis, growth and metastasis of breast cancer via inhibition of the AKT and p38 signaling pathways. Oncol Rep. 2016 Dec;36(6):3311-3316.

Zhang Z, Qi H, Hou S, Jin X. TIPE2 mRNA overexpression correlates with TNM staging in renal cell carcinoma tissues. Oncol Lett. 2013c Aug;6(2):571-575.

Zhao L, Jiang L, Du P, Zhang D, Liu Z, Li K, Zhang B. Expression of SKA1 and MMP-9 in primary salivary adenoid cystic carcinoma: Correlation with tumor progression and patient prognosis. Acta Otolaryngol. 2016 Jun;136(6):575-9.

Zhao Q, Zhao M, Dong T, Zhou C, Peng Y, Zhou X, Fan B, Ma W, Han M, Liu S. Tumor necrosis factor-α-induced protein-8 like-2 (TIPE2) upregulates p27 to decrease gastic cancer cell proliferation. J Cell Biochem. 2015 Jun;116(6):1121-9.

Zhou J, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, Margolick JB, Liotta LA, Petricoin E 3rd, Zhang Y. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc Natl Acad Sci U S A. 2007 Oct 9;104(41):16158-63.

Zhou Z, Li Z, Shen Y, Chen T. MicroRNA-138 directly targets TNFAIP8 and acts as a tumor suppressor in osteosarcoma. Exp Ther Med. 2017 Oct;14(4):3665-3673.

Zhu Y, Tao M, Wu J, Meng Y, Xu C, Tian Y, Zhou X, Xiang J, Zhang H, Xie Y. Adenovirus-directed expression of TIPE2 suppresses gastric cancer growth via induction of apoptosis and inhibition of AKT and ERK1/2 signaling. Cancer Gene Ther. 2016 Apr;23(4):98-106.

LIST OF ABBREVIATIONS

4E-BPs	: eIF4E-binding proteins
4NQO	: 4-nitroquinoline-1-oxide
6-OHDA	: 6-hydroxydopamine
AMPK	: AMP-activated protein kinase
ANXA1	: Annexin A1
AP-1	: Activator protein 1
AST	: Aspartate aminotransferase
BaP	: Benzo[a]pyrene
BARD1	: BRCA1 associated RING domain 1
Bcl-2	: B-cell lymphoma 2
Bcl-xL	: B-cell lymphoma-extra large
BSA	: Bovine serum albumin
CASP10	: Caspase 10
CASP8	: Caspase 8
cIAP1	: Cellular Inhibitor of Apoptosis Protein 1
CLP	: Cecal ligation and puncture
Cox-2	: Cyclooxygenase-2
CXCR-4	: C-X-C chemokine receptor type 4
D2S	: Dopamine D2 receptor short form
DAB	: 3,3'-diaminobenzidine
DED	: Death effector domain
DLBCL	: Diffuse large B-cell lymphoma
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl sulfoxide
DN	: Diabetic nephropathy
DNA	: Deoxyribonucleic acid
DSF	: Dextran sodium sulfate
DTT	: Dithiothreitol
EAE	: Experimental autoimmune encephalomyelitis
EBV	: Epstein-barr virus
EC	: Endometrial cancer
ECL	: Enhanced chemiluminescence
EDTA	: Ethylene diamine tetraacetic acid
EGF	: Epidermal growth factor
EGFR	: Epidermal growth factor receptor
eIF4E	: eukaryotic initiation factor-4E
EMT	: Epithelial–mesenchymal transition
EOC	: Epithelial ovarian cancer
ERK	: Extracellular signal-regulated kinase
ESCC	: Esophageal squamous cell carcinoma
FBS	: Fetal bovine serum
FBXW5	: F-Box and WD Repeat Domain Containing 5
FGF	: Fibroblast growth factor
FLIP	: Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
GSK3	: Glycogen synthase kinase 3
GTP	: Guanosine-5'-triphosphate
GVHD	: Graft-versus-host disease
HBV	: Hepatitis B virus

HCC	: Hepatocellular carcinoma
HCV	: Hepatitis C virus
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIF-1α	: Hypoxia-inducible factor alpha
HNSCC	: Head and neck squamous cell carcinoma
HPV	: Human papillomavirus
HRP	: Horseradish peroxidase
HSP70	: Heat shock protein 70
HSV	: Herpes simplex viruses
hTERT	: Human telomerase reverse transcriptase
IDC	: Invasive ductal carcinoma
IFN	: Interferon
IGA	: Intestinal-type gastric adenocarcinoma
IgE	: Immunoglobulin E
IHC	: Immunohistochemistry
ILKAP	: ILK Associated Serine/Threonine Phosphatase
ILs	: Interleukins
ING5	: Inhibitor of Growth Family Member 5
IκBα	: IκB kinase
JNK	: c-Jun NH2-terminal kinase
LATS1	: Large tumor suppressor kinase 1
LC3	: Microtubule-associated protein 1A/1B-light chain 3
LPS	: Lipopolysaccharide
LRP1B	: Low-Density Lipoprotein Receptor-Related Protein 1B
MAPK	: Mitogen-activated protein kinase
MDM2	: Mouse double minute 2 homolog
miR	: microRNA
mL	: Milliliter
mm	: Millimeter
mM	: Millimolar
MMP-1	: Matrix metalloproteinase-1
MMP-9	: Matrix metalloproteinase-9
mTOR	: Mammalian target of rapamycin
MTT	: (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide)
nAChRs	: Nicotinic acetylcholine receptors
NaCl	: Sodium chloride
NADPH	: Nicotinamide adenine dinucleotide phosphate reduced
NDED	: NF-κB-inducible DED-containing protein
NF-κB	: Nuclear factor kappa-B
ng	: Nanogram
NHL	: Non-Hodgkin's lymphoma
nM	: Nanomolar
NNK	: 4 [methylnitrosoamino]-1-[3- pyridyl]-1-butanone
NNN	: N-nitrosonornicotine
NOS2	: Nitric oxide synthase 2
NPC	: Nasopharyngeal carcinoma
NSCLC	: Non-small-cell lung carcinoma
OLP	: Oral lichen planus
OSCC	: Oral squamous cell carcinoma
OSF	: Oral submucous fibrosis
PAH	: Polycyclic aromatic hydrocarbons

PAI-1	: Plasminogen activator inhibitor-1
PBS	: Phosphate-buffered saline
PD-ECGF	: Platelet-derived endothelial cell growth factor
PE	: Phosphatidylethanolamine
PGF	: Placental growth factor
PI	: Propidium iodide
PMSF	: Phenylmethylsulfonyl fluoride
PPP1R7	: Protein Phosphatase 1 Regulatory Subunit 7
PTEN	: Phosphatase and tensin homolog
PTM	: Post translational modification
Rac1	: Ras-related C3 botulinum toxin substrate 1
Rb	: Retinoblastoma
RCC	: Renal cell carcinoma
REDD1	: Regulated in development and DNA damage responses -1
RT	: Room temperature
RTK	: Receptor tyrosine kinase
SCC	: Squamous cell carcinomas
SDS	: Sodium dodecyl sulfate
SLE	: Systemic lupus erythematosus
SLT	: Smokeless tobacco
SMAD4	: SMAD4 Family Member 4
SNP	: Single nucleotide polymorphism
STAT3/5	: Signal transducer and activator of transcription 3/5
STZ	: Streptozotocin
Tbil	: Total bilirubin
TCR	: T cell receptor
TE	: Tobacco extract
TGF-β	: Transforming growth factor beta
TIMPs	: Tissue inhibitors of metalloproteinases
TLR	: Toll-like receptor
TMA	: Tissue micro array
TNFAIP8/TIPE	: Tumor necrosis factor-α induced protein 8
TNFAIP8L1/TIPE1	: Tumor necrosis factor-α induced protein 8 Like 1
TNFAIP8L2/TIPE2	: Tumor necrosis factor-α induced protein 8 Like 2
TNFAIP8L3/TIPE3	: Tumor necrosis factor-α induced protein 8 Like 3
TNF-α	: Tumor necrosis factor-α
TSC2	: Tuberous sclerosis complex 2
TSNAs	: Tobacco specific N'-nitrosamines
uPA	: Urokinase-type plasminogen activator
VEGF	: Vascular endothelial growth factor
VEGF-R	: Vascular endothelial growth factor receptor
VSMCs	: Vascular smooth muscle cells
WHO	: World Health Organization
XIAP	: X-linked inhibitor of apoptosis protein
YAP1	: Yes associated protein 1
Z-VAD-FMK	: Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-Fluoromethylketone
µg	: Microgram
µM	: Micromolar

LIST OF FIGURES

LIST OF TABLES

LIST OF PUBLICATIONS AND PRESENTATIONS

Research articles, reviews and book chapters:

1. **Padmavathi G**, Banik K, Monisha J, Bordoloi D, Shabnam B, Arfuso F, Sethi G, Fan L, Kunnumakkara AB. Novel tumor necrosis factor-α induced protein eight (TNFAIP8/TIPE) family: Functions and downstream targets involved in cancer progression. Cancer Lett. 2018 Sep 28;432:260-271. doi: 10.1016/j.canlet.2018.06.017. Epub 2018 Jun 18. PubMed PMID: 29920292.

2. Bordoloi D, Banik K, Shabnam B, **Padmavathi G**, Monisha J, Arfuso F, Dharmarajan A, Mao X, Lim LHK, Wang L, Fan L, Hui KM, Kumar AP, Sethi G, Kunnumakkara AB. TIPE Family of Proteins and Its Implications in Different Chronic Diseases. Int J Mol Sci. 2018 Sep 29;19(10). pii: E2974. doi: 10.3390/ijms19102974. Review. PubMed PMID: 30274259; PubMed Central PMCID: PMC6213092.

3. Banerjee K, **Padmavathi G**, Bhattacherjee D, Saha S, Kunnumakkara AB, Bhabak KP. Potent anti-proliferative activities of organochalcogenocyanates towards breast cancer. Org Biomol Chem. 2018 Nov 21;16(45):8769-8782. doi: 10.1039/c8ob01891j. PubMed PMID: 30403243.

4. Monisha J, Roy NK, **Padmavathi G**, Banik K, Bordoloi D, Khwairakpam AD, Arfuso F, Chinnathambi A, Alahmadi TA, Alharbi SA, Sethi G, Kumar AP, Kunnumakkara AB. NGAL is Downregulated in Oral Squamous Cell Carcinoma and Leads to Increased Survival, Proliferation, Migration and Chemoresistance. Cancers (Basel). 2018 Jul 10;10(7). pii: E228. doi: 10.3390/cancers10070228. PubMed PMID: 29996471; PubMed Central PMCID: PMC6071146.

5. 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. doi: 10.1016/j.ejmech.2018.08.088. Epub 2018 Aug 30. PubMed PMID: 30368201.

6. **Padmavathi G**, Roy NK, Bordoloi D, Arfuso F, Mishra S, Sethi G, Bishayee A, Kunnumakkara AB. Butein in health and disease: A comprehensive review. Phytomedicine. 2017 Feb 15;25:118-127. doi: 10.1016/j.phymed.2016.12.002. Epub 2016 Dec 12. Review. PubMed PMID: 28190465.

7. **Padmavathi G**, Rathnakaram SR, Monisha J, Bordoloi D, Roy NK and Kunnumakkara AB, Potential of Butein, a Tetrahydroxychalcone to Obliterate Cancer, Phytomedicine 22(2015): 1163–1171. PubMed PMID: 26598915.

8. Shabnam B, **Padmavathi G**, Banik K, Girisa S, Monisha J, Sethi G, Fan L, Wang L, Mao X, Kunnumakkara AB. Sorcin a Potential Molecular Target for Cancer Therapy. Transl Oncol. 2018 Dec;11(6):1379-1389. doi: 10.1016/j.tranon.2018.08.015. Epub 2018 Sep 11. Review. PubMed PMID: 30216763; PubMed Central PMCID: PMC6134165.

9. Singh YP, Girisa S, Banik K, Ghosh S, Swathi P, Deka M, **Padmavathi G**, Kotoky J, Sethi G, Fan L, Mao X, Halim CE, Arfuso F, Kunnumakkara AB.

Potential application of zerumbone in the prevention and therapy of chronic human diseases. J Funct Foods. 2019 February;53: 248–258. Doi: 10.1016/j.jff.2018.12.020.

10. Ranaware AM, Banik K, Deshpande V, **Padmavathi G**, Roy NK, Sethi G, Fan L, Kumar AP, Kunnumakkara AB. Magnolol: A Neolignan from the Magnolia Family for the Prevention and Treatment of Cancer. Int J Mol Sci. 2018 Aug 10;19(8). pii: E2362. doi: 10.3390/ijms19082362. Review. PubMed PMID: 30103472; PubMed Central PMCID: PMC6121321.

11. 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. doi: 10.3389/fphar.2018.00686. eCollection 2018. Review. PubMed PMID: 30127736; PubMed Central PMCID: PMC6087759.

12. Khwairakpam AD, Damayenti YD, Deka A, Monisha J, Roy NK, **Padmavathi G**, Kunnumakkara AB. Acorus calamus: a bio-reserve of medicinal values. J Basic Clin Physiol Pharmacol. 2018 Mar 28;29(2):107-122. doi: 10.1515/jbcpp-2016-0132. Review. PubMed PMID: 29389665.

13. 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. doi: 10.1016/j.phrs.2018.02.017. Epub 2018 Feb 27. Review. PubMed PMID: 29496592.

14. Roy NK, Monisha J, **Padmavathi G**, Das A, Gupta S, Ramakrishnan E, Kotoky J and Kunnumakkara AB. Rapid Biosynthesis of Gold Nanoparticles Using Aqueous-ethanoic Leaf Extract of Heartleaf Moonseed: Characterization and Effect of pH on its Synthesis. Current Nanomaterials. 2017; 2(1):3-10.

15. Kunnumakkara AB, Bordoloi D, **Padmavathi G**, Monisha J, Roy NK, Prasad S, Aggarwal BB. Curcumin, the golden nutraceutical: multitargeting for multiple chronic diseases. Br J Pharmacol. 2017; 174(11):1325-1348. PubMed PMID: 27638428.

16. Monisha J, Roy NK, Bordoloi D, Kumar A, Golla R, Kotoky J, **Padmavathi G**, Kunnumakkara AB. Nuclear Factor Kappa B: A Potential Target to Persecute Head and Neck Cancer. Curr Drug Targets. 2017;18(2):232-253. doi: 10.2174/1389450117666160201112330. PubMed PMID: 26844566.

17. Roy NK, Bordoloi D, Monisha J, **Padmavathi G**, Kotoky J, Golla R, Kunnumakkara AB. Specific Targeting of Akt Kinase Isoforms: Taking the Precise Path for Prevention and Treatment of Cancer. Curr Drug Targets. 2017;18(4):421-435. doi: 10.2174/1389450117666160307145236. PubMed PMID: 26953242.

18. Monisha J, **Padmavathi G**, Roy NK, Deka A, Bordoloi D, Anip A, Kunnumakkara AB. NF-κB Blockers Gifted by Mother Nature: Prospectives in Cancer Cell Chemosensitization. Curr Pharm Des. 2016;22(27):4173-200. Review. PubMed PMID: 27291284.

19. Bordoloi D, Roy NK, Monisha J, **Padmavathi G**, Kunnumakkara AB. Multi-Targeted Agents in Cancer Cell Chemosensitization: What We Learnt from Curcumin Thus Far. Recent Pat Anticancer Drug Discov. 2016;11(1):67-97. Review. PubMed PMID: 26537958.

20. Thomas D, Govindhan S, Baiju EC, **Padmavathi G**, Kunnumakkara AB, Padikkala J. Cyperus rotundus L. prevents non-steroidal anti-inflammatory drug-induced gastric mucosal damage by inhibiting oxidative stress. J Basic Clin Physiol Pharmacol. 2015 Sep;26(5):485-90. PubMed PMID: 25915081.

21. Khwairakpam AD, Shyamananda MS, Sailo BL, Rathnakaram SR, **Padmavathi G**, Kotoky J and Kunnumakkara AB. ATP citrate lyase (ACLY): a promising target for cancer prevention and treatment. Curr Drug Targets. 2015;16(2):156-63. PubMed PMID: 25537655.

22. Monisha J, **Padmavathi G**, Bordoloi D, Roy NK and Kunnumakkara AB, Neutrophil Gelatinase-Associated Lipocalin (NGAL): A Promising Biomarker for Cancer Diagnosis and A Potential Target for Cancer Therapeutics, Journal of Cell Science & Molecular Biology, 2014;1(2).

23. Bordoloi D, Sailo BL, Manteghi N, **Padmavathi G** and Kunnumakkara AB, (2018). Introduction and Basic Concepts of Cancer, In AB Kunnumakkara, D Bordoloi and J Monisha (Eds.), Cancer cell chemoresistance and chemosensitization (pp. 1-14). New Jersey: World Scientific.

24. **Padmavathi G**, Bordoloi D, Banik K, Monisha J, Singh AK and Kunnumakkara AB, (2018). Mechanism of Chemoresistance in Bone Cancer and Different Chemosensitization Approaches, In AB Kunnumakkara, D Bordoloi and J Monisha (Eds.), Cancer cell chemoresistance and chemosensitization (pp. 81-106). New Jersey: World Scientific.

25. Harsha C, Bordoloi D, Prakash J, Manteghi N, **Padmavathi G**, Monisha J and Kunnumakkara AB, (2018). Different Chemosensitization Approaches in Gastric Cancer, In AB Kunnumakkara, D Bordoloi and J Monisha (Eds.), Cancer cell chemoresistance and chemosensitization (pp. 267-320). New Jersey: World Scientific.

26. Monisha J, Roy NK, Sharma A, Banik K, **Padmavathi G**, Bordoloi D and Kunnumakkara AB, (2018). Chemoresistance and Chemosensitization in Melanoma, In AB Kunnumakkara, D Bordoloi and J Monisha (Eds.), Cancer cell chemoresistance and chemosensitization (pp. 479-528). New Jersey: World Scientific.

27. **Padmavathi G**, Monisha J, Banik K, Thakur KK, Harsha C, Bordoloi D and Kunnumakkara AB, (2018). Different Chemosensitization Approaches to Overcome Chemoresistance in Prostate Cancer, In AB Kunnumakkara, D Bordoloi and J Monisha (Eds.), Cancer cell chemoresistance and chemosensitization (pp. 583-614). New Jersey: World Scientific.

28. **Padmavathi G**, Bordoloi D, Banik K and Kunnumakkara AB, 'Cancer Biomarkers: Important Tools for Cancer Diagnosis and Prognosis', In P Chandra,

YN Tan and SP Singh (Eds.), Next generation point-of-care biomedical sensors technologies for cancer diagnosis. Springer.

29. Roy NK, Bordoloi D, Monisha J, Anip A, **Padmavathi G** and Kunnumakkara AB, (2017). Cancer- an overview and molecular alterations in cancer. In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.1-15). New Jersey: World Scientific.

30. **Padmavathi G**, Roy NK, Bordoloi D, Monisha J and Kunnumakkara AB, (2017). Basic concepts of fusion genes and their classification, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.17-58). New Jersey: World Scientific.

31. Roy NK, **Padmavathi G**, Bordoloi D and Kunnumakkara AB, (2017). Techniques available to identify novel fusion genes and to detect known fusion genes, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.59-79). New Jersey: World Scientific.

32. **Padmavathi G**, Thakur KK, Anip A, Bordoloi D and Kunnumakkara AB, (2017). The receptor tyrosine kinase ALK; its fusion partners and their implication in various cancers, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.81-109). New Jersey: World Scientific.

33. **Padmavathi G**, Banik K, Roy NK, Monisha J, and Kunnumakkara AB, (2017). Role of BCR-ABL fusion kinase in the development of leukemia, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.111-127). New Jersey: World Scientific.

34. **Padmavathi G**, Bordoloi D, Banik K and Kunnumakkara AB, (2017). BRD4-NUT fusion oncoprotein and its significance in the initiation and progression of NUT midline carcinoma (NMC), In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.129-135). New Jersey: World Scientific.

35. **Padmavathi G**, Harsha C, Bordoloi D, Thakur KK and Kunnumakkara AB, (2017). Importance of CBFB-MYH11- a chimeric transcriptional regulator in leukemia, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.137-146). New Jersey: World Scientific.

36. **Padmavathi G**, Monisha J, Banik K, Harsha C, Bordoloi D and Kunnumakkara AB, (2017). Rearrangements involving ETS family of genes and their role in different cancers, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.147-162). New Jersey: World Scientific.

37. **Padmavathi G**, Bordoloi D, Anip A, Thakur KK and Kunnumakkara AB, (2017). Translocation of FET family members with various partner genes and their role in cancer development, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.163-188). New Jersey: World Scientific.

38. **Padmavathi G**, Monisha J, Harsha C and Kunnumakkara AB, (2017). Translocations of FGF and FGFR proteins and their effect in cancer, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer

(pp.189-199). New Jersey: World Scientific.

39. **Padmavathi G**, Banik K, Thakur KK and Kunnumakkara AB, (2017). IG/MYC and its implication in cancer, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.201-208). New Jersey: World Scientific.

40. **Padmavathi G**, Banik K, Bordoloi D, Harsha C and Kunnumakkara AB, (2017). Chimeric RAF kinases in the development of cancer, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.209-220). New Jersey: World Scientific.

41. **Padmavathi G**, Harsha C, Bordoloi D, Banik K and Kunnumakkara AB, (2017). Mucoepidermoid carcinoma (MEC) and associated MAML2 fusion genes, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.221-230). New Jersey: World Scientific.

42. **Padmavathi G**, Harsha C and Kunnumakkara AB, (2017). Mixed Lineage Leukemia/AF9 fusion and associated leukemia, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.231-243). New Jersey: World Scientific.

43. **Padmavathi G**, Thakur KK and Kunnumakkara AB, (2017). MYB-NFIB fusion gene- hallmark of adenoid cystic carcinoma (ACC), In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.245-251). New Jersey: World Scientific.

44. **Padmavathi G**, Thakur KK and Kunnumakkara AB, (2017). Translocations involving PAX family genes and their effect in cancer, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.253-270). New Jersey: World Scientific.

45. **Padmavathi G**, Monisha J, Anip A, Thakur KK and Kunnumakkara AB, (2017). Retinoic acid receptor alpha (RARα) fusion genes in leukemia, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.271-285). New Jersey: World Scientific.

46. **Padmavathi G**, Bordoloi D, Anip A, Harsha C and Kunnumakkara AB, (2017). RET/PTC translocations and thyroid malignancies, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.287-296). New Jersey: World Scientific.

47. **Padmavathi G**, Monisha J, Banik K, Harsha C, Bordoloi D and Kunnumakkara AB, (2017). RUNX1 or AML1 fusion genes in leukemia and other cancers, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.297-313). New Jersey: World Scientific.

48. **Padmavathi G**, Bordoloi D, Monisha J, Roy NK, Harsha C and Kunnumakkara AB, (2017). Recently discovered fusion genes and their implications in cancer, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.315-348). New Jersey: World Scientific.

49. Roy NK, Bordoloi D, **Padmavathi G** and Kunnumakkara AB, (2017). Targeting

fusion genes for cancer therapy, In AB Kunnumakkara, G Padmavathi and NK Roy (Eds.), Fusion genes and cancer (pp.349-371). New Jersey: World Scientific.

50. Guruvayoorappan C, Sakthivel KM, **Padmavathi G**, Bakliwal V, Monisha J and Kunnumakkara AB, 'Cancer Preventive and Therapeutic Properties of Fruits and Vegetables: An Overview', In Anticancer Properties of Fruits and Vegetables: A Scientific Review. World Scientific Publications, 2015.

51. Aranjani JM, **Padmavathi G**, Kunnumakkara AB and Mathew A, 'Cancer Chemopreventive and Therapeutic Properties of Fruits and Vegetables Against Head and Neck Malignancies'. In Anticancer Properties of Fruits and Vegetables: A Scientific Review. World Scientific Publications, 2015.

52. Monisha J, **Padmavathi G**, Bakliwal V, Katre N, Padikkala J and Kunnumakkara AB, 'Cancer Preventive and Therapeutic Properties of Fruits and Vegetables Against Commonly Occurring Cancers in Humans'. In Anticancer Properties of Fruits and Vegetables: A Scientific Review. World Scientific Publications, 2015.

53. **Padmavathi G**, Monisha J, Banik K, Bordoloi D, Kunnumakkara AB. Inhibition of TNFAIP8L2 (TIPE2) reduced survival, proliferation and migration of oral cancer cells through downregulation of Akt/mTOR/S6/NF-κB/STAT3 signaling axis. [Manuscript submitted]

54. **Padmavathi G**, Banik K, Bordoloi D, Khwairakpam AD, Monisha J, Kunnumakkara AB. Involvement of Akt-independent mTOR/STAT3 signaling in TNFAIP8L3 (TIPE3) mediated regulation of oral cancer cell survival, proliferation and migration. [Manuscript submitted]

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

56. Bordoloi D, Monisha J, Roy NK, **Padmavathi G**, Banik K, Harsha C, Fan L, Wang H, Arfuso F, Sethi G, Kunnumakkara AB. An investigation on the therapeutic potential of butein, a tretrahydroxychalcone against human oral squamous cell carcinoma. [Manuscript under review]

57. Roy NK, Monisha J, **Padmavathi G**, Lalhruaitluanga H, Kumar NS, Singh A, Bordoloi D, Arfuso F, Fan L, Wang H, Wang LZ, Kumar AP, Sethi G, Kunnumakkara AB. Isoform-specific role of Akt in oral squamous cell carcinoma. [Manuscript under review]

58. **Padmavathi G**, Monisha J, Bordoloi D, Banik K, Roy NK, Kunnumakkara AB. Suppression of TNFAIP8 (TIPE) expression reduced survival, proliferation and migration of oral cancer cells through downregulation of Akt/mTOR/S6 signaling axis. [Manuscript under revision]

59. **Padmavathi G**, Bordoloi D, Monisha J, Roy NK, AK singh, Banik K, Kunnumakkara AB. Knockout of TNFAIP8L1 (TIPE1) activated Akt/mTOR pathway and enhanced survival, proliferation and migration of oral cancer *in vitro*.

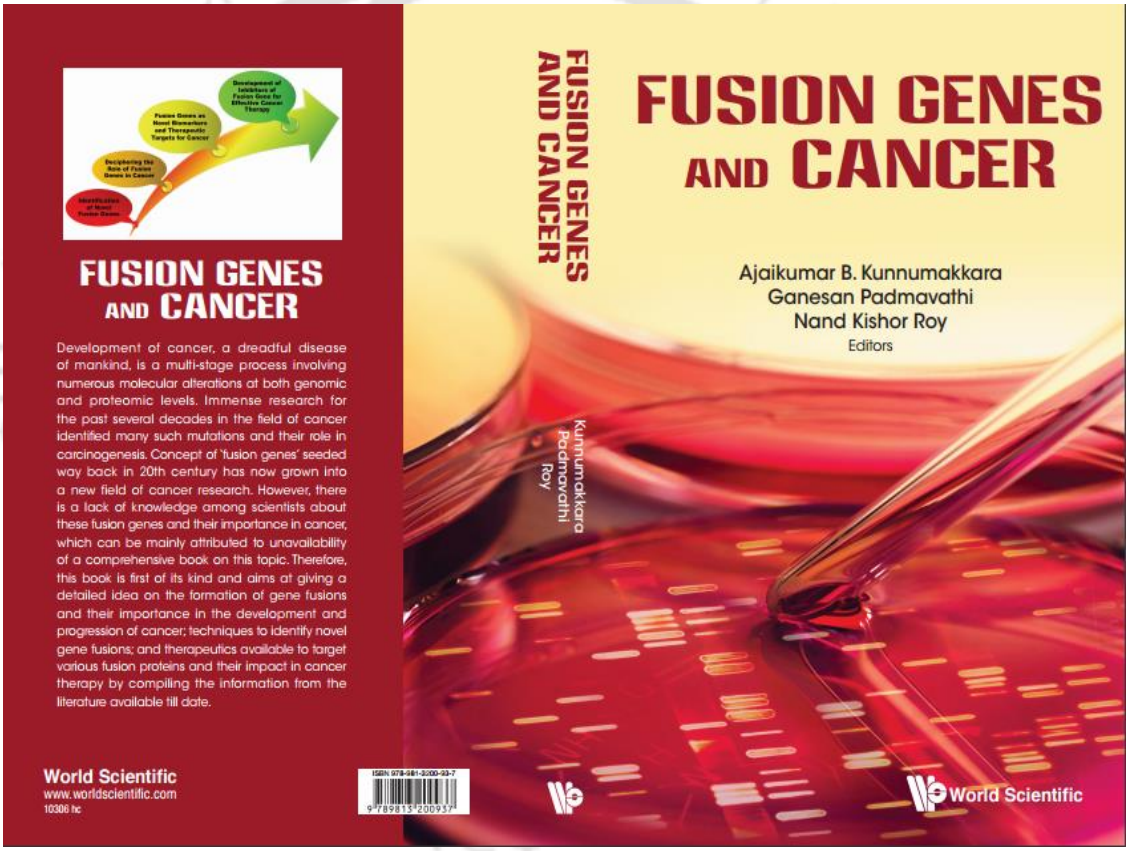
[Manuscript under revision]

60. Koodalingam D, **Padmavathi G**, Kunnumakkara AB, Anandalakshmi R. Microwave-assisted synthesis of cellulose/zinc-sulfate-calcium-phosphate (ZSCAP) nanocomposites for biomedical applications. [Manuscript under revision]

61. Roy NK, Monisha J, Singh A, **Padmavathi G**, Kunnumakkara AB. Isoform-specific Role of Akt Kinase in Cancer and its Selective Targeting by Potential Anticancer Natural Agents. [Manuscript under revision]

Book:

Fusion Genes and Cancer. Editors: AB Kunnumakkara, **G Padmavathi**, NK Roy. (http://www.worldscientific.com/worldscibooks/10.1142/10306), Publisher: World Scientific, Published in April 2017.



Abstracts presented in conferences:

1. **Padmavathi G**, Sajin Fransis K, Javadi Monisha, Mangalam S. Nair, Ajaikumar B Kunnumakkara. Exploring the anticancer potential of epoxyazadiradione (EAZA), a novel neem limonoid against oral cancer, Translational Cancer Research, Chennai, India, 2018.
2. **Padmavathi G**, Javadi Monisha, Devivasha Bordoloi, Nand K Roy, Ajaikumar B Kunnumakkara. TNFAIP8 family as possible diagnostic biomarkers for oral cancer, 5th AIST International Imaging Workshop, held at Biomedical Research Institute, Tsukuba Science city, Japan, January 21-30, 2018.
3. **Padmavathi G**, Sajin Fransis K, Javadi Monisha, Mangalam S. Nair, Ajaikumar

B Kunnumakkara. A novel neem limonoid exhibits antineoplastic activity against oral cancer cells through the induction of cell cycle arrest and apoptosis, International Conference on Nutraceuticals and Chronic Diseases, Goa, India, 2017.

4. **Padmavathi G**, Monisha J, Bordoloi D, Khwairakpam AD and Kunnumakkara AB. Differential Expression of TNFAIP8 Family of Proteins In Oral Cancer Tissues, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.
5. **Padmavathi G**, Monisha J, Bordoloi D, Roy NK, Sailo BL and Kunnumakkara AB. Putative Post Translational Modifications (PTMs) of TNFAIP8 Family of Proteins, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.
6. **Padmavathi G**, Roy NK, Bordoloi D, Thakur KK, Monisha J and Kunnumakkara AB. Introduction to Fusion Genes and Their Classification, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.
7. Roy NK, Bordoloi D, Harsha C, **Padmavathi** G and Kunnumakkara AB. Fusion genes as target for cancer therapy, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.
8. **Padmavathi G**, Banik K, Roy NK, Monisha J and Kunnumakkara AB. Identification of BCR-ABL fusion kinase- a breakthrough in the management of leukemia, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.
9. Singh MS, **Padmavathi G**, Fransis KS, Raju RS, Monisha J, Roy NK, Nair MS and Kunnumakkara AB. Novel labdane type diterpene isolated from *Hedichium coronarium* as a potential anticancer agent against triple negative breast cancer, 36th Annual Conference of Indian Association for Cancer Research, Amala Cancer Research Centre, Thrissur, India, 2017.
10. **Padmavathi G**, Simon PS, Roy NK, Bordoloi D, Monisha J, Padikkala J, Kunnumakkara AB. An Investigation on the Cancer Preventive Potential of the Spice *Carum copticum* (Ajwain) Against Azoxymethane Induced Colon Carcinogenesis, First 'International Conference on Nutraceuticals and Chronic Diseases', Kochi, India, 2016.
11. **Padmavathi G**, Simon PS, Roy NK, Bordoloi D, Monisha J, Padikkala J, Kunnumakkara AB. Prevention of Azoxymethane Induced Colon Carcinogenesis by the Spice *Carum copticum* (Ajwain), 'Research Conclave', IIT Guwahati, India, 2016.
12. **Padmavathi G,** Simon PS, Roy NK, Bordoloi D, Monisha J, Padikkala J, Kunnumakkara AB. Prevention of Azoxymethane Induced Colon Carcinogenesis by the Spice *Carum copticum* (Ajwain), 'Translational Cancer Research', Ahmedabad, India, 2016.

Abstracts in conference proceedings:

1. Ajaikumar B. Kunnumakkara, Devivasha Bordoloi, Bethsebie Lalduhsaki sailo, **Ganesan Padmavathi** and Javadi Monisha. 'Fruits, Vegetables and Their Components in Cancer Prevention: What We Learned Thus Far?', 8th International aromatherapy conference, San Fransisco, November 6-8, 2015, 145-88.
2. Bethsebie Lalduhsaki sailo, Javadi Monisha, **Ganesan Padmavathi** and Ajaikumar B. Kunnumakkara. 'Tocotrienols: The Analogues of Vitamin E, Gifted By Mother Nature'. National Seminar on Emerging trends in Herbal Technology, Thrissur, India, October 7-9, 2015.

Conferences, workshops and trainings attended:

1. Particpated in **5th AIST International Imaging Workshop** held in January 21-30, 2018 at AIST Tsukuba, Japan.
2. 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.
3. Participated in the National conference on **'Recent Developments in Medical Biotechnology and Structure Based Drug Designing'** organized by Department of Biosciences and Bioengineering, IIT Guwahati, India, held on 6th & 7th December, 2015.
4. Participated in a 9 day advanced research training workshop on **'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approach'** sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kalyani, Kolkata held during October 5-13, 2015.
5. Participated in a 6 day research training workshop on **'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approach'** sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kolkata and Department of Biotechnology, Assam University, Silchar held during April 6-11, 2015 and **Selected** for the advanced level workshop.
6. Participated in a 5 day national course on **'Theoretical and Practical aspects of Cancer Research'** conducted under the Technical Education Quality Improvement Programme sponsored by the Ministry of Human Resource Development, Govt. of India, from February 4th – 8th, 2015.
7. Participated in a 2 day national workshop on **'Flow Cytometry Data Analysis'** organized by Department of Biotechnology, Indian Institute of Technology Guwahati from 23rd – 24th January, 2015.
8. Participated in the National Conference on **'Recent Advances in Cancer Biology and Therapeutics'** organized by Department of Biotechnology, IIT Guwahati, India, held on 5th December, 2014.
9. Participated in a 4 day national workshop on **'Next Generation Sequencing and Data Analysis'** organized by Biotech Hub, Centre for the Environment, Indian Institute of Technology Guwahati held during May 14-17, 2014.

Co-curricular activities:

1. Member, local organizing committee of the "**International conference on nutraceuticals and chronic diseases 2017 (INCD-2017)**, Goa, India, 2017.
2. Member, organizing committee of the workshop on "**Animal cell culture, molecular techniques in cancer diagnosis and drug discovery**" held at Department of Biosciences and Bioengineering, IIT Guwahati during Research conclave 2017.
3. Member, organizing committee of the Indo-Japan symposium "**Hope from herbs: Research- based care and cure potentials**" jointly organized by IIT Guwahati and AIST Japan at IIT Guwahati, 2017.
4. Member, organizing committee of the pre-conference workshop at "**AMBICON 2017**", IIT Guwahati, 2017.
5. Member, local organizing committee of the "**International conference on nutraceuticals and chronic diseases 2016 (INCD-2016)**, Kerala, India, 2016.

6. Member, organizing committee of the workshop on "**Theoretical and practical aspects of cancer research**" conducted under the Technical Education Quality Improvement Programme sponsored by the Ministry of Human Resource Development, Govt. of India, 2015.
7. Member, organizing committee of the National Conference on "**Recent advances in cancer biology and therapeutics**" organized by Department of Biotechnology, IIT Guwahati, 2014.

Awards:

1. Received '**Best Poster Presentation Award**' for the paper entitled "Exploring the anticancer potential of epoxyazadiradione (EAZA), a novel neem limonoid against oral cancer" at the 7th International conference on Translational Cancer Research, Chennai, India, 2018.
2. Selected as one among the top 20 candidates and awarded fellowship for attending the **5th AIST International Imaging Workshop** held in January 21-30, 2018 at AIST Tsukuba, Japan.
3. Received '**Best Oral Presentation Award**' for the paper entitled "A novel neem limonoid exhibits antineoplastic activity against oral cancer cells through the induction of cell cycle arrest and apoptosis" at the Second International Conference on Nutraceuticals and Chronic Diseases, Goa, India, 2017.
4. Received '**Best Poster Presentation Award**' in the departmental level for the paper entitled "Differential Expression of TNFAIP8 Family of Proteins in Oral Cancer Tissues" at Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.
5. Received '**Best Outstanding Poster Presentation Award**' for the paper entitled "Prevention of Azoxymethane Induced Colon Carcinogenesis by the Spice Carum copticum (Ajwain)" at the 6th International conference on Translational Cancer Research, Ahmedabad, India, 2016.