A Study on the Role of Lipogenic enzymes in Oral Cancer

A thesis submitted for the degree of

Doctor of Philosophy

To

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

By

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June, 2021.

Dedicated to

My Mother

For her encouragement, support and

love that endures through all

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING
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DECLARATION

I hereby declare that the contents of the research work described in this thesis titled **"A study on the Role of Lipogenic enzymes in 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 Prof. Ajaikumar B. Kunnumakkara.

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

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CERTIFICATE

This is to certify that the work described in the thesis titled **"A study on the Role of Lipogenic enzymes in Oral Cancer"**, submitted by Khwairakpam Amrita Devi (Roll no: 136106028) 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.

14 August, 2021

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Acknowledgement

Firstly, I would like to thank the "Almighty God" for all his blessings throughout the journey of my life.

I would like to take this opportunity to acknowledge all those people without whom this incredible journey of my life would have never been possible.

I convey my profound gratitude to my thesis supervisor Prof. Ajaikumar B. kunnumakkara for his patience, invaluable guidance, motivation, enthusiasm, immense knowledge, and insights for my research throughout my PhD. I shall be forever indebted for his esteemed guidance that has helped me tremendously during my Ph.D.

I am also extremely grateful to my Doctoral committee chairperson Dr. Manish Kumar, Dr. Lalit Mohan Pandey, Prof. Sandip Paul for their intuitive comments and valuable suggestions that have contributed immensely in refining my thesis.

I thank the Department of Biosciences and Bioengineering for providing the infrastructure and facilities, and the Ministry of Human Resources and Development, New Delhi, Government of India, for assisting me with the financial aid during my Ph.D.

I would like to acknowledge the present and past Heads of the Department of Biosciences and Bioengineering for providing the facilities necessary for conducting my research.

I also take this opportunity to thank all the staff members of the IIT Guwahati, securities, and janitors for their tireless help and assistance during my Ph.D.

My heartfelt appreciation also extends to my present and past labmates of the Cancer Biology Laboratory, Dr. Monisha, Dr. Nand Kishor, Dr. Padmavathi, Dr. Devivasha, Dr. Anuj, Dr. Babita, Dr. Elina, Bethsebie, Kishore, Harsha, Krishan, Sosmitha, Parama, Shabnam, Rajesh, Aviral Kumar, Uzini, Elika, Jyothsna, Ramya, Aswin, Soham, Nikunj, Shyamananda, Raju, Nafiseh, Anip, Aviral, Poonam, Rooplekha, Bidisha, Joydeep, Shubhrojyoti, Anindita for all their immense help, encouragement and fun amidst work.

I will forever owe my achievements to my loving mother, my siblings (Ranju, Ranita, Reema) and my husband for their untiring love, unflinching support and constant encouragement to pursue my interests. Their motivation, blessings and love has helped me to reach here.

I value and treasure all my friends at IIT Guwahati. My special thanks to Devi, Anushree, Babita, Elina, Monika, Anju, Shanti, Binota, Devarani, Sundar, Sophia, Bebina, Shrutika, and Pooja, for their love, empathy, and relentless support.

I thank all the teachers of my school and colleges for inspiring and encouraging me.

Last but not the least, my sincere thanks go to all my well-wishers who have supported me directly or indirectly in this journey of my life.

Khwairakpam Amrita Devi

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Introduction and Review of Literature

Chapter 1

1.1. Introduction

Oral cancer is the sixth utmost prevalent cancer in the world. According to World Health Organization (WHO), nearly 650,000 new oral cancer cases and over 330,000 deaths have been reported annually [Bodhade et al., 2013, Chen et al., 2016, Gupta et al., 2016, Khurshid et al., 2018]. Oral cancer is rampant in the countries of South Asia such as India, Srilanka, Pakistan and Bangladesh [Gupta et al., 2016, Scully et al., 2005]. The International Agency for Cancer Research GLOBOCAN-2018 has reported that oral cancer incidence and mortality is the second highest cancer type in India [IARC GLOBOCAN-2018]. Notably, oral cancer in India is over 30% of all cancer types stated in the country [Coelho, 2012]. In developing countries like India people had a higher risk of oral cancer as majority of them consumed tobacco and are deprived of oral hygiene [Das and Nagpal, 2002]. Sharma et al., has stated that the overall risk of developing tobacco-related cancer was maximum in the northeastern India when compared to other parts of India [Sharma et al., 2018]. Moreover, the second maximum incidence of mouth cancer was detected among females in the northeast region [Sharma et al., 2018]. Further, chewing of betal nuts, deficit intake of vitamin A, B2 and iron, human papilloma virus (HPV) infection are the important risk factors of tumor of the oral cavity [Podlodowska et al., 2012]. In addition, HPV (16 & 18) subtype and Epstein-Barr virus (EBV) have been found to cause tumor on oral epithelia [Gupta and Gupta, 2015]. Studies have found that the precancerous lesions such as leukoplakia and erythroplakia arise as a result of the smoking/nonsmoking of tobacco in combination with or without alcohol. Also, chewing of betal nut along with or without tobacco caused oral submucous fibrosis [Goel et al., 2014]. Primarily, there are several treatment strategies for oral cancer such as surgery, radiotherapy, chemotherapy

(methotrexate, 5-fluorouracil, cisplatin, carboplatin, docetaxel), targeted therapy (cetuximab), gene therapy (use of DNA as an agent to treat disease), and neutraceuticals (curcumin, green tea, resveratrol) [Rao et al., 2015]. Despite this, there are serious oral health complications related to tumor of the oral cavity and its treatment which includes mucosities, mucosal neuropathy, taste alteration, loss of taste, trismus, halitosis, limited movement of the jaws, tongue, lip aperture, neck, shoulder and necrosis of the soft tissue and bones, advanced attachment loss in mobility of the periodontal region etc.[Rao et al., 2015]. Studies have revealed a recurrence rate of 32.7% and also 40%-50% advanced disease recurrence in OSCC [Bavle et al., 2016, Chen et al., 2016]. In addition, it has been found that OSCC frequently metastasizes to cervical lymph nodes [Sasabe et al., 2017]. This supports the line that OSCC is related with the significant mortality and morbidity. Further, resistance to chemotherapy confines the efficacy of treatment with anti-cancer drugs [Longley, 2005]. Chemoresistance to common chemotherapeutic drugs, radioresistance and poor prognosis are drawbacks related with the treatment of oral cancer [Wang, 2016]. Shukla et al., has reported that the mean age of people with oral cancer in India is 36 years and peak at 55–60 years [Shukla et al., 2012]. Besides, the molecular complexity and clinical behavior lessen the chances for early detection of the disease. As such significant markers are essential for the primary detection of OSCC [Randhawa et al., 2015].

Various inflammatory pathways such epidermal growth factor receptor (EGFR), ras homolog gene family member C (RhoC), cyclooxygenase-2 (COX-2), p38a mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), peroxisome proliferator-activated receptor gamma (PPARy), nuclear factor kappa B (NF-kB) etc. are found to play an important role in the oral squamous cell

tumorigenesis [Sarode et al., 2015]. Considerable upregulation in the expression of insulin-like growth factor type 1 receptor (IGF-1R), glycolysis-related proteins (lactate dehydrogenase A (LDHA), hexokinase 2 (HK2), glucose transporter 1 (GLUT-1), transketolase-like protein 1 (TKTL1)), and mitochondrial enzymes like succinate dehydrogenase complex subunit A (SDHA) and B (SDHB), ATP synthase was observed in the tumorigenesis of OSCC. It has also been studied that oxygen deprived metabolism of glucose and oxidative phosphorylation in the mitochondria regulates the tumorigenesis of OSCC [Hamakawa et al., 2008]. Researchers have identified that the downregulation of pentose phosphate pathway (PPP), glycolysis and ROS generation is essential for the treatment of OSCC [Hamakawa et al., 2008]. Occurrence of increased lipogenic and glycolytic enzymes, thereby elevated rate of glycolysis takes place in cancerous cells due to formation of *de novo* fatty acids [Kuhajda et al., 2000, Jackowski et al., 2000]. The rate of synthesis of fatty acids is lesser in normal cells while in tumor cells elevated *de novo* lipogenesis (DNL) occurs for large amounts of fatty acids are required by the actively proliferating cells [Kuhajda et al., 2000, Jackowski et al., 2000]. ACLY, a key enzyme identified to have involved in cancer metabolism [Zu et al., 2012]. It is the first enzyme that catalyzes the first step of DNL [Wellen et al., 2009, Migita et al., 2013] and accountable for the production of oxaloacetate and acetyl-CoA in the cytosol [Qian et al., 2015]. Further, the produced acetyl-CoA takes part in production of fatty acid [Migita et al., 2013, Ameer et al., 2015]. In various cancer types such as glioblastoma, breast cancer, bladder cancer, colorectal cancer, liver cancer, lung cancer, prostate cancer and ovarian cancer, the expression and activity of the enzyme ACLY was found to be highly upregulated [Turyn et al., 2003, Zu et al, 2012, Beckner et al., 2012, Migita et al., 2012,

Khwairakpam et al., 2015]. The synthesis of lipid is deregulated in tumorous cells thereby, an increased expression of ACLY occurs enabling the survivability of the cancerous cells [Zaidi et al., 2012]. It is also found to play an important role in histone acetylation other than de novo lipogenesis [Kuhajda et al., 2000, Jackowski et al., 2000]. In a study conducted by Qian et al., the expression of ACLY was found to be considerably high in gastric adenocarcinoma tissue in comparison to the nearby normal tissues and that the increased ACLY associates with the advanced stages and lymph node metastasis. It therefore indicates the potential of ACLY as a biomarker to speculate the progression and prognosis of patients with GA [Qian et al., 2015]. Therefore, there is indeed, a need to focus on lipogenetic enzymes and the role of ACLY in the process of tumorigenesis of oral cancer. Understanding it will absolutely assist in the development of a prognostic marker and also in providing the novel targets against which highly potent cancer drugs can be assessed to overcome this terrible life threatening disease OSCC.

1.2.Oral cancer

Oral cancer mostly refers to squamous cell carcinoma of the oral or mouth cavity [Epstein et al., 2008]. The oral squamous cell carcinoma (OSCC) is the most common neoplasm of head and neck cancer [Chen et al., 2013, Almangush et al., 2017]. 95% of all types of head and neck cancer are OSCC [Rivera et al., 2014, Elango et al., 2006]. Oral cancer involves tumor growth in the lips, cheeks, hard palate, soft palate and uvula, gingiva, gums, floor of the mouth, tongue, teeth, tonsils and retromolar trigone [Jemal et al.,2006, Radhakrishnan et al., 2012]. In Asia, oral cancer is highly prevalent and India ranks first in oral cancer among all the various cancers [Kao et al., 2015] and constitutes a major public health problem [Shenoi et al., 2012, Tanaka et al., 2011].

Unfortunately, around 33% of tobacco related oral cancers occurs in the North-east region of India [Bhattacharjee et al., 2006]. The epidemiological studies of OSCC cases in India from January 2008 to September 2010 determine the male to female ratio of 4.18:1 and 49.73 years of mean age [Shenoi et al., 2012]. It has been revealed that tobacco and alcohol are important oral mutagens [Johnson et al., 2001, Scully et al., 2005]. Aforementioned, the most important risk factors of OSCC includes tobacco, alcohol, betel quid, and human papillomavirus infection [Zheng et al., 2020]. Additionally, the infective agents and genetic factors also takes part in the oral cancer development [Scully et al., 2005]. It has been found that the frequency of oral cancer is vast in India due to the geographical factors, cultural, ethnic and prominent style of addiction [Byakodi et al., 2012]. Mortazavi et al., have reported that oral squamous cell carcinoma appeared from potentially malignant disorders (PMDs) that arises from the uncared signs and symptoms and also a delay in diagnosis [Mortazavi et al., 2014]. Moreover, the epidemiological studies have reported that the oral cancer patients in tumour lymph node metastasis (TNM) stage III and IV survived 5-year lesser than those who are in earlier stage I and II after chemotherapy [Kao et al., 2015]. The patients with oral cancer have an increased risk of recurrence restricted to the local region and evolving new primary tumors consequently nevertheless, the threat of far off recurrence is usually lesser [Montero et al., 2015].

1.3.Types of oral cancer

(Cancer Research UK, Cancer Treatment Centers of America

1.3.1. Squamous cell carcinoma

95% of the oral cancer is SCC. This tumor type originates in the squamous cells. These squamous cells popularly has flat shape cells that protects the inside of the mouth, nose, larynx and throat [Lamichhane et al., 2015].

1.3.2. Verrucous carcinoma

Verrucous carcinoma comprises around 5% of oral cancer. This cancer type grows slow and are made up of squamous cells which may invade the nearby tissue but hardly metastasize to the distant organs [Lamichhane et al., 2015].

1.3.3. Minor salivary gland carcinomas

Tumors that arises in the minor salivary glands which are present all over the lining of the mouth and oropharynx. The common sites for this type of tumors are located over the lining of the mouth and oropharynx. The Adenoid cystic carcinoma, polymorphous low-grade adenocarcinoma and mucoepidermoid carcinoma etc. are some of the types of salivary gland carcinomas [Lamichhane et al., 2015].

1.3.4. Lymphoma

Lymphomas are tumors that arises in the lymph tissue. For example, tonsils and the base of the tongue that consists of lymph tissue where tumor growth and proliferation may occur [Lamichhane et al., 2015].

1.3.5. Melanoma

Oral mucosal melanoma is exceedingly uncommon disease that accounts only 0.5 % of oral tumors and 1–2 % of total melanomas. It is a erratic neoplasm which develops from the uncontrolled proliferation of melanocytes mostly of oral mucosal epithelium that are mainly present in the basal layers. As compared to other tumors of the oral cavity, melanoma is exceedingly malignant and are

able to metastasize or quickly invade the tissues locally. The common spots for oral melanoma consists of maxillary gingiva and hard palate [Lamichhane et al., 2015].

1.4. Development of oral cancer

The tumorigenesis of oral cancer is usually a multistage process and includes precancerous lesions, invasion and metastasis [Tanaka et al., 2011, Rivera et al., 2014,]. Montero et al., has reported that the development of OSCC is related with a variety of premalignant lesions, which includes leukoplakia, erythroplakia, oral lichen planus (OLP), and oral submucous fibrosis [Montero et al., 2015]. Moreover, the WHO have graded dysplasia (premalignant lesions) into mild, moderate, severe, and carcinoma *in situ* [Izumo et al., 2011, Montero et al., 2015].

Precancerous lesions

Precancerous lesion is a morphologically altered tissue where the tumorous growth is possible to occur than the nearby normal tissues [Radhakrishnan et al., 2012]. Aforemention, the most common premalignant lesions includes leukoplakia, erythroplakia, lichen planus and submucous fibrosis [Villa et al., 2011, Montero et al., 2015]. In addition, it has been observed that this premalignant lesions have erratic ability for malignant transformation [Montero et al., 2015].

1.4.1. Leukoplakia

As defined by WHO, leukoplakia is a "white patch or plaque that cannot be characterized clinically or pathologically as any other disease" [Montero, 2015]. This type of lesion is generally associated with smoking and drinking of alcohol. The incidence of leukoplakia is nearly 2% worldwide and the dysplastic changes was

observed in 2–5% of patients. In addition, the malignant transformation rate of leukoplakia is 1% annually [Montero et al., 2015]. The main risk factors for malignant transformation include incidence of dysplasia, female gender, lengthy duration of leukoplakia, size more than 2cm, position on the floor of mouth or tongue, leukoplakia in non-smokers, and non-homogeneous type [Montero et al., 2015]. It has been shown that closely 70% of oral leukoplakias were observed on the lip vermilion, buccal mucosa, and gingivae [Mortazavi et al., 2014]. Further, the proliferative verrucous leukoplakia has been found to be resistant to treatment and exhibits higher rate of recurrence and malignant transformation [Parlatescu et al., 2014]. It has been reported that the risk of developing tumor is 8-10 times more in people with oral leukoplakia compared to normal [Wang et al., 2009]. Moreover, it has been observed that the malignant transformation rate of proliferative leukoplakia is 70.3% (mean follow-up of 11.6 years) [Lee et al., 2000]. Histologically, leukoplakia involves hyperkeratosis, parakeratosis and acanthosis [Mehta et al., 1993]. Montero et al. noted that the absolute way for the correct diagnosis and treatment of leukoplakia includes avoidance of tobacco, alcohol and excision [Montero et al., 2015].

1.4.2. Erythroplakia

Fournier and Darier was the first to describe erythroplakia as a malignant dyskeratosis with unknown cause in 1893 and termed it as epitheliome papillaire [Mortazavi et al., 2014]. The WHO has defined oral erythroplakia as any lesion of the mucosal area of the oral cavity that appears like a bright red velvety plaques which cannot be characterized clinically or pathologically as any other condition [Villa et al., 2011]. In erythroplakia, keratin is usually absent and possess atrophic epithelium. Further, hyperplasia is also seen at times [Villa et al., 2011]. It has been reported that 1 per 2500

adults suffers from oral erythroplakia [Mortazavi et al., 2014]. In addition, erythroplakia often occurs on ventral tongue, tonsillar fauces, floor of mouth, soft palate, and retromolar pad [Villa et al., 2011, Mortazavi et al., 2014].

1.4.3. Oral Lichen Planus:

Lichen planus (LP) was first described in 1869 [Mortazavi et al., 2014]. It mainly occurs in tongue, gingivae, palate, posterior buccal mucosa, and vermilion border of the oral cavity [Mortazavi et al., 2014, Buajeeb et al., 2015]. OLP is a common chronic, inflammatory, immunologically regulated muco-cutaneous disease that occurs in the buccal mucosa [Munde et al., 2013, Mortazavi et al., 2014]. The reported rate of malignant transformation of OLP is of 0.4-5.3%, as a result of which the WHO categorizes OLP as a potentially malignant disorder [Munde et al., 2013]. Further, the characteristic features of LP are white papules, white plaques, erythema, erosions or blisters [Sugerman et al., 2002]. Clinically, OLP is characterized by white keratotic lesions usually painless to erosions and ulcerations that are painful [Mortazavi et al., 2014].

1.4.4. Oral Submucous Fibrosis (OSMF):

Goel et al., have defined OSMF as a chronic, progressive, scarring, a premalignant condition which are characterized by inflammation and progressive fibrosis of submucosa of the oral cavity that subsequently resulted into rigidity and trismus [Goel et al., 2014, Mortazavi et al., 2014, Passi et al., 2017]. OSCC that originates from OSMF are found to be more aggressive and metastatic than the OSSC that arenot originated from OSMF [Mortazavi et al., 2014]. It is a premalignant condition caused by chewing areca-nut with or without tobacco. Further, the conversion rate of OSMF

to malignany has been reported to be 7.6% in India and 4-13% globally [Rajalalitha and Vali, 2005]. The areas of the oral cavity often affected by OSMF includes soft palate, retromolar area, oral mucosa, and tongue. Further, people with OSMF showed signs and symptoms like limited jaw movement, ulcerations, melanoses, vesicles, petechiae, xerostomia, and burning sensation. Additionally, it has been estimated that OSMF patients will have 19 times higher chances to develop OSCC compared to healthy people [Mortazavi et al., 2014]. Furthermore, the characteristic feature of OSMF also includes mucosal rigidity, decrease in vasculature, atrophy of surface epithelium, and dysphagia [Karemore et al., 2012, Passi et al., 2017].

1.5.TNM (Tumour lymph node metastasis) Staging:

The staging of oral cancer is highly critical for proper prognosis and therapy. It is performed mostly by using the TNM technique, where T indicates the size of the primary tumor, N indicates status of the regional lymph nodes, and M indicates the presence or absence of distant metastasis [Radhakrishnan et al., 2012]. Shown below are the TNM clinical classification for tumor of the lip and oral cavity.

Table 1.1. TNM stage classification for oral cancer

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor 2cm or less in its greatest dimension

T2	Tumor more than 2cm but not more than 4cm in greatest dimension
T3	Tumor more than 4cm in greatest dimension
T4a (Lip)	Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth or skin (Chin or nose)
T4b(Oral cavity)	Tumor invades through cortical bone, into deep / extrinsic muscles of the tongue (genioglossus, hyoglossus, palatoglossus and styloglossus), maxillary sinus or skin of face
T4b(Lip and oral cavity) –	Tumor invades through masticator space, pterygoid plates, or skull base or encases internal carotid artery
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3cm or less in greatest dimension.
N2	Metastasis as specified in N2a, 2b, 2c below
N2a	Metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6 cm or less in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
Distant metastasis (M)	
MX	Distant metastasis cannot assessed

M0	No evidence of distant metastasis
M1	Distant metastasis is present

1.6. Stage grouping:

Stage 0	Tis	NO	MO
Stage I	T1	NO	MO
Stage II	T2	NO	MO
Stage III	T1,2,3	N1	MO
Stage IVA	T1,2,3 T4a	N2 NO, N1, N2	MO
Stage IV B	Any T T4b	N3 Any N	MO
Stage IVC	Any T	Any N	M1

1.7.Risk factors of oral cancer

The case of oral cancer is highly positive in India compared to other countries in the world [Gupta et al., 2013]. Byakodi et al., has reported that India ranks first in having the highest incidence of oral cancer among men and third among women [Byakodi et al., 2012]. The common risk factors of oral cancer includes tobacco and tobacco related products, consumption of areca nut, alcohol, HPV infection, and increasing age, male gender and socioeconomic factors, genetic predisposition and hormonal factors [Byakodi et al., 2012, Gupta et al., 2013]. In addition, chronic immunosuppression after solid organ transplant, hematopoietic cell transplant, human

immunodeficiency viruses (HIV) infection and acquired immunodeficiency syndrome (AIDS) are also some of the causal agents [Epstein et al., 2008]. The main risk factors of oral cancer are described as follows:

1.7.1. Tobacco

Oral cancer is known to be a man-made disease correlated with tobacco consumption since centuries [Kao et al., 2015, Sanner et al., 2015]. All forms of tobacco are said to be risk factors for oral cancer [Johnson, 2001, Warnakulasuriya et al., 2005]. More than 90% of the head and neck cancer occurs worldwide due to consumption of tobacco, alcohol and person who are deprived of essential diets [Johnson, 2001]. Nearly 4000 chemicals are found occurring in tobacco products [A Report of the Surgeon General, CDCP, 2010]. It has been reported that 7000 chemicals are found present in cigarette smoke of which 69 are known carcinogens and introduction to it causes DNA mutation [A Report of the Surgeon General, CDCP, 2010]. For instance, substances like polycyclic hydrocarbons and tobacco-specific N-nitrosamines (TSNA), are carcinogenic in nature and are responsible for the tobacco associated cancer [Sanner et al., 2015]. In addition, N-nitrosonornicotine (NNN), 4[methylnitosoamino]-1-[3-6-pyridyl]-1-butanone (NNK) are identified Tobacco-specific-nitrosamines (TSNA) which are some of the active carcinogens present in tobacco. Additionally, nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine found in oral products when consumed is absorbed by the mucosa of the oral cavity slowly and easily due to the alkalinity of nicotine. Further, carcinogenic compound like benzo[a]pyrene (Bap) along with TSNA are found to present in the smoke of cigarette [A Report of the Surgeon General, CDCP, 2010]. Studies have shown that the presence of nicotine regulates certain stages of tumor growth and might be the reason of

cancer recurrence. It is also said that inside the body, nicotine may give rise to TSNA [Sanner et al., 2015].

1.7.2. Betal quid and areca nut

Chewing of betal quid causes the chronic malignancies like OSCC [Liao et al., 2011]. The chewing of betal nuts and products derived from it, affects the oral soft tissues that gives rise to precancerous changes such as oral submucous fibrosis (OSMF) and when eaten with tobacco causes leukoplakia [Anand et al., 2014]. Studies have found that chewing of betal quid along with areca nut and tobacco causes an increasing risk of oral cancer 8-15 times whereas consumption of quid without tobacco has 1-4 times [Manjari et al., 1996]. Studies have shown that areca nut (betel nut) consists of 11%–12% tannins and 0.15%–0.67% alkaloids like arecoline, arecaidine, guvacine, and guvacoline and chewing of it is known to be one of the main cause of OSMF. Besides, arecaidine (active metabolite) promotes fibroblast stimulation and proliferation, and also instigated the production of collagen [Passi et al.,2017]. Arecaidine has been reported to induce development of tumor [Jeng et al., 2001]. Studies have demonstrated the hydrolysis of arecoline to arecaidine upon inclusion of slaked lime ($Ca[OH]_2$) to areca nut thereby, making it accessible in the oral environment [Passi et al., 2017]. Moreover, areca nut alkaloids have been found to show mutagenicity as well as genotoxicity [Jeng et al, 2001]. The autooxidation of the polyphenols present in areca nut occurs in saliva of the chewer generates ROS that in turn causes gene alterations and genotoxicity which assists the growth and proliferation of the cancereous cells [Jeng et al., 2001]. In addition, it affects the proteins of the salivary gland and oral mucosa and allows the entry of other ingredients of betal quid and environmental toxicants [Jeng et al., 2001].

1.7.3. Alcohol

Tobacco consumed along with alcohol synergistically affects all upper aerodigestive tract SCC (mouth, larynx and esophagus) [Johnson, 2001, Sanner et al., 2015]. Alcohol increases the permeability of toxin and carcinogens. The presence of acetaldehyde, production of ROS and nitrogen species plays a critical role in tumorigenesis [Boffetta et al., 2006]. The immunosuppressive effect of alcohol when accompanied by deficit in required nutrients stimulates the process of tumorigenesis [Radhakrishnan et al., 2012].

1.7.4. Human papilloma virus (HPV)

Some of the viruses such as EBV, HPV, Retroviruses and, Herpes Simplex viruses (HSV), are involved in tumorigenesis of the oral cavity [Radhakrishnan et al., 2012]. Among them, HPV is identified to have higher risk factor in the initiation of oral malignancies [Radhakrishnan et al., 2012, Chattopadhyay et al., 2019]. It has been identified that HPV-E6 protein together with E6 linked protein (E6/E6-AP) complex binds to the tumor suppressor p53 and inactivates the gene expression of p53. More importantly, a viral infection aided by carcinogenic substances is highly efficient for the formation of oral tumors [Radhakrishnan et al., 2012]. Additionally, HPV-16 and HPV-18 has been identified in up to 22 % and 14 % of oral cancer cases respectively [Neville et al., 2002].

1.7.5. Other factors

Various other factors like poor hygienic condition of the oral cavity are also known to increased the risk of oral cancer [Das et al., 2002, Podlodowska et al., 2012]. Immunity of a person also regulates the tumorigenesis of the mouth [Kim et al., 2004, Jakymiw

et al., 2010]. Deficiency of vitamin A, riboflavin and iron, therapy which are immunosuppressive in nature also increases the pervasiveness of oral carcinoma [Podlodowska et al., 2012]. Furthermore, orodental factors such as sharp teeth, incorrect restorations, and improper fitting of dentures are responsible for tumor of the oral cavity [Podlodowska et al., 2012].

1.8. Molecular Alterations of Oral cancer

The characteristics of the tumor cells are altered due to various genetic alterations. It has been studied in oral cancer cell lines that there is an elevation in the expression of anti-apoptotic proteins belonging to B-cell lymphoma 2 (Bcl-2) family such as B-cell lymphoma-extra large (Bcl-xL) and myeloid cell leukemia-1 (Mcl-1) [Mehrotra et al., 2006]. In p53 gene, loss of heterozygosity has been reported in nearly 22% of premalignant oral lesions and 20% of OSCCs [Mehrotra et al., 2006]. Moreover, caspase-8 (CASP8), caspase 10 (CASP10), low-density lipoprotein receptor-related protein 1B (LRP1B), protein phosphatase 1 regulatory subunit 7 (PPP1R7), BRCA1-associated RING domain protein 1 (BARD1), ILK Associated Serine/Threonine Phosphatase (ILKAP), and inhibitor of growth family member 5 (ING5) are the tumor suppressor genes known to be expressed in several cell lines of OSCC often influenced by the continual deletions at the regions of 2q21-24, 2q33-35, and 2q37 [Yanamoto et al., 2007]. In addition, the abnormalities in 4q, 5q21-22, 11q, 18q, 21q and 22q13 regions and allelic lose in 9p21 region encoding p16 and p14, the suppressors of cyclin dependent kinase (CDK) was observed in many stages of oral cavity carcinoma [Moles et al., 2008, Ohta et al., 2009]. Further, oral tumorigenesis was found to be associated with abnormal expression of the proto-oncogene, cyclin D1, epidermal growth factor receptor (EGFR), c-myc, hst-1, int-2, parathyroid adenomatosis 1 (PRAD-1), bcl-2,

and members of the Ras family [Moles et al., 2008]. In addition, elevated expression of the erbB-1, erbB-2 and cyclin B was also observed in tissues of oral cancer [Kushner et al., 1999, Tsantoulis et al., 2007]. The epithelial cells are transformed to malignant cells due to the impairment of human cyclin G2 (hCG2), also cell proliferation occurs by the reduced expression of let-7b as a result of the increased levels of DICER [Kim et al., 2004, Jakymiw et al., 2010]. The increase in MVD (microvessel density) affects VEGF thereby regulating the progression of OSCC [Tsantoulis et al., 2007]. Several proangiogenic factors are also found to regulate angiogenesis in OSCC. They are HIF1α, COX-2, VEGF-R, PD-ECGF, NOS2, FGF-2, and PGF-3 [Wakulich et al., 2002, Schliephake et al., 2003]. Furthermore, a member of VEGF, Flt-4 has been found to intricate in lymph node metastasis. On the other hand, membrane-bound MMPs (MT1-MMP), collagenases like MMP-1 and -13, the gelatinases such as MMP-2 and -9, and stromelysins like MMP-3, -10 and -11 regulated the OSCC progression [Thomas et al., 1999]. Studies have found that the molecular complexity and clinical behavior lessen the chances for early detection of the OSCC [Randhawa et al., 2015]. Interestingly, various inflammatory pathways played a critical role in tumorigenesis of OSCC such as COX-2, NF-kB, PPARy, p38a MAPK, STAT, RhoC etc. [Sarode et al., 2015]. In addition, the mitochondrial enzymes like SDHA, SDHB, ATP synthase, glycolysis-related proteins (like GLUT-1, HK 2, LDHA, TKTL1) and IGF-1R also lessens the chance of early detection of the OSCC [Grimm et al., 2014, Randhawa et al., 2015]. It has also been studied that oxygen deprived metabolism of glucose and oxidative phosphorylation in the mitochondria regulates the tumorigenesis of OSCC [Hamakawa et al., 2008]. It has been investigated that downregulation of pentose phosphate pathway (PPP), glycolysis and ROS generation is essential for the treatment

of OSCC [Hamakawa et al., 2008]. Bavle et al., has stated that in spite of recent advances in treatment of oral cancer such as chemotherapy, targeted therapy, radiotherapy, and surgery, the prognosis of OSCC is still deprived mainly as a result of tumor invasion, metastasis and recurrence in OSCC [Bavle et al., 2016].

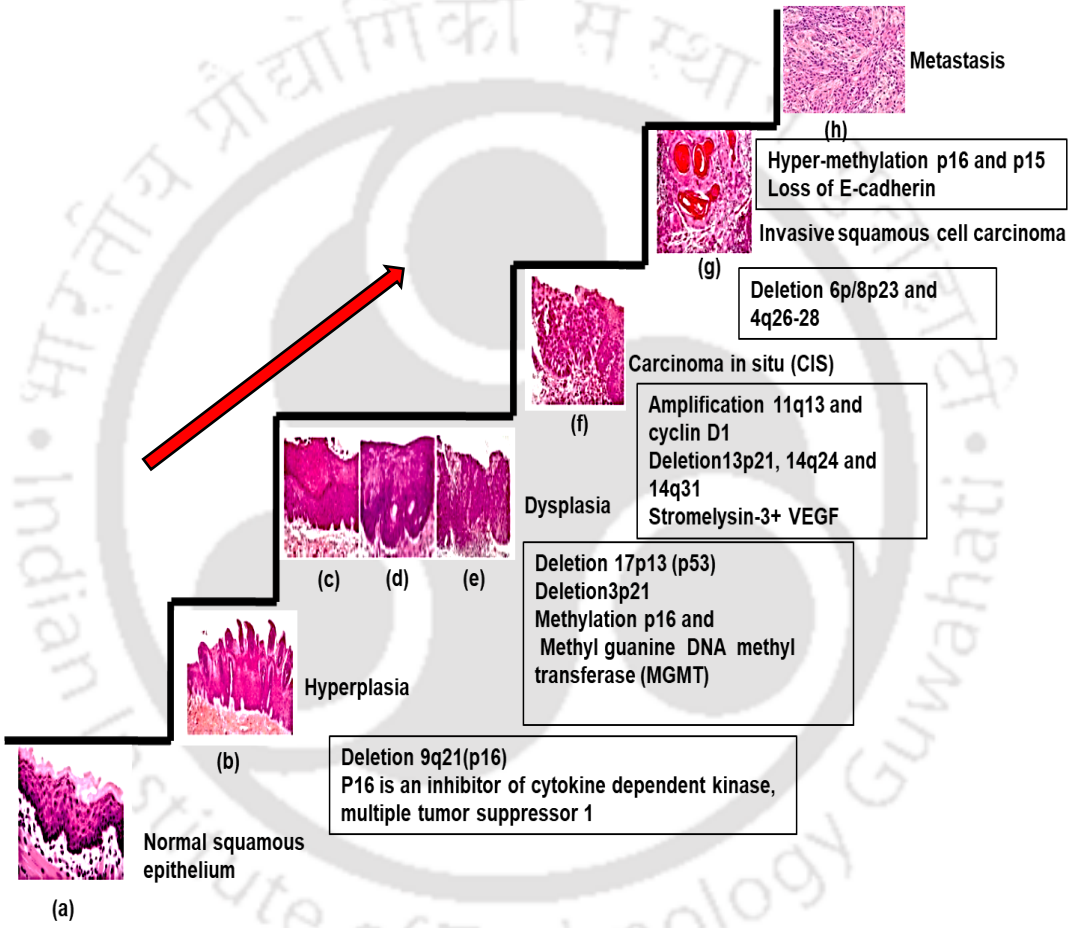


Figure 1.1. Genetic alterations of oral tumorigenesis; (a) Normal squamous epithelium, (b) papillary hyperplasia, (c) middle dysplasia, (d) moderate dysplasia, (e) severe dysplasia, (f) carcinoma in situ, and (g) invasive squamous cell carcinoma (Tanaka T et al., 2011)

1.9.Therapies available for cancer of the oral cavity

1.9.1. Surgery:

Surgery is the most commonly used for the treatment oral cancer [Rao et al., ,2015]. The need for surgical treatment arises depending on the location of tumor, its size, proximity to bone, and complexity of infiltration [Epstein et al., 2012]. In Stage I and II of OSCC, the tumors are accessible and smaller in size; here surgery is usually done in order to remove the tumor and the thin layer of healthy tissue that encloses it. Some of the important works of surgery consists of maxillectomy, tracheotomy, removal of part or all of the jaw, elimination of lymph nodes and other tissue in the neck, and dental surgery. Tumor invaded bones are removed by excising the tumor along with the segment of the bone. Moreover, it has been observed that the chances of occult cervical metastasis is 10%-26%, therefore an alternative therapy is essential for the treatment of the disease [Alvi et al., 1996].

1.9.2. Radiation therapy:

Radiation therapy is mainly endorsed in small tumours or in the initial stages of tumour. The high energy electromagnetic radiations such as X-rays or γ- rays are often utilized to kill or diminish the dimension of the tumor. The nearby normal cells can be protected from the radiation by using fractionation of radiation. Two different fractionations can be accomplished: hyper-fractionation where at seperate time intervals small doses of radiation are specified in a single day; Accelerated fractionation where the rate of weekly dose accretion is 20 to 50% rapid than the standard fractionation thereby decreasing the duration of treatment and recurrence of cancer that may occur amid fractions [Awwad et al., 2002, Budach et al., 2006]. The best sited dose as suggested by National Comprehensive Cancer Network for the treatment of OSCC

is 66-74 Gy. Occasionally, for better prognosis of the disease radiation therapy can also be combined with chemotherapy [David et al., 2011].

1.9.3. Chemotherapy:

In chemotherapy various natural or synthetic chemicals are used to suppress proliferation, progression and invasion thereby promoting cell death in cancer cells. Drugs that are available commercially for treatment of oral cancer include bleomycin, carboplatin, cisplatin, fluorouracil, paclitaxel, methotrexate, ifosfamide, cetuximab, and docetaxel etc. [Fotedar et al., 2013]. Chemotherapy can also be used as neoadjuvant therapy where the chemotherapeutic drug is utilized formerly of any other therapy or by combining with chemoradiation where the drug is given along with the radiation instantaneously [Jain et al., 2008]. As far as the thing is concerned, none of these treatment approaches are 100% efficacious. The treatment of cancer cells with radiation also affects the nearby normal tissues worsening the situation. The chemotherapeutics utilized are likely to cause ischemic heart disease, hepatic dysfunction, myelosuppression, bone marrow, malaise, vomiting, hypertension etc. are some of the side effects that are likely to occur due to chemotherapies [Liu et al., 2014, Tahover et al., 2014]. Also, majority of the cancer cells develop resistance towards the therapy and recurrence of tumor makes it hard to manage this disease [Wang et al., 2012]. As such there is need to find out the actual molecular target for the treatment of OSCC.

1.10. Problems associated with therapies

1.10.1. Chemoresistance

Chemotherapy is one of the most significant approaches used to fight against cancer. However, due to the chemoresistance develop by the cancer cells; it fails to put

an end to cancer growth and proliferation [Longley et al., 2005]. Cancerous cells may be inherently resistant to chemotherapy or it may attain through the treatment processes that were once sensitive to the treatment. Furthermore, after developing resistance to the chemotherapy the treatment becomes limited leading to metastasis in 90% of patients. Several factors that leads to the development of chemoresistance includes rise in drug efflux, drug activation and inactivation, variations in drug target, DNA, methylation , processing drug-induced damage and evasion of apoptosis [Wilson et al., 2006]. Studies have found that most OSCCs are receptive to drugs that are cytotoxic in nature which may be either due to the limiting of the transport of the agents into the cells or a halt with their intracellular molecular targets [Warnakulasuriya et al., 1996].

1.10.2. Side effects

The two methods that are often used for oral cancer treatment are radiotherapy and chemotherapy which may gives rise to side effects and other complications. Problems like mucositis, xerostomia, osteoradionecrosis, microbial infection, ageusia, osteoradionecrosis and dental caries [Dose et al., 1995, Zlotolow et al., 1998]. An important nonhematologic complication of cytotoxic chemotherapy and radiotherapy is oral mucositis that is liable for subsequent dehydration and malnutrition, odynodysphagia, pain, morbidity, and dyseugia [Bitran et al., 1996].

1.10.3. Tumor recurrence

The major problem in the treatment of OSCC patients is tumor recurrence. It has been proven by histopathological examination that around 20-30% of OSCC cases broader than 5mm removed *via* surgery, the tumor-free margins can develop into local or contiguous regional “recurrence”. And moreover, the remained neoplastic

keratinocytes at the margins of the surgical wound or as a result of the large area of precancerized epithelium that consists of keratinocytes which are premalignant at unlike stages of transformation that have not been removed surgically. Studies have found that in the precancerous tissue, cytogenic variations such as loss of heterozygosity and mutations or epigenetic alterations in methylations of tumor-suppressor and DNA repair may take place. Further, the continual genetic alterations in the keratinocytes may lead to the transformation of the keratinocytes into tumorous cells forming a new area of tumor nearby the resected primary tumor [De Vries et al., 1986, Gallo et al., 1995, Braakhuis et al., 2002, Goldenberg et al., 2004, Levi et al., 2006]. Inspite of the advances in oral cancer treatment, tongue malignancy is found to be related with relentless morbidity and long-term survival lower than 50%. Here, the survivability rate of patients becomes low for there is an increased risk of evolving a subsequent primary tumor [Tanaka et al., 2011].

1.11. Lipogenic enzymes

It is well established that fatty acids are vital ingredients of all biological membrane lipids, and are essential substrates for vitality of body metabolism [Menendez et al., 2007]. It forms the main source of energy in human body whose function is to store the excess energy derived from glucose metabolism in the form of triacylglycerols, which, when required, provide energy *via* β-oxidation [Menendez et al., 2007, Bian et al., 2015]. For animal metabolism, two sources of FAs are available such as dietary FAs which are obtained exogenously and synthesized FAs obtained endogenously [Menendez et al., 2007]. Studies have found that the lipogenic enzymes catalyzes the series of reactions for the *de novo* synthesis of fatty acid in the cytosol [Wang et al., 2009]. It has been shown that DNL is normally active during the process

of embryogenesis as well as in fetal lungs where the FAs are utilized for the generation of lung surfactant [Menendez et al., 2007]. Some of the main lipogenic enzymes are Fatty acid synthase (FAS), Acetyl CoA carboxylase (ACACA), ATP citrate lyase (ACLY) that are involed in the production of fatty acids [Chypre et al., 2012, Bian et al., 2015]. The liver is a major lipogenic organ that is of central importance in energy storage and conversion [Wang et al., 2009]. ACLY is the first lipogenic enzyme that converts the citrate (present in the cytoplasm) to acetyl-CoA in an ATP consuming reaction, where glucose metabolism links with fatty acid biosynthesis [Wang et al., 2009, Migita et al., 2014]. Acetyl-CoA is then, converted to malonyl-CoA with the help of ACACA which forms the rate limiting step in synthesis of *de novo* fatty acid [Chypre et al., 2012]. In the consecutive reactions, the FAs are produced from malonyl-CoA by FAS, long-chain fatty acid elongase (ELOVL6), and stearoyl-CoA desaturase 1 (SCD1). Thereafter, TG are generated which are catalyzed by a chain of enzymatic reactions involving glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) [Wang et al., 2009, Chypre et al., 2012].

In normal tissues, the rate of fatty acid biosynthesis take place at a lower rate as lipids supports the necessities of the cells [Kuhajda et al., 2000]. However, the synthesis of fatty acids was found to be elevated in tumorous cells [Kuhajda et al., 2000]. Further, C^{14} glucose studies have proved that all the FAs in tumor cells are derived from *DNL* in spite of an ample availability of extracellular lipids [Ookhtens et al., 1984]. It has been reported that more than 93% of triacylglycerol FAs in tumor cells are produced by *de novo* process and the synthesized fatty acids assisted the growth and development of cancerous cells [Menedez et al., 2007, Wellen et al., 2009]. Moreover, this synthesized FAs has been found to correlate with the augmented glycolysis as revealed

by the increase in glycolytic and lipogenic enzymes in tumor cells [Menendez et al., 2007]. Further, in the tumor cells, the excess pyruvate (finale product of glycolysis) takes part in *de novo* biosynthesis of FAs which therefore maintains the regular supply of lipids for membrane synthesis and lipid based post-translational modification of proteins [Menedez et al., 2007]. Several researchers have found that fatty acid metabolism is impaired in many human diseases including cancer [Bian et al., 2015]. In addition, the increased expression of the glycolytic enzymes like hexokinases, phosphofructokinase 1 (PFK1), and pyruvate dehydrogenase kinase was found to be activated by c-MYC, hypoxia-inducible factor 1 (HIF-1) and mTOR oncogenes in breast, colon, prostate and lung. Moreover, upregulation in the expression of ACLY, ACACA, FAS and malic enzyme (ME) was observed in neoplastic cells [Menedez et al., 2007, Csanadi et al., 2015, Flaveny et al., 2015].

The profound cancerous growth and survival of the immortalized epithelial cells is maintained by the increased lipogenesis thereby maintaining the physiological levels of FA biosynthesis endogenously [Menedez et al., 2007]. Therefore, the lipogenic enzymes might work as an intermediate of oncogenesis that correlates that cellular anabolism with malignant transformation. A lipogenic categorization of the tumors would give a novel molecular rationale which will enable in the identification and development of new therapeutics that strongly inhibits the initiation, progression and metastasis of tumor cells.

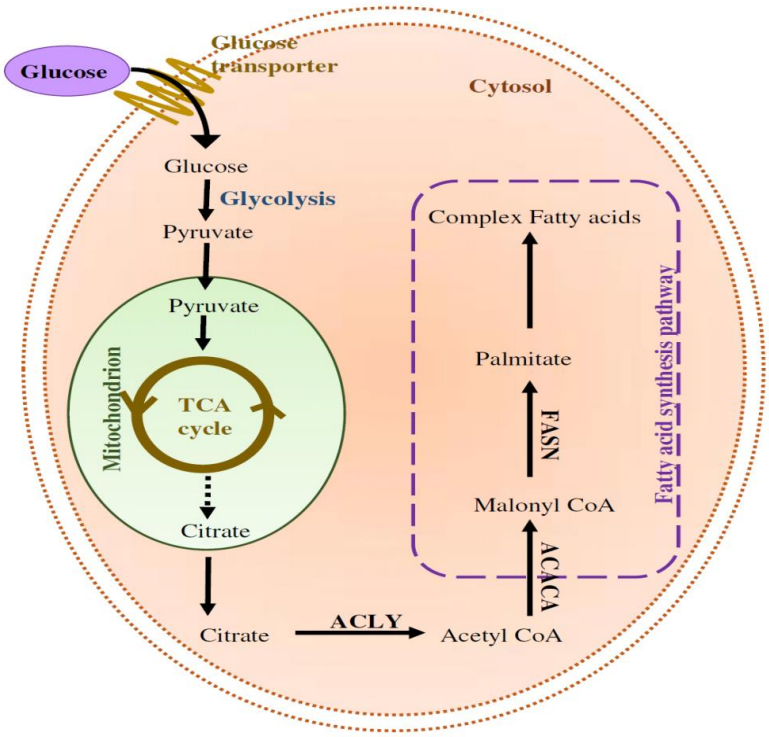


Figure 1.2. Denovo lipogenesis, *Fatima Ameer et al., 2014*

1.11.1. ATP citrate lyase (ACLY)

Generally the expression of ACLY was found to be available abundantly in liver, white adipose tissue and pancreatic beta cells while it is lesser in brain, heart, small intestine and muscles [Wang et al., 2010, Chu et al., 2010, Chypre M et al., 2012]. ACLY being the first enzyme that is involved in the first step of lipogenesis is known to be upregulated in many cancers [Elshourbagy et al., 1990, Migita et al., 2014]. Pyruvate, the product of glycolysis goes into the tricarboxylic acid cycle (TCA Cycle) giving citrate. In the cytosol, ACLY acts on citrate to produce OAA and acetyl-CoA in the presence of Coenzyme A and ATP [Baggetto, 1992, Kaplan et al., 1993].The produced acetyl-CoA is found to be a precursor for both fatty acid synthesis and mevalonate synthesis pathway (MVA) (synthesis of cholesterol and isoprenoids) [Zaidi et al., 2012]. Other than its role in synthesis of fatty acids,

expression of *ACLY* was found to be responsible for the production of acetyl-CoA for acetylcholine synthesis. *In vivo* studies have also shown that ACLY is essential for embryonic development in mice [Beigneux et al., 2004]. ACLY also takes part in histone acetylation at the time of cell differentiation and in the availability of growth factors as well as insulin [Wellen et al., 2009]. Acetylation of histone proteins has a great role in maintaining the chromatin structure and involves in the expression of genes that are specifically mean for glucose metabolism [Wellen et al., 2009]. Many studies have proven that ACLY inhibitors dowregulated the expression of ACLY [Hatzivassiliou et al., 2005]. When ACLY is inhibited, activation of reactive oxygen species (ROS) occurs and hamper the mitochondrial FA oxidation as well as the elongation of fatty acid in the endoplasmic recticulum [Wellen et al., 2009, Zaidi et al., 2012, Migita et al., 2013, Gao et al., 2014]. The development and proliferation of cancer cells are inhibited by ACLY knockdown and statin treatment which is due to the obstruction of MAPK and PI3K/AKT pathways [Hanai et al., 2012]. Upon silencing of ACLY, hexokinase-2, PFK1 and lactate dehydrogenase that are known to involve in glycolysis are inhibited and also suppressed the mRNA expression of GLUT-4 (insulin-responsive glucose transporter) [Alexander et al., 1981, Brownsey et al., 1984, Reinhart and Roehrig, 1987, Emmerson et al., 1992, Kim et al., 1996, Bollu et al., 2014]. Evidences have shown that EGF, TGF- β, glucose metabolites and insulin helps in the upregulation of ACLY in normal hepatocytes of rat. It has also been reported that insulin is involved in the increased phosphorylation of ACLY in the normal fat cells of human by elevation of protein kinase [Sánchez-Solana et al., 2014]. MORC-2 is found to enhance the ACLY regulation in the cytosol which leads to increase in lipogenesis,

cholesterol synthesis, histone acetylation and the development of pre-adipocytic cells [Sánchez-Solana et al., 2014]. Evidences have shown that the genetic variants in *ACLY* gene possess a significant effect on the prognosis of patients with advanced stage colorectal cancer (CRC) [Xie et al., 2015]. Mis-sense single nucleotide polymorphism (SNP) rs2304497, caused modifications of the ACLY protein leading to changes in structure and activity of ACLY while another SNP (rs9912300) which is present in the transcription-factor-binding site (TFBS), affects the ACLY expression *via* transcription of *ACLY* [Xie et al., 2015].Therefore, dissimilar levels of ACLY expression in CRC cells due to gene polymorphisms occurred. It therefore, gives rise to alteration of metabolic system and other biological properties that culminates distinct prognosis in CRC patients [Xie et al., 2015].

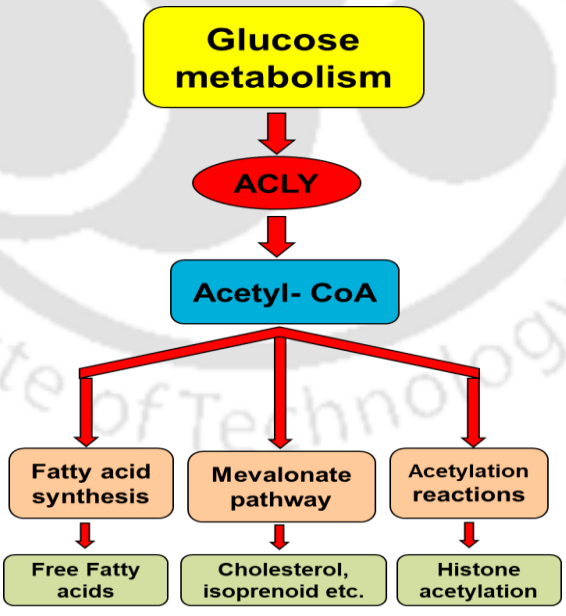


Figure 1.3. ATP-citrate lyase is at the crossroads of several pathways such as synthesis of fatty acids, mevalonate pathway and acetylation reactions, *Chypre et al.,* 2012

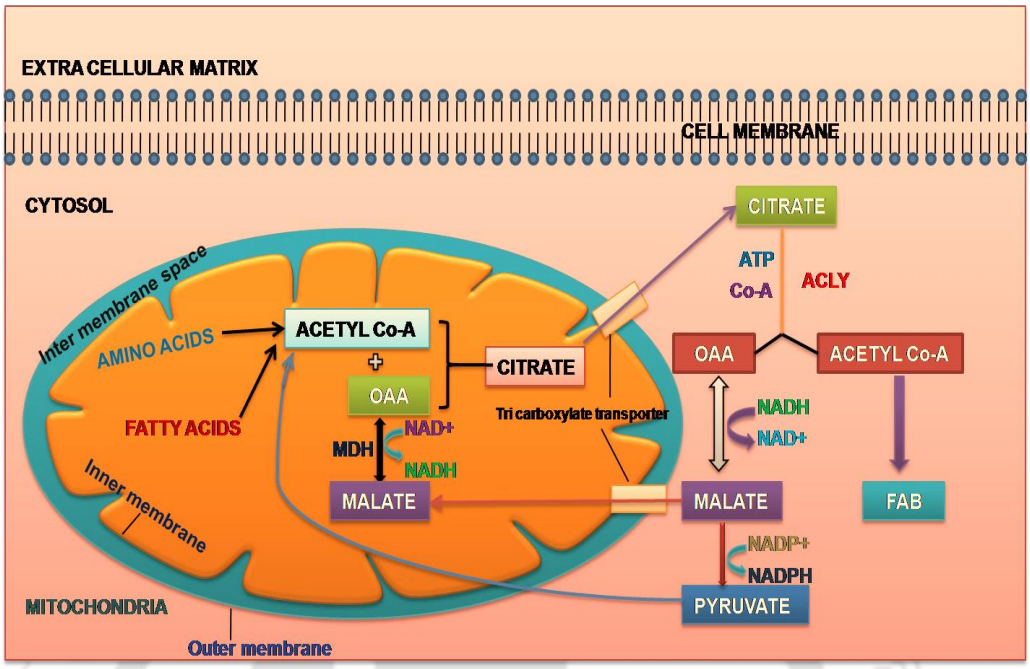


Figure 1.4. The pathway in which ACLY catalyzed the conversion of citrate to OAA and Acetyl-CoA in the presence of ATP & CoA, *Khwairakpam et.al*, *2015.*

1.11.2. Structure and Distribution of ACLY in body tissues

ACLY is a homotetramer, a member of the acyl Co-A synthase super-family. It consists of 1101 amino acid residues and protein size is 480 kDa. Moreover, it is found to be located at chromosome 17q21.2. [Singh et al., 1976, Elshourbagy et al., 1990, Sanchez et al., 2000, Wojnarowicz et al., 2008, Khwairakpam et al., 2015, Pinkosky et al., 2017, Wei, 2019, Verschueren et al., 2019]. Elshourbagy et al. has reported that the entire sequences of ACLY cDNA of human and rat has 96.3% peculiarity [Elshourbagy et al., 1992].

It also exhibited great similarity of the sequence with succinyl-CoA synthetase and citrate synthase [Sun et al., 2010]. Additionally, ACLY forms a homotetramer *via* the C-terminus to simplify the CoA binding and production of acetyl-CoA [Bazilevsky et al., 2019]. It is active only in its tetramer form [Wei et al., 2019]. ACLY comprises of

six domains, CoA-binding site is in domain 1, His760 catalytic phos-phorylation site in domain 2, the ATP binding site is at domains 3 and 4, domain 5 provides the binding of citrate, while the CS domain forms the central core of ACLY [Sun et al., 2010, Khwairakpam et al., 2015, Wei et al., 2019, Verschueren et al., 2019]. Studies have found that the CS domain of ACLY and citrate synthase have homology at the C-terminal segment and is known to take part in catalyzing the production of OAA and acetyl-CoA from citryl-CoA [Verschueren et al., 2019]. Wei et al. have shown the presence of five N-terminal domained crystal structures of ACLY alone forming complex with ligands like citrate or Mg-ADP [Wei et al., 2019]. Moreover, Hu et al. investigations have observed the phosphorylation of His760 as a part of the catalytic behaviour [Hu et al., 2017]. It has also been reported that His760 mutagenesis of ACLY ceased the activity of the enzyme, which indicates the involvement of a catalytic histidine [Fan et al., 2012]. The supplementation of high glucose is known to initiate acetylation of ACLY at three lysine residues such as 540, 546, and 554 (3K) through P300/calcium-binding protein (CBP)-associated factor (PCAF) acetyl transferase. It inhibited the ubiquitinylation and degradation of ACLY. The active site of ACLY, consist of histidine that are phosphorylated at the time of catalysis [Kreil et al., 1964]. There is a marked resemblance at the carboxy terminal of ACLY and citrate synthase that is considered to stimulate the transformation of citrate to oxaloacetate and acetyl-CoA [Sun et al., 2010].

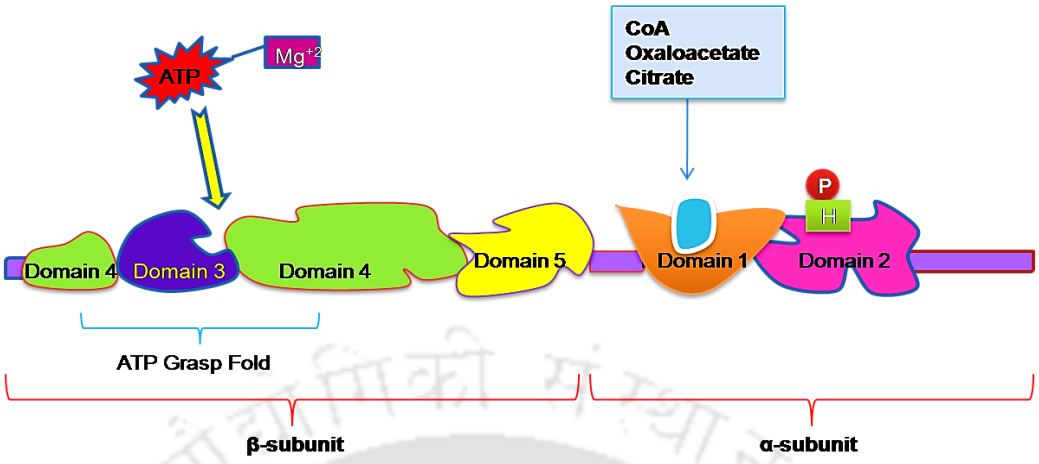


Figure 1.5. Structure of ATP Citrate lyase (1101 amino acid residue), showing 5 different domains with the binding side of different proteins, *Khwairakpam et.al*, 2015.

ACLY is an extra mitochondrial enzyme which primarily occurs in the cytoplasm and nucleus of cells [Hildebrandt et al., 1995, Li et al., 2007, Chu et al., 2010, Khwairakpam et al., 2015, Teng et al., 2018, Verschueren et al., 2019]. Investigations have found that over 80% of the extra mitochondrial acetyl-CoA obtained from pyruvate are supplied *via* the ACLY pathway in the ratliver [Khwairakpam et al., 2015].

ACLY is commonly expressed in a small number of prokaryotes and all eukaryotes excluding oleaginous yeasts [Zhang et al., 2014]. ACLY is critical in autotrophic prokaryotes for the reverse krebs cycle (reductive tricarboxylic acid (TCA) cycle) [Sun et al., 2010]. Studies have found that mammalian cells depend on the expression of ACLY for histone acetylation while the single cell eukaryotes rely on the acetyl-CoA synthetase enzymes which transforms acetate to acetyl-CoA [Sato et al., 2000]. Besides, the increase in the expression of ACLY was observed in cholinergic neurons, adipose tissue, lactating mammary glands, and liver [Wellen et al., 2009].

1.11.3. Pathways served by ACLY

ACLY is an indispensable metabolic enzyme that interlinks glucose and lipid metabolism [Migita et al., 2008, Zu et al., 2012, Wei et al., 2019]. ACLY has been found to play a vital role in biosynthesis of lipids and histone acetylation [Chypre et al., 2012, Gopal and Pizzo, 2017, Wei et al., 2019]. Aforementioned, ACLY plays a catalytic role in the conversion of citrate and CoA to OAA and acetyl-CoA in the presence of an ATP molecule [Sanchez et al., 2000, Chen et al., 2016, Ference et al., 2019]. The citrate synthesized in the TCA cycle is transferred *via* the citrate transport protein from the mitochondria to the cytoplasm and produced the nuclear or cytoplasmic acetyl-CoA from or fatty acids, glucose or glutamine [Ference et al., 2019 , Elshourbagy et al., 1990, , Granchi et al., 2018]. The acetylCoA is the main building block for DNL and mevalonate pathway [Beigneux et al., 2004]. It is also involved in the production of acetylcholine and acetylation of histones and proteins [Verschueren et al., 2019]. The acetyl CoA in the cytoplasm is carboxylated to malonyl-CoA by ACACA. Further, the malonyl-CoA obtained is then converted the acetyl-CoA and malonyl-CoA to long-chain FAs *via* a condensation reaction [Zaidi et al., 2012, Knowles ,2008, Lee et al., 2014]. Moreover, acetyl-CoA is also a substrate for the MVA. In the nucleus acetyl-CoA behaves as a substrate for histone acetyltransferases (HATs) and helps in the modification of histone proteins and alteration of chromatin structure [Rathmell et al., 2011]. The synthesized FAs and cholesterol are crucial for producing the structural components of the cell membrane bilayer, and also transportation and storage of energy. Further, it is essential for the synthesis of signaling molecules and substrates necessary for the post-translational modification of signaling proteins [Pinkosky et al., 2017, Abramson, 2011]. Studies have found that ACLY

recruits phosphate-carboxylate anhydrides, that are required for the activation of phosphate-carboxylate alike glutamine synthetase [Dolle et al., 1992]. It has been demonstrated that knockout of ACLY using CRISPR/Cas9 reduced the acetylation of histone proteins in human THP-1 macrophages [Namgaladze et al., 2018]. Further, the change in the availability of ACLY instigated the site specific modulation of histone H3 Lys27 acetylation which is concomitant with the integrin signaling and cell adhesion [Lee et al., 2018].

Additionally, it has been demonstrated that silencing of ACLY and inhibition of poly (ADP-ribose) polymerase (PARP) triggered apoptosis and genomic instability [Sivanand et al., 2017]. Reduction in the expression of carnitine palmitoyl transferase 1A (CPT1A), a transporter of mitochondrial FAs occurred on decreasing the expression of ACLY. Conversely, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) correlated with the mitochondrial FA oxidation (FAO), reduced the TG accumulation mediated by ACLY depletion [Migita et al., 2014].

1.11.4. Regulation in the expression of ACLY

It has been evidenced that several signaling molecules such as GSK3, Akt, insulin, SREBP regulated the activities of ACLY [Sato et al., 2000]. In addition, the elevation in the phosphorylation of ACLY triggers the stabilization of the protein *via* kinases such as PKA, Akt, GSK-3, and cAMP-dependent protein kinase [Pinkosky et al., 2017, Sanchez-Solana et al., 2014]. Moreover, stimulation of ACLY was observed in mouse model upon phosphorylation of ACLY at Thr446, Ser450, and Ser454 residues [Sanchez-Solana et al., 2014].

In addition, evidences have found that SREBP-1c regulated the expression of ACACA, FAS, ACLY, and acetyl-CoA synthetase 1 (ACS1) [Beigneux et al., 2004 , Sato et al., 2000, Bhalla et al., 2012]. SREBP is known to regulate the transcription of fatty acid and cholesterol synthesis [Shimano, 2002 , Bhalla et al., 2012]. Studies have found that ACLY inhibited the function of AMPK by interacting with the catalytic unit of AMPK. Additionally, knockdown of ACLY stimulate AMPK thereby inducing p53 activation necessary for cellular senescence [Lee et al., 2015]. Suppression of ACLY using SB-204990 or RNAi has shown to reduced tumor growth and proliferation and induced differentiation [Hatzivassiliou et al., 2005]. Further, phosphorylation of ACLY takes place at Ser454 *via* cAMP-dependent protein kinase and protein kinase B/Akt which led to the covalent activation of ACLY [Pinkosky et al., 2017, Ramakrishna et al., 1981]. Besides, the activation of ACLY through its phosphorylation at ser454 also occurs upon binding of insulin to the insulin receptor [Berwick et al., 2002]. Sanchez-Solana et al., has mapped 300 to 630 amino acid sequence of ACLY where the interaction of ACLY with MORC2 occurred [Sanchez-Solana et al., 2014]. In addition, MORC2 has been found to enhanced the enzymatic activity of ACLY by stimulating its phosphorylation at ser454 [Sanchez-Solana et al., 2014]. It has been evidenced that there is a rise in the branched-chain α-keto acid dehydrogenase kinase (BDK) in the liver with the increased level of ACLY and activated the process of DNL [White et al., 2018]. Studies have found α2-Macroglobulin (α2M*) to signal through tumor cell Surface GRP78 (CS-GRP78). The α2M*/CS-GRP78 axis activated the expression of ACLY via Akt signaling, and upregulated histone acetylation and synthesis of acetyl-CoA [Gopal and Pizzo, 2017]. It has been demonstrated that acetate also regulated the expression of ACLY through a feedback loop mechanism in an Akt-dependent manner

[Gopal and Pizzo, 2017]. Further studies have found that the deletion of ACLY in Kras-mutant acinar cells strongly reduced the acetylation of histone H4 [Carrer et al., 2019]. Additionally, suppression in the phosphorylation of ACLY at ser455 was observed on impeding the expression of mTORC2 through inhibition of IGF-1 [Chen et al., 2016]. Moreover, IGF-1 is known to activate the expression of ACLY that leads to the increase level of cardiolipin and also the activities of mitochondrial complex and supercomplex [Das et al., 2015, Li & Sartorelli , 2018].

The level and activity of the transcription factor ETV4 was found to be reduced in lack of ACLY. In addition, silencing of ETV4 has been found to prevent cells from hypoxia mediated apoptosis [Keenan et al., 2015]. It has been demonstrated that the simultaneous siRNA knockdown of both ACSS2 and ACLY in primary goat mammary epithelial cells decreased the mRNA expression of the important enzymes that take part in TAG synthesis such as diacylglycerol O-Acyltransferase (DGAT1), DGAT2, glycerol-3-phosphate acyltransferase 1 (GPAM), 1-acylglycerol-3-phosphate o-acyltransferase 6 (AGPAT6), FAS, ACACA, and SCD1 [Xu et al., 2018]. Furthermore, cells with higher levels of ACLY showed upregulated expression of snail (stimulate epithelial-mesenchymal transition (EMT) and stemness) [Hanai et al., 2013]. Besides, studies have shown RIPK3 to activate fibrogenesis *via* stimulation of ACLY in Akt dependent manner [Imamura et al., 2018]. Additionally, Cullin3 (CUL3) (tumor suppressor) has been found to interrelate with ACLY *via* its adaptor protein, Kelch-like family member 25 promoting ubiquitination and degradation of ACLY in the cells [Zhang et al., 2016]. It has been demonstrated that the ACLY knockdown in rat insulinoma 832/13 cells reduced the cytosolic OAA and malonyl-CoA levels [Joseph et al., 2007].

In skeletal muscles, ACLY has been found to regulate the mitochondrial function along with glucose and lipid metabolism [Das et al., 2015, Li et al., 2018, Das et al., 2017]. It has been observed that the rise in the level of MYOD expression *via* ACLY induced alterations of H3(K9/14) and H3(K27) at the MYOD locus [Das et al., 2017]. Besides, ACLY promotes histone acetylation at the double-strand break sites and impeded localization of 53BP1 and recruited breast cancer type1 (BRCA1) [Sivanand et al., 2017]. Furthermore, decrease in the mRNA expression of ACLY was observed in PU.1 treated cultured myeloid cells [Rhee et al., 2019]. Steeg et al., has revealed that ACLY imparted to the antimetastatic effect of Nm23-H1 (first discovered metastasis suppressor gene) as a result of the histidine kinase activity of Nm23-H1 towards ACLY [Steeg et al., 2008].

1.11.5. Inhibition of ACLY

Several evidences have shown that increased expression of ACLY associates with tumorigenesis in various tumor types. Interestingly, the controlled expression of ACLY instigated inhibition in the proliferation and increased apoptosis in the tumor cells without being cytotoxic to noncancerous cells [Wang et al., 2010]. Wang et al., have demonstrated that the knockdown of hepatic ACLY downregulated the expression of acetyl-CoA and reduced the levels of TG and free fatty acids, irrespective of the dietary intake of fat [Wang et al., 2010 238]. The diminution of ACLY, the Drosophila ortholog of human ACLY caused chromosome breaks (CBs) which shows the partial requirement of ACLY in chromosomal stability in mitotic cells [Morciano et al., 2019 239]. Studies have shown molecular compounds such as BMS-303141, radicicol, bempedoic acid, metformin, 4–6 10,11-dehydrocurvularin (DCV), and hydroxycitrate (HCA) inhibited the expression of ACLY [Ki et al., 2000, Pietrocola et al., 2016, Deng

et al., 2019, Zagelbaum et al., 2019]. In addition, it has been investigated that DCV (a fungus derived natural-product macrolide) is a novel irreversible inhibitor of ACLY which showed potent anti-neoplastic effect [Deng et al., 2019]. For instance, the treatment of metastatic human cervical tumor cells with the anti-diabetic drug metformin and caffeic acid (CA, trans-3,4- dihydroxycinnamic acid) downregulated the expression of ACLY thereby, reducing the growth of the tumor cells [Tyszka-Czochara et al., 2017]. Further, bempedoic acid (or ETC-1002 (8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid) monotherapy or the combined treatment with ezetimibe, or statin/statin-intolerant hypercholesterolemic patients decreased the LDL-C in phase 2 clinical trials, indicating that ACLY could be a potent target for cardiovascular diseases [Bilen & Ballantyne, 2016, Burke & Huff, 2017]. Supporting this, Pinkosky et al. have also reported that bempedoic acid lowered the levels of LDL-C thereby, reducing the chances of atherosclerosis independent of AMPK [Lemus & Mendivil, 2015, Pinkosky et al., 2016].

It has been found that SB-204990 inhibited the expression of ACLY thereby, suppressing the biogenesis of fatty acids and cholesterol in both *in vitro* and *in vivo* studies [Pearce et al., 1998]. Another compound (3R,5S)-omega-substituted-3-carboxy-3, 5- dihydroxyalkanoic acid is known to suppress the recombinant human form of ACLY [Gribble et al., 1998]. *In vivo* studies have shown that the combined treatment of lipoic acid and HCA, along with cisplatin or methotrexate strongly inhibited ACLY and pyruvate dehydrogenase kinase [Guais et al., 2012]. Further, the inhibition of ACLY *via* shRNA-mediated ACLY silencing in several cell lines of different cancer types have been found to induced cell cycle arrest and apoptosis [Zaidi et al., 2012]. The levels of phosphocholine and total choline was found to be minimized

affecting the protein expression of choline kinase alpha, FAS, and phosphorylated ACLY upon treatment of human PTEN null PC3 prostate and PIK3CA mutant HCT116 colon carcinoma cells with PI-103, an isoform-selective class I PI3K and mTOR inhibitor [Al-Saffar et al., 2010]. The phosphorylation of ACLY was found to be regulated by mTORC2 thereby, instigating the synthesis of acetyl-CoA and DNL. These process was found to be reversed upon treatment with an mTORC1/mTORC2 kinase inhibitor (mTOR-KI) or cellular depletion of mTORC2 or ACLY. Moreover, mTOR-KI inhibited IGF-1-mediated phosphorylation of ACLY [Chen et al., 2016]. It has also been demonstrated that the administration of the anti-tumoral Rh(III) complex caused reduction in ACLY, ACACA, and FAS in the livers of rats with thioacetamide-induced tumors [Cascales et al., 1986].

The MicroRNAs or small non-coding RNAs played a critical role in the initiation and tumor progression of various cancer types [Duan et al., 2013]. MiRs are known to negatively regulate the expression of proteins thereby, suppressing the translation by binding to protein-coding mRNAs [Cheng et al., 2017]. Evidences have shown that miRs regulated the expression of ACLY [Pinweha et al., 2016]. It has been reported that ACLY reduced the level of DNA methyltransferase 1 (DNMT1) partially by stimulating miR-148a during adipocyte differentiation [Londono Gentile et al., 2013]. Further, lowered expression of miR-126 was observed in patients with malignant mesothelioma (MM). There was an inverse correlation of miR126 levels with insulin receptor substrate-1 (IRS1) and ACLY expression [Tomasetti et al., 2014]. In addition, miR-126 is known to downregulated in metastatic breast tumor thereby, impeding tumor development [Wang et al., 2008]. Also, miR-22 has been found to inhibit ACLY and fatty acid elongase 6 that leads to the suppression of FA synthesis and tumor cell

elongation [Koufaris et al., 2016]. Further studies have found that miR-22 downregulated the expression of ACLY and reduced the tumor proliferation, invasion and instigate apoptosis in various types of cancer such as lung, cervical, prostate and osteosarcoma [Xin et al., 2016]. Besides, a high level of ACLY was observed in lower levels of miR-22 which indicates that ACLY and miR-22 expression has a negative association between them [Xin et al., 2016]. Moreover, *in vivo* studies have shown downregulation in the ACLY expression in miR-22-treated mice that led to the development of reduced tumor type showing fewer chances of metastasis and equitably longer survival [Xin et al., 2016]. Interestingly, studies have found abnormally expressed miR-22 in the paired breast tumor tissues and adjacent non-tumor tissues. Liu et al. works showed that miR-22 reduced the ACLY expression thereby, inhibiting the proliferation and metastasis in tumor cells and regulated cell- differentiation [Liu et al., 2018]. Studies have also found significant reduction of miR-133b by 93.55% particularly in CRC tissues of metastatic tumors [Duan et al., 2013]. Another study has also found increased expression of ACLY along with decreased miR-133b and nuclear distribution of PPARγ in human gastric cancer tissues and cell lines. Furthermore, high miR-133b reduced the transcriptional activity of ACLY in a PPARγ-dependent manner [Cheng et al., 2017]. It has been demonstrated that miR-182 downregulated PDH kinase 4 (PDK4) expression thereby, instigating lung tumorigenesis. The silencing of ACLY reduced the effect of miR-182-PDK4 in tumor growth [Li et al., 2017]. Overall, ACLY inhibition might be helpful in exploring different treatment strategies for various chronic diseases.

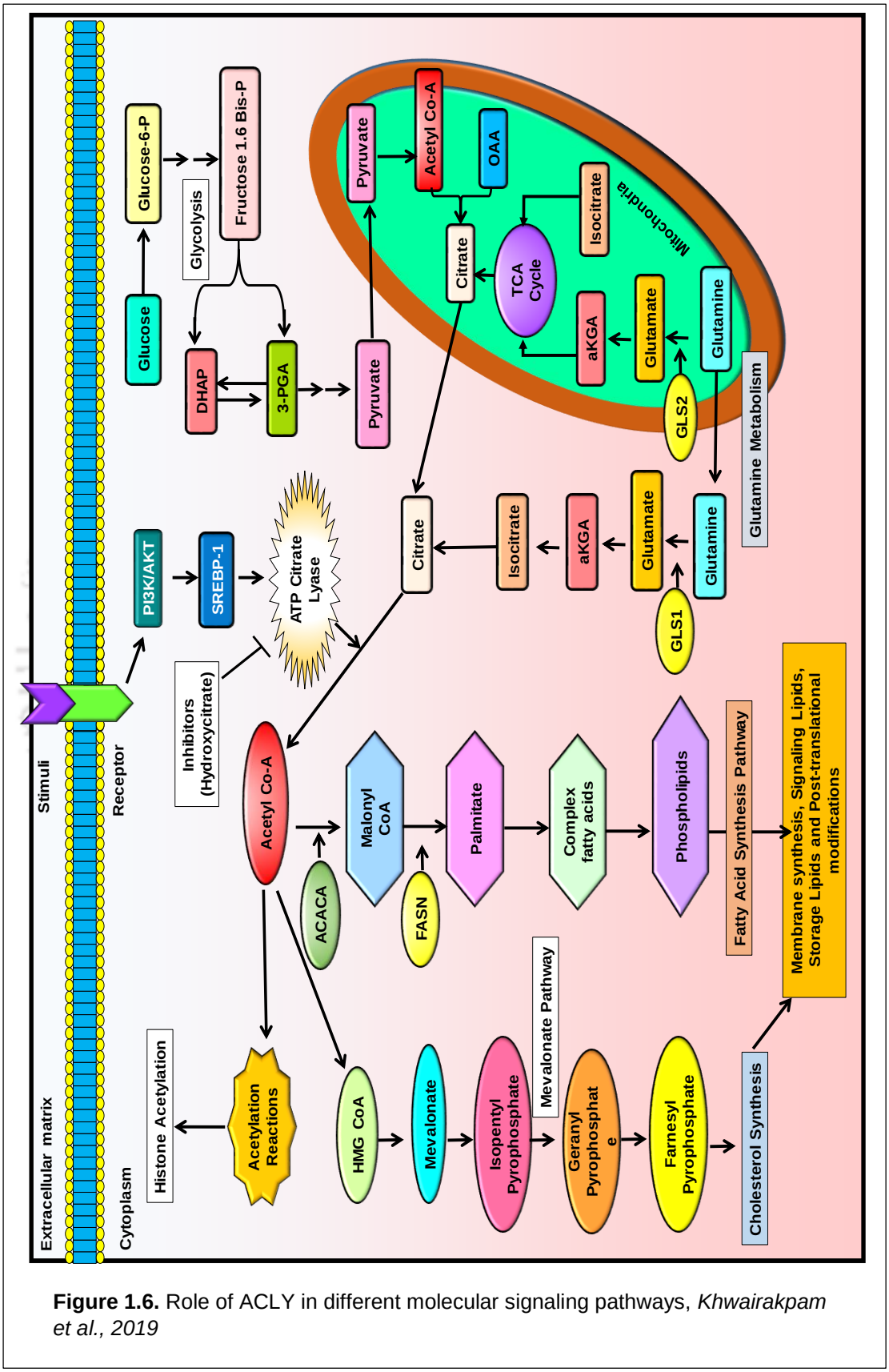


Figure 1.6. Role of ACLY in different molecular signaling pathways, *Khwairakpam et al., 2019*

1.12.ATP citrate lyase and cancer

Globally, cancer is the second foremost cause of death 1 in 6 deaths occur worldwide due to cancer [WHO, Jung et al., 2018]. Studies have found that cancer is accountable for an estimated 9.6 million deaths in 2018 [WHO]. Metabolic alternations and abnormal regulation of multiple signaling pathways have been noted in tumor cells [Bauer et al.. 2005, Sethi et al., 2014]. Some of the metabolic pathways such as glycolysis and lipogenesis are critical for cancer growth and maintenance [Khwairakpam et al., 2019]. It has been reported that alterations in DNL led to dysregulation of enzymes involved in fatty acid synthesis as the increasingly proliferating cells needed a high amount of energy required for the membrane synthesis and cell metabolism [Szutowicz et al., 1979, Wang et al., 2017, Jung et al., 2018]. Moreover, upregulation in the expression of ACLY has been known to linked with the rise in lipid synthesis and tumor growth in various types of human cancer [Rajendran et al., 2011, Jung et al., 2018, Zagelbaum et al., 2019]. It has been demonstrated that the stable knockdown of ACLY diminished the glucose dependent lipid synthesis and augmented the mitochondrial membrane potential. It also triggered a reduction of cytokine-stimulated cell proliferation [Qian et al., 2015]. The reduction in ACLY expression both in the protein and mRNA level has been found to reduce cell viability and inhibited tumor growth, invasion, and metastasis, thereby inducing apoptosis in different cancer types [Szutowicz et al., 1979, Migita et al., 2013, Sanchez-Solana et al., 2014, Volinsky et al., 2015, Ozkaya et al., 2015]. The activities of ACLY in various cancer types are menstioned as follows.

1.12.1. Breast cancer

Breast cancer is the second widespread disease among women and accounts for nearly 30% of female cancers [Siegel et al., 2020]. High expression of ACLY was observed in human breast carcinoma tissue samples as compared to normal tissues [Migita et al., 2013, Volinsky et al., 2015]. The introduction of insulin in breast cancer cells has been found to increase accretion of lipid synthesised due to the actvation of ACLY [Zhou et al., 2013]. The increased expression of ACLY has been found to increase the snail expression thereby, improving the association between ACLY and snail that instigated cancer growth and cancer stemness in HMLER breast cancer cells [Hatzivassiliou et al., 2005]. In addition, the silencing of endogenously expressed ACLY in MCF-7 cells *via* siRNA suppressed the tumor cell viability and induced apoptosis [Volinsky et al., 2005]. It has been revealed that hyperphosphorylation of ACLY occurred in human breast cancer. Upregulation of DNL enhanced the synthesis of acetyl-CoA due to the phosphorylation of ACLY at ser455 stimulated by mTORC2 in HER2+/PIK3CAmut cells [Chypre et al., 2012]. Besides, inhibition in cancer growth in breast cancer cells was observed on reduction of ACLY level that leads to mitochondrial hyperpolarization [Chypre et al., 2012]. In addition, inhibition of ACLY induce reduction of the cytosol citrate level thereby, reducing the proliferation of breast tumor cells [Tomaszewicz et al., 2003].

1.12.2. Colon cancer

Colorectal cancer (CRC) is the third foremost cause of cancer death in the world [Rawla et al., 2019]. Xie et al. has reported ACLY as a significant therapeutic target

in colon cancer [Xie et al., 2015]. The expression of ACLY was found to be augmented in CRC cells as well as chemoresistant CRC cells when compared to normal cells and chemo-naïve cells of CRC [Zhou et al., 2013]. The exogenous upregulation in the expression of ACLY has been found to instigate chemoresistance of CRC cells to SN38. However, knockdown of ACLY sensitized the CRC cells again to SN38 [Board et al., 1996]. Studies have revealed that SNPs which occurs in the ACLY gene function as an independent prognostic marker for the advanced stage CRC patients. Additionally, the increased expression of ACLY and FAS was found to be associated with progressive stage of CRC and liver metastasis [Schwartz et al., 2010]. Moreover, *in vivo* studies have shown that suppression of FAS induced relapse of cancer growth and inhibited hepatic metastasis and development of secondary metastasis. The lowered expression of the lipogenic enzymes downregulated the expression of important molecules such as the tyrosine kinase receptor (MET), Akt, focal adhesion kinase, and paxillin that stimulates adhesion, migration, and invasion of cancerous cells [Schwartz et al., 2010].

1.12.3. Bladder cancer

Bladder cancer or urological cancer is the 10th most prevalent cancer in the world. There are reports that the incidence rate of bladder cancer have been increasing progressively [Saginala et al., 2020]. Turyn et al. has reported in bladder cancer that the activity of GAPDH subsidizes towards the source of glycerol 3-phosphate for lipid biosynthesis either directly through FAS or indirectly *via* ACLY [Turyn et al., 2003]. Moreover, the combination study of alpha lipoic acid and calcium

hydroxycitrate has been found to induce cellular cytotoxicity to the tumor cells however, unaffected to normal cells, thereby instigating regression of tumor growth and survival of bladder cancer *in vivo* conditions [Schwartz et al., 2010]. In addition, the reduced expression of ACLY demonstrated anti-tumorogenic effects by enhancing the intracellular ROS and phosphorylation of AMPK in both *in vitro* and *in vivo* conditions [Xie et al., 2015]. It has been revealed that the diminuition of ACLY led to reduction in the level of intracellular ROS and phosphorylation of AMPK thereby, exerting anticancer effects against bladder cancer [Xie et al., 2015].

1.12.4. Gastric cancer

Gastric cancer is a deadliest disease and ranks fifth in the world. Nearly 1,033,701 new cases and 782,685 deaths takes place 2018 worldwide due to gastric cancer [Hamashima, 2020, Ozkaya et al., 2015]. Studies have revealed that around 90% of all gastric cancer are neoplastic, and 95% of it comprises gastric adenocarcinoma [Ozkaya et al., 2015]. The Immunohistochemical analysis of patient samples showed remarkably increased ACLY expression in the gastric adenocarcinoma as compared to the nearby normal tissues [Ozkaya et al., 2015]. These elevated expression of ACLY is associated with the advanced stage of the disease, lymph node metastasis, and reduction of the survival time [Ozkaya et al., 2015]. Studies have shown that augmentation in the expression of miR-133b inhibited the expression of ACLY and decreases the cancer cell proliferation and invasion [Cheng et al., 2017, Carrer et al., 2019]. These inhibition of ACLY has been found to induce elevated expression PPARγ [Cheng et al., 2017, Carrer et al., 2019].

1.12.5. Brain cancer

In glioblastoma cells, the rate of glycolysis is found to be higher than the normal brain cells thereby the level of ACLY is also elevated [Beckner et al., 2010]. Studies have revealed that the upregulated expression of ACLY and enolase 1 in the pseudopodia of glioblastoma cells. Moreover, suppression of ACLY using HCA lessen the tumor cell migration, clonogenicity, and invasion in glycolytic conditions [Icard and Lincet, 2016]. It has been found that in glioblastoma, the synthesis of acetyl-coA instigates migration and adhesion of the cancerous cells to the extracellular matrix. The acetyl-CoA-dependent gene regulation and cell adhesion occurred through modulations of Ca^{2+}signals mediated *via* nuclear factor of activated T cells-1 transcription factor [Sanchez-Solana, 2014]. It has been demonstrated that administration of citrate or an ACLY inhibitor, inhibited Mcl-1 expression and also reversed the cell dedifferentiation and improved sensitivity of the tumor cells to cisplatin [Szutowicz et al., 1983]. Moreover, it has been investigated that dibutyryl cyclic AMP and butyrate inhibited the growth of neuroblastoma cells by upregulating the activity of choline acetyltransferase and ACLY [Tajima et al., 2016]. It has also been demonstrated that Antizyme (AZ) enhanced the activity of ACLY and knockdown of it significantly reduces the ACLY activity and levels of acetyl-CoA and cholesterol [Yahagi et al., 2005].

1.12.6. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) ranks fourth in cancer-related deaths in the world and generally, accounts for 90% of primary liver cancer [Kim and Viatour, 2020]. Increased mRNA levels of FAS, ACACA, and ACLY was observed in the samples

of HCC patients who had undergone surgical resection [Wu et al., 2015]. It has been reported that SNP rs9912300 in the ACLY gene have been found to be associated with the overall survival of HCC patients with a lower alpha-fetoprotein (AFP) level [Li et al., 2017]. Upserge in the enzymatic activity of ACLY with the rise in the expression of LMW-E leads to the formation of lipid droplet crucial for tumor cell growth and proliferation [Lee et al., 2014]. Moreover, it has been demonstrated epigallocatechin gallate and epicatechin inhibited the expression of ACLY and caused activation of CPT-1 thereby inducing cell death in HCC cells [Szutowicz et al., 1979]. It has been revealed that knockdown of SLC13A5 reduced the intracellular levels of ACLY along with citrate, the ATP/ADP ratio and phospholipid content [Lligona-Trulla et al., 1997]. Furthermore, the diminution of SLC13A5 has been found to activate AMPK, thereby reducing the expression of oncogenic mTOR signaling pathway [Lligona-Trulla et al., 1997]. Experimental studies have found that hydroxycitrate upregulated the alteration of acetyl-L-carnitine to lipid and dramatically reduced the flux of glucose to lipid [Berkhout et al., 1990]. Additionally, the treatment of hydroxycitrate on HCC cells suppressed the activity of ACLY and caused reduction in the synthesis of cholesterol by 27% [Yuan et al., 2009]. Besides, decrease in the ACLY expression and few other enzymes that takes part in lipid metabolism occurred on treatment with tetrazanbigen [Csanadi et al., 2015].

1.12.7. Lung cancer

Lung cancer has been reported as the leading cause of cancer incidence and mortality [Bray, 2018]. Investigations have shown that the expression of ACLY correlated with the local tumor stage of NSCLC [Hanai et al., 2012]. It has been

reported that upregulation in the expression of ACLY and malic enzyme linked with the poorer survival rate in older patients as compared to young patients with NSCLC [Hanai et al., 2012]. Moreover, clinical studies in patients with NSCLC showed a significant correlation of the increased expression of ACLY or GLUT1 with poor prognosis in node-negative patients. Also, the expression of ACLY and GLUT1 has been identified as an independent prognostic factor for the overall survival of node-negative NSCLC patients [Bauer et al., 2005]. In lung adenocarcinoma cells, ACLY knockdown *via* RNA interference has been found to induce growth arrest and decrease tumor proliferation through alteration of PI3K-AKT signaling pathway in both *in vitro* and *in vivo* conditions [Li et al., 2007]. Another *in vitro* study, also showed reduction in the activation of PI3K/AKT that resulted in the suppression of tumor cell proliferation and survival. In addition, *in vivo* studies showed differentiation and apoptosis of tumor cells and also inhibition in the growth of the tumor cells [Sun et al., 2015]. Further, reduction in the expression of ACLY along with the decrease expression of VEGF and MMP-2 was observed upon knockout of pyruvate kinase M2 (PKM2) [Deng et al., 2019]. Notably, truncated CUL3 expression correlated with an augmented expression of ACLY and poor prognosis in human lung cancer. Additionally, treatment with SB-204990, an ACLY inhibitor, excluded the stimulating effect of CUL3 significantly that leads to decrease in the FA synthesis, tumor growth and proliferation [Rajendran et al., 2011]. Furthemore, the SNP rs9912300 in ACLY gene was found to be correlated with the increase death risk in patients with lung cancer [Zagelbaum et al., 2019].

1.12.8. Prostate cancer

Prostate cancer is known to be one of the main cause of cancer-related deaths in males [Gao et al., 2014]. Studies have found elevated *de novo* fatty acid synthesis as a targetable aspect of prostate cancer in human [Singh et al., 2018]. An inhibition in apoptosis was observed in prostate tumor cells with the increased expression of lipogenic enzymes, SREBP1-c, and Glut-1 [Misra and Pizzo, 2015]. Experimental studies have showed that the occurrence of huge amount of triacylglycerols, lesser citrate, and acidic mucins related with the increased ACLY activity in prostate adenocarcinoma [Shah et al., 2016]. Bertilsson et al., have reported varied expression of ACLY with alterations in the citrate levels in prostate cancer patients [Halliday et al., 1988]. Moreover, PPARγ has been found to activate tumor metastasis *via* rise in ACLY expression and othe lipogenic enzymes such as FAS and ACACA [Bertilsson et al., 2012]. The combined action of AR antagonist, activation of AMPK and inhibition of ACLY has been found suppressed the AR levels thereby, inhibiting growth and proliferation and also induced apoptosis in castration-resistant prostate cancer (CRPC) cells [Ahmad et al., 2016]. A positive correlation of the AR mRNA levels and the expression of ACLY and metabolism of fatty acid was observed [Ahmad et al., 2016]. Both *in vitro* and *in vivo* studies have found that cucurbitacin B (CuB) inhibited the phosphorylation of ACLY [Gao et al., 2014]. Furthermore, treatment with PI-103 (PI3K inhibitor) also reduced the phosphorylation of ACLY (Ser454) in PC3 cells [Wagner et al., 1997].

1.12.9. Ovarian cancer

Ovarian cancer is the most prevalent cause of gynaecological cancer-associated death [Gordon Jayson, 2014]. Upregulated expression of phosphorylated ACLY has been observed in tissues of ovarian cancer [Wang et al., 2012,]. Wang et al. studies have shown that the increment of the mRNA expression of ACLY to 3.7 fold in tissues of ovarian cancer as compared to normal [Wang et al., 2012]. Evidences have found specific deubiquitination of ACLY *via* ubiquitin-specific peptidase13 (USP13) which leads to the increased expression of ACLY [Al-Saffaret al., 2010. Moreover, reduction of USP13 suppressed the progression of tumor significantly and sensitized the ovarian tumor cells to PI3K/AKT inhibitors [Al-Saffar et al., 2010]. It has also been demonstrated that knockdown of ACLY inhibited tumor proliferation and instigate cell cycle arrest in ovarian tumor cells [Wang et al., 2012].

1.13.Importance of the study

The 5-years survival rate of patients with OSCC are still lesser then 60%. Moreover, tumor recurrence and metastasis of oral cancer are observed in patients with OSCC. Studies have shown that fatty acids played a crucial role in many cellular processes of the body. Glucose is mainly utilized by the tumor cells for the synthesis of important cellular modules such as nucleotides, aminoacids, and fatty acids. Large amount of energy required for cancer cell proliferation is found to be compensated by the increased glycolysis and lipogenesis. Here, ACLY is a positive regulator of both. It is an important enzyme involved in fatty acid biosynthesis, plays a major role in promotion of tumor growth in different types of cancer. Remarkably high ACLY expression and activity was observed in cancer cells. However, the role of ACLY in

oral cancer is still unknown. Being an enzyme that catalyzes the first step of *de novo* lipogenesis, targeting ACLY might help in the reduction of tumor growth of the oral cavity. Therefore, understanding the role of ACLY in oral carcinogenesis might prove significant for the early detection and prevention of oral malignancy. Having found ACLY to play a great role in tumorigenesis thus signifies its possibility to behave as a significant biomarker in oral cancer detection.

1.14. Objectives

I. To determine the expression of ACLY in normal epithelium as well as in different stages of OSCC in human tissues and oral cancer cell lines.

II. To examine the effect of tobacco extract and various components of tobacco on the expression of ACLY in OSCC.

III. To determine the role of ACLY in different processes involved in the development of OSCC.

Chapter 2

Differential expression of ACLY in oral cancer

CHAPTER 2

2.1. Introduction

Aforementioned, oral cancer is the deadliest disease which is a form of Head and Neck cancers known to be the most prevalent cancer in the Northeast region of India. It is well established that the growth and development of oral cancer is a multistep process which includes several genetic alterations controlled by genetic susceptibility and environmental influence [Pérez-Sayáns et al., 2009]. Moreover, this disease is usually diagnosed at a late stage and medications are quite expensive and ineffective [Mignogna et al., 2004]. As discussed in the previous chapter, dysregulation in *de novo* lipogenesis occurred in tumorigenesis leading to increment in fatty acid production in order to meet the growing ultimatum of fatty acids needed for membrane formation by the actively proliferating tumor cells [Khwairakpam et al., 2015, Khwairakpam et al., 2019]. The inhibition of ACLY in turn suppressed the proliferation of tumor cells *via* induction of apoptosis and cell cycle arrest indicating its crucial role in tumor cell progression [Zaidi et al., 2012]. In addition, tumor cell migration and invasion were also attenuated in ACLY-deficient colon tumor cells [Wen et al., 2019]. Hatzivassiliou et al. have demonstrated that the inhibition of ACLY in human lung adenocarcinoma reduced the tumor cell proliferation and survival *in vitro*. In addition, he also showed that reduction in the expression of ACLY suppressed the tumor growth and induce differentiation *in vivo* conditions [Hatzivassiliou et al., 2005]. More importantly, a detailed review of literature has found increased expression of ACLY in various tumors such as breast cancer, bladder cancer, brain cancer, colon cancer, liver, prostate, ovarian cancer etc. [Khwairakpam et al., 2015]. However, studies on the expression of ACLY in oral cancer has not been examined till now. Accordingly, we hypothesized that the ACLY proteins might play a crucial function in the initiation and progression of the cancer of the oral cavity. Therefore, in order to evidence the above mentioned

hypothesis, we primarily studied the genetic alteration of ACLY in head and neck carcinoma. 530 samples and another 279 samples assimilated from the open data portal of The Cancer Genome Atlas dataset (TCGA) [Cerami et al., 2012, Gao et al., 2013] and cBioPortal platform (http://www.cbioportal.org). The Kaplan-Meier survival curve was generated for analyzing ACLY expression on prognostic of head and neck cancer patient. Then, we investigated the expression of ACLY in normal human cell line and OSCC cell lines using western blot and reverse transcriptase PCR. Moreover, immunohistochemical analysis was used in order to analyze the differential expression of ACLY in oral cancer tissues as well as normal tissues by utilizing oral cancer tissue micro array (TMA).

2.2. Materials and Methods

2.2.1. Genetic alteration of ACLY in head and neck carcinoma analyzed in The Cancer Genome Atlas dataset (TCGA)

The genetic alteration of ACLY was studied in head and neck carcinoma. 530 samples and another 279 samples were obtained from the open data portal of TCGA and cBioPortal platform [Cerami et al., 2012, Gao et al., 2013] (http://www.cbioportal.org). The Kaplan-Meier survival curve was generated for analyzing ACLY expression on prognostic of head and neck cancer patient. On the website select www.cbioportal.org, "Query" option, then select "Head and Neck Carcinoma (TCGA, Nature 2015)" firstly for analysis and then "Head and Neck Carcinoma (TCGA, Provisional)" secondly. Both studies were done separately. Then, in "Select Genomic Profiles", select the options "Mutations, Putative copy-number alterations and mRNA expression Z-scores". In "Enter Gene set", type "ACLY", then click "Submit Query". The "Oncoprint" tab will appear. Next, click "Mutation" tab to detect the protein change and mutation type.

Lastly, click the "Survival" tab, then the overall survival Kaplan-Meier Estimate and the disease free survival appeared.

2.2.2. Cell culture

The OSCC cell lines such as SCC-9, HSC-3, SAS, (tongue carcinoma), T.Tn (an esophageal carcinoma cell line) and HaCaT (immortalized human keratinocyte) cells were utilized for our studies. The HaCaT and SCC-9 cell lines were presented by Dr. V.G.M. Naidu from NIPER Guwahati, India. The SAS cell line was procured from Rajiv Gandhi University of Biotechnology (RGCB) Trivandrum, India. While the T.Tn and HSC-3 cell lines were contributed by Dr. Renu Wadhwa from AIST, Japan. The cell lines HSC-3 and SCC-9 were cultured in DMEM/F12 and MEM medium respectively. Further, the HaCaT and SAS cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), and the T.Tn cells at 45% DMEM with 45% Ham's F12 medium with 10% fetal calf serum, at 37 °C in a humidified atmosphere with 5% CO_2. The different cells types were sub-cultured with 0.25% trypsin-EDTA after washing with 1× PBS at pH 7.5 in order to disassociate the cells. The cells commonly in the logarithmic phase of growth were used for various experimental studies.

2.2.3. RNA preparation and reverse transcription-PCR

The comparative mRNA expression of ACLY on SAS and HaCaT cell lines was studied using reverse transcription-polymerase chain reaction (RT-PCR). The cells were seeded in culture plates with a concentration of $5x10^5$ cells per well for 24hr. The cell in logarithmic phase of growth were taken for the RNA extraction and the isolation of RNA was done by using TRI Reagent® (SIGMA® Life Science) according to manufacturer's protocol. The total RNA was utilized for synthesis of first-strand cDNA utilizing a high-capacity cDNA Reverse Transcriptase kit (Cat No. 4368814, Applied

Biosystems™, USA). The amplification of the gene expression of ACLY was done in 35 cycles of PCR using exact primers. Here, 1µL of cDNA was used as template, and 2x Hot Start Taq Master Mix (Cat No. M0496L) obtained from New England Biolabs® (NEB, USA). Further, the obtained PCR products or the amplicons were resolved in 1% agarose gel electrophoresis and the band intensity was determined by Image lab software. 100 bp DNA Ladder (Cat No. N3231S, NEB, USA) was run as a standard. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal gene control. Gene-specific primer was obtained from Integrated DNA Technologies (IDT ®) (Table 2.1). The PCR was carried out in 96-well plates using the Veriti (Applied Bioscience).

Table 2.1. Primer sequence for reverse transcriptase PCR

Primer	Sequence	Product size	Annealing temp
ACLY-F	5′ -TACGGGTGATGGGAGAAGTC- 3′	413bp	54^0C
ACLY-R	5′ -CCCAGTGAAAGGGTAGACCA- 3′		
GAPDH-F	5'- AGG TCG GAG TCA ACG GAT TTG -3'	532bp	60^0C
GAPDH-R	5'- GTG ATG GCA TGG ACT GTG GT -3'		

2.2.4. Western blot analysis

The expression of ACLY was examined in different oral cancer cell lines versus normal cell line using western blot analysis. Here, we seeded $5x10^5$ cells of each cell type in 35mm sterile petridish and incubated for 24h for isolation of proteins. Then, the protein lysate was isolated using lysis buffer (20 mM HEPES buffer, 2 mM EDTA, 0.1% (v/v) Triton-X100, 2 µg/mL Aprotinin, 250 mM NaCl and 1 mM PMSF, 1 mM DTT, and 2 µg/mL Leupeptin hemisulfate). Bovine serum albumin (BSA) was used as the standard for estimation of protein and the concentration of the isolated protein was quantified

using Bradford's reagent. Then, equal amount of protein (20µg) was loaded to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using polyacrylamide gel with 5x Laemmli Buffer (5% β-mercaptoethanol, 250 mM Tris-HCl, 30% Glycerol, 0.02% Bromophenol blue and 10% SDS) at a voltage of 90V. Further, the resolved proteins were relocated to a nitrocellulose membrane (NCM) using a semi-dry transfer cell (Trans-blot Turbo, Bio-Rad) and Ponceau-S stain (HiMedia) was used to visualized the transferred proteins. 5% non-fat skimmed milk/BSA was used to block the non-specific binding sites of the proteins. The NCM membranes were incubated with primary antibodies at 4°C overnight. Subsequently, the blots were washed with 1x Tris-buffered saline (TBS) and Polysorbate 20 (Tween 20) (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2hr at room temperature. Lastly, the blots were established with opti-ECL kit (BIO-RAD), and the ChemiDoc™ XRS System (Bio-Rad, California, USA) was used to scanned the protein bands. The housekeeping gene GAPDH was utilized as a loading control. Table 2.2 summarizes the primary and secondary antibody details utilized for the analysis.

Table 2.2. Details of the primary and secondary antibodies used for Western blot

Protein	**Cat.no**	**Company**	**Dilutions used**
ACLY	13390BC	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Phospho-ACLY (T447+S451)	Ab53007	Cell Signalling Technologies, Danvers, MA, USA	1:2000
GAPDH	2118S	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Secondary Anti-rabbit	Ab97023	Abcam	1:12

2.2.5. Tissue micro array (TMA):

For our study, oral cavity disease spectrum (oral cavity carcinoma progression) tissue microarray (TMA) that consists of paraffin-embedded normal, preneoplastic and neoplastic oral cavity tissues (US Biomax, Inc., Cat. No. OR802) have been used. The TMA slide comprises of 80 tissues from 79 different individuals (table2.3) and each tissue core is 1.5 mm in diameter and 5 µm thick (figure 2.1). It consists of 28 cases of squamous cell carcinoma, 4 adenocarcinoma, 8 mucoepidermoid carcinoma, 2 basal cell carcinoma, 4 metastatic carcinoma, 8 adamantinoma, 6 hyperplasia, 5 each of adjacent tissue, inflammation, adjacent normal tissue and normal tissue, single core per case. In addition, 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). The tissue samples are of patients of different age groups and include both the sexes.

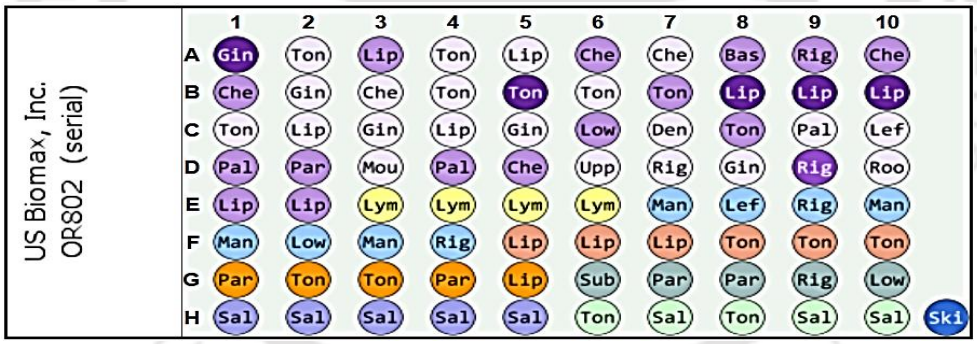


Figure 2.1. Microarray Panel Display, **Bas** - Base of tongue, **Che** - Cheek, **Den** - Dental alveoli, **Gin** - Gingiva, **Lef** - Left mandible, **Lip** - Lip, **Low** - Lower lip, **Lym** - Lymph node, **Man** - Mandible, **Mou** - Mouth floor, **Pal** - Palate, **Par** - Parotid gland, **Rig** - Right cheek, **Roo** - Root of tongue, **Sal** - Salivary gland, **Sub** - Submaxillary gland, **Ton** - Tongue, **Upp** - Upper lip , - AT, - Benign tumor, - Hyperplasia, - Inflammation, - Malignant tumor, - Malignant tumor (stage I), - Malignant tumor (stage II), - Malignant tumor (stage III), - Malignant tumor (stage IV), - Metastasis, - NAT, - Normal tissue

Table 2.3. Oral cavity disease spectrum (oral cavity cancer progression) tissue array with normal tissue, including TNM, clinical stage and pathology grade, 79 cases/80 cores

Position	Age	Sex	Organ	Pathology diagnosis	TNM	Grade	Stage	Type
A1	40	M	Gingiva	Squamous cell carcinoma	T4N0M0	1	IV	Malignant
A2	47	F	Tongue	Squamous cell carcinoma	T1N0M0	1	I	Malignant
A3	81	M	Lip	Squamous cell carcinoma	T2N0M0	1	II	Malignant
A4	57	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I	Malignant
A5	52	F	Lip	Squamous cell carcinoma	T1N0M0	2	I	Malignant
A6	53	M	Cheek	Squamous cell carcinoma	T2N0M0	1	II	Malignant
A7	62	F	Cheek	Squamous cell carcinoma	T1N0M0	1	I	Malignant
A8	48	M	Base of tongue	Squamous cell carcinoma (salivary gland)	T2N0M0	-	II	Malignant
A9	68	M	Right palate	Squamous cell carcinoma	T2N0M0	1	II	Malignant
A10	56	F	Cheek	Squamous cell carcinoma	T2N0M0	1	II	Malignant
B1	79	M	Cheek	Squamous cell carcinoma	T2N0M0	1	II	Malignant
B2	60	M	Gingiva	Squamous cell carcinoma	T1N0M0	1	I	Malignant
B3	55	M	Cheek	Squamous cell carcinoma	T1N0M0	1	I	Malignant
B4	66	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I	Malignant
B5	46	F	Tongue	Squamous cell carcinoma	T1N0M1	1	IV	Malignant
B6	39	F	Tongue	Squamous cell carcinoma	T1N0M0	1	I	Malignant
B7	78	M	Tongue	Squamous cell carcinoma	T2N0M0	1	II	Malignant

B8	78	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV	Malignant
B9	54	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV	Malignant
B10	75	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV	Malignant
C1	60	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I	Malignant
C2	73	M	Lip	Squamous cell carcinoma	T1N0M0	1	I	Malignant
C3	60	M	Gingiva	Squamous cell carcinoma	T1N0M0	2	I	Malignant
C4	78	M	Lip	Squamous cell carcinoma	T1N0M0	2	I	Malignant
C5	55	M	Gingiva	Squamous cell carcinoma	T1N0M0	2--3	I	Malignant
C6	47	M	Lower mandible	Squamous cell carcinoma	T2N0M0	3	II	Malignant
C7	41	M	Dental alveoli	Squamous cell carcinoma	T1N0M0	3	I	Malignant
C8	60	M	Tongue	Squamous cell carcinoma	T2N0M0	3	II	Malignant
C9	40	F	Palate	Adenoid cystic carcinoma	T1N0M0	-	I	Malignant
C10	45	M	Left lower mandible	Adenoid cystic carcinoma (sparse)	T1N0M0	-	I	Malignant
D1	64	M	Palate	Adenoid cystic carcinoma	T2N0M0	-	II	Malignant
D2	66	M	Parotid gland	Acinic cell carcinoma	T2N0M0	-	II	Malignant
D3	71	M	Mouth floor	Mucoepidermoid carcinoma	T1N0M0	1	I	Malignant
D4	57	M	Palate	Mucoepidermoid carcinoma	T2N0M0	1	II	Malignant
D5	50	F	Cheek	Mucoepidermoid carcinoma	T2N0M0	1	II	Malignant
D6	57	M	Upper lip	Mucoepidermoid carcinoma (skeletal muscle and blood vessel)	T1N0M0	-	I	Malignant
D7	48	F	Right lower mandible	Mucoepidermoid carcinoma	T1N0M0	2	I	Malignant
D8	55	M	Gingiva	Mucoepidermoid carcinoma	T1N0M0	3	I	Malignant
D9	60	M	Right lower mandible	Mucoepidermoid carcinoma	T3N0M0	3	III	Malignant
D10	50	M	Root of tongue	Mucoepidermoid carcinoma (sparse)	T1N0M0	-	I	Malignant
E1	79	F	Lip	Basal cell carcinoma (sparse)	T2N0M0	-	II	Malignant
E2	48	F	Lip	Basal cell carcinoma	T2N0M0	-	II	Malignant

E3	70	F	Lymph node	Metastatic squamous cell carcinoma of neck from cheek	-	2	-	Metastasis
E4	79	M	Lymph node	Metastatic squamous cell carcinoma of neck from tongue	-	1	-	Metastasis
E5	59	F	Lymph node	Metastatic squamous cell carcinoma of neck from mandible	-	2	-	Metastasis
E6	40	F	Lymph node	Metastatic mucoepidermoid carcinoma of neck from mandible	-	3	-	Metastasis
E7	11	M	Mandible	Adamantinoma	-	-	-	Benign
E8	28	M	Left mandible	Adamantinoma	-	-	-	Benign
E9	51	M	Right mandible	Adamantinoma	-	-	-	Benign
E10	64	M	Mandible	Adamantinoma (fibrous tissue and blood vessel)	-	-	-	Benign
F1	37	F	Mandible	Adamantinoma	-	-	-	Benign
F2	40	M	Lower mandible	Adamantinoma	-	-	-	Benign
F3	47	F	Mandible	Adamantinoma	-	-	-	Benign
F4	70	F	Right jaw bones	Adamantinoma	-	-	-	Benign
F5	67	M	Lip	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F6	40	M	Lip	Mild atypical hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F7	82	M	Lip	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F8	46	M	Tongue	Hyperplasia of squamous epithelium (skeletal muscle and blood vessel)	-	-	-	Hyperplasia
F9	60	F	Tongue	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
F10	3	F	Tongue	Hyperplasia of squamous epithelium	-	-	-	Hyperplasia
G1	68	M	Parotid gland	Cancer adjacent tissue (with squamous cell carcioma sparse)	-	-	-	AT
G2	53	F	Tongue	Cancer adjacent tissue	-	-	-	AT
G3	53	M	Tongue	Cancer adjacent tissue (hyperplasia of squamous epithelium)	-	-	-	AT
G4	70	M	Parotid gland	Cancer adjacent tissue (with mucoepidermoid carcinoma)	-	2	-	AT
G5	63	M	Lip	Cancer adjacent tissue (chronic inflammation of fibrous tissue and blood vessel)	-	-	-	AT
G6	43	M	Submaxillary gland	Chronic submaxillaritis	-	-	-	Inflammation
G7	23	F	Parotid gland	Chronic parotitis	-	-	-	Inflammation
G8	66	F	Parotid gland	Chronic parotitis	-	-	-	Inflammation

G9	40	F	Right cheek	Chronic inflammation of mucosa	-	-	-	Inflammation
G10	75	F	Lower lip	Chronic inflammation of mucosa of No. 20	-	-	-	Inflammation
H1	48	F	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H2	48	F	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H3	37	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H4	63	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H5	56	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-	NAT
H6	42	F	Tongue	Normal tongue tissue	-	-	-	Normal
H7	38	F	Salivary gland	Normal salivary gland tissue	-	-	-	Normal
H8	48	M	Tongue	Normal tongue tissue	-	-	-	Normal
H9	50	M	Salivary gland	Normal salivary gland tissue	-	-	-	Normal
H10	22	M	Salivary gland	Normal salivary gland tissue	-	-	-	Normal

2.2.2. Immunohistochemical Analysis:

It is well known that immunohistochemistry (IHC) is a widely accepted method equally in the clinical and experimental areas of medical science [Fedchenko et al., 2014]. In our study, immunostaining of the TMA slide was done using the Histostain-Plus Broad Spectrum (Invitrogen, Cat. No. 859043) and Metal enhanced DAB Substrate Kit (Invitrogen, Cat No. 34065). Moreover, IHC was accomplished as per the manufacturer's protocol starting with removing the paraffin wax from the tissue (deparaffinization), rehydration, peroxidase quenching, blocking, primary antibody incubation, secondary antibody-peroxidase conjugate incubation, addition of DAB chromogen and counterstaining with hematoxylin. Then, the slide was dehydrated and mounted with coverslip using D.P.X. mountant. Antibodies such as ATP- Citrate Lyase (D1X6P) (Cat. no. 13390BC) and Phospho- ATP- Citrate Lyase (Cat. no. 4331BC)

obtained from Cell Signaling Technologies and used in the dilutions of 1:50. Finally, analysis of the TMA was done using Nikon YS100 Upright microscope.

2.2.3. Scoring and Statistical analysis:

The brown colored stained tissues are measured as positive showing the occurrence of antigen of interest and according to the staining intensity and the number of positive cells a score is given. Scoring of the percentage of positive cells was marked from 0 to 4+ while the staining intensity was measured from 1 to 3 as given in table 2.4 [McDonald et al., 1999]. The statistical analysis was performed using simple Student's t-test and p-value ≤ 0.05 was considered as statistically significant.

Table 2.4. 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 score Q= P*I	
Intensity of Staining	weak staining	moderate staining	strong staining		

2.3. Results and Discussion

2.3.1. Genetic alteration of ACLY studied in the open data portal of The Cancer Genome Atlas dataset (TCGA)

Here, a study has been made in 279 patient samples from TCGA samples, Nature 2015. Alterations in ACLY was found in 13 (5%) of 279 patients. However, no mutation was observed in this case. The Kaplan-Meier survival curve (figure2.2) showed that patients with alteration in ACLY had lesser median month survival than the patients without alterations in ACLY gene (Table 2.5).

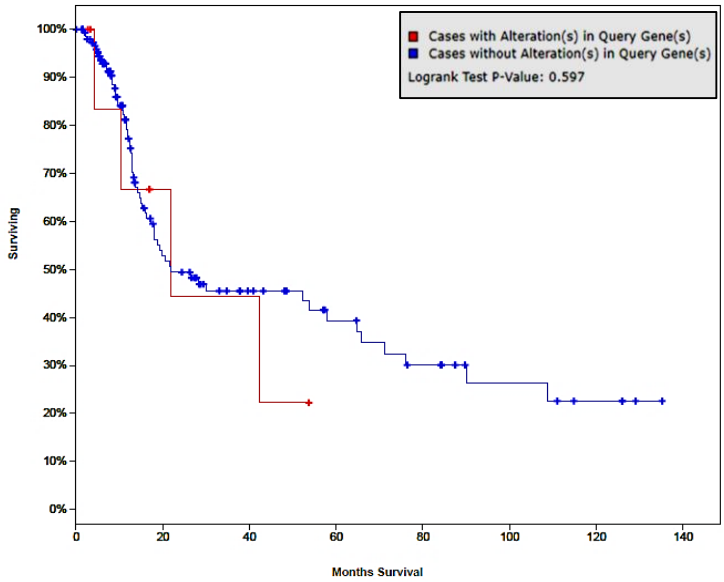


Figure 2.2. Kaplan-Meier survival curve of Head and Neck Carcinoma (TCGA, Nature 2015)

Table 2.5. Median month survival of Head and Neck Carcinoma (TCGA, Nature 2015)

	Total cases	**Cases deceased**	**Median months Survival**
Cases with Alteration(s) in Query Gene(s)	8	4	21.75
Cases without Alteration(s) in Query Gene(s)	151	65	21.85

Similarly, another study was also done in 530 samples from Head and Neck Carcinoma (TCGA, Provisional). Here, alterations in ACLY was found in 25 (5%) of the 528 patients. In these, three types of missense mutation P480R near the CoA binding domain, qwG665S and E718Q in the CoA ligase domain and also one type of splice mutation X798_splice near the CoA ligase domain was observed on ACLY protein. P480R mutation has been reported in mucopolysaccharidosis type II (MPS II) patients, a disease due to disorder in lysosomal storage [Emma et al., 2005]. Here, we found that

HNSCC patients having alterations in the ACLY gene showed less median month survival rate as compared to the HNSCC patients without alterations (Table 2.6).

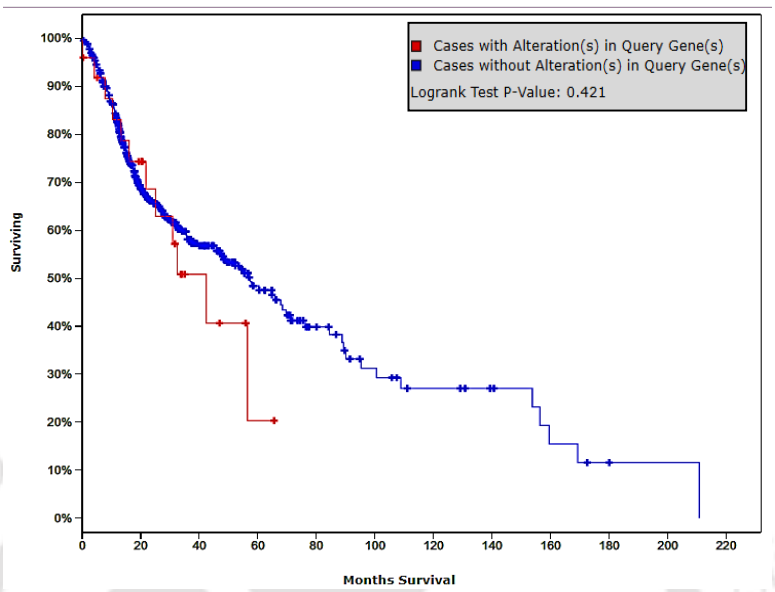


Figure 2.3. Kaplan-Meier survival curve of Head and Neck Carcinoma (TCGA, Provisional)

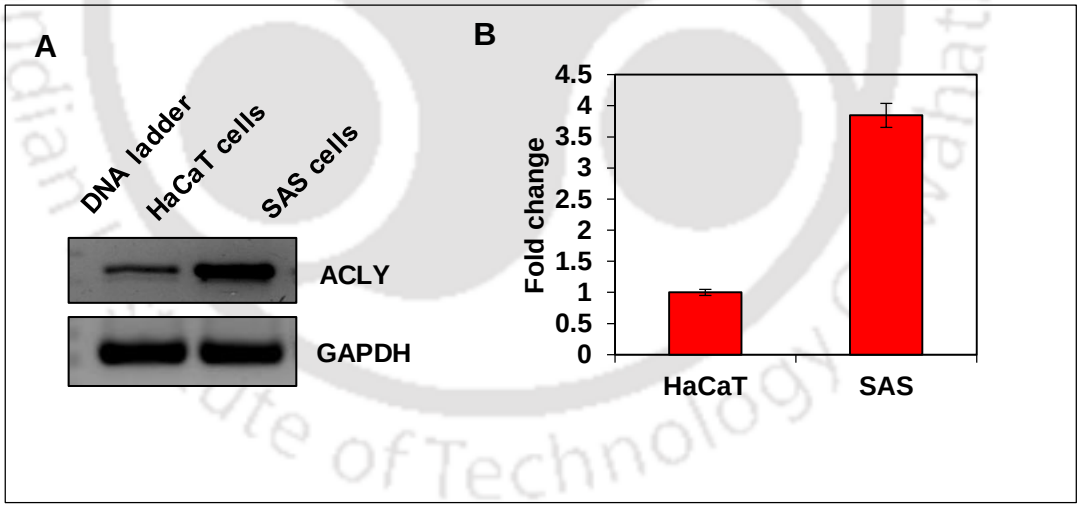
Table 2.6. Median month survival of Head and Neck Carcinoma (TCGA, Provisional)

	Total cases	Cases deceased	Median months Survival
1. Cases with Alteration(s) in Query Gene(s)	25	12	42.35
2. Cases without Alteration(s) in Query Gene(s)	501	211	57.42

From the analysis, we can conclude that ACLY plays an important role in the clinical outcome of HNSCC patients. Further studies are therefore necessary to determine the involvement of the ACLY mutation in the tumorigenesis of oral cancer.

2.3.2. Expression analysis of ACLY in normal and oral cancer cell lines

In the above case, we observed that the genomic alterations in ACLY played a crucial role in the tumorigenesis of HNSCC. In our study, we first determined the gene expression of ACLY in HaCaT, a normal cell line versus SAS, an oral cancer cell line. The expression of ACLY was found to be significantly higher in the SAS cells (figure 2.4 A and B). Then, we comparatively examined the protein expression of ACLY in HaCaT, SAS, HSC3, and T.Tn using western blot analysis. The level of ACLY expression was highest in SAS cells followed HSC3 cells as compared to the HaCaT and T.Tn cells (figure 2.4 C). These initial findings showed an association of ACLY with oral tumorigenesis. In order to prove our findings there is a need to examine the ACLY expression in the clinical samples of oral cancer tissues as well as in normal oral tissues.



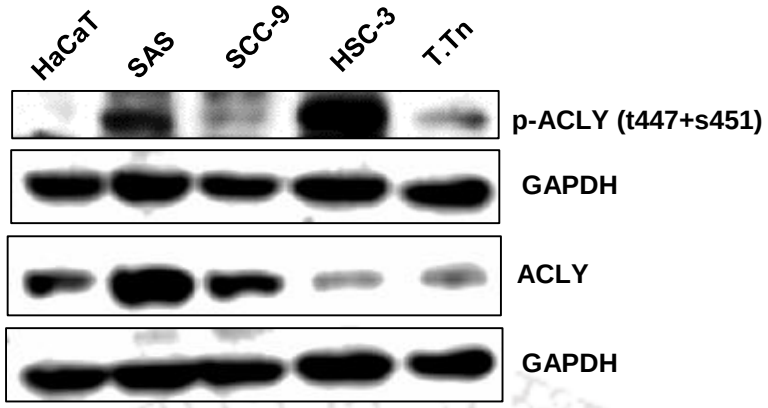


Figure 2.4. Expression of ACLY and p-ACLY in oral cancer cells in comparison with normal cells. A. mRNA expression of ACLY in SAS as compared to HaCaT cells, B. Graph representing the differential increase in the gene expression of ACLY.C. Western blot analysis of ACLY expression in different oral cancer cell lines as compared to the normal HaCaT cells.

2.3.3. Expression analysis of ATP citrate lyase (ACLY) and phospho-ACLY in normal oral tissues and oral cancer tissues

To authenticate the increased expression of ACLY in oral cancer cells, the expression of ACLY was examined in the normal as well as in oral tumor tissues. The upregulation in the expression of ACLY has been found to be associated with cancer growth and proliferation in various types of cancers such as bladder cancer, breast cancer, colorectal cancer, glioblastoma, lung cancer, ovarian cancer and prostate cancer [Khwairakpam et al., 2015]. Higher expression ACLY is known to be related with the rise in the lipid synthesis thereby, stimulating uncontrolled growth of tumor cells [Khwairakpam et al., 2015]. It has been observed that expression of ACLY increased considerably in breast cancer tissue compared with normal tissue [Wang et al., 2017].

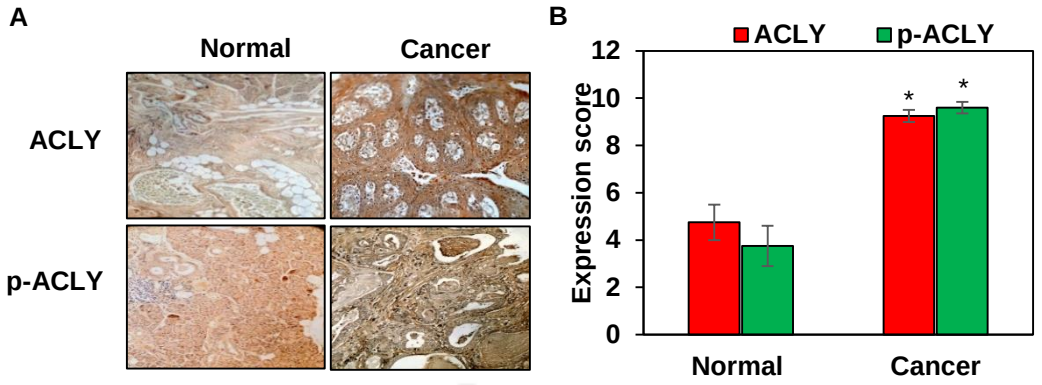


Figure 2.5. Expression of ACLY and p-ACLY in oral cancer tissue in comparison with normal oral tissue. A. Represntative images for immunohistochemical staining of ACLY in normal versus oral cancer tissues. B. Graphical representation of the quantification of the expression ACLY in normal versus tumor tissues of the oral cavity. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

Similarly, in our study, the immunohistochemical analysis of the TMA of oral cancer showed a significant increase in the expression of ACLY and phospho- ACLY as compared to normal tissues (figure 2.5 A and B). Therefore, it can be hypothesized that upregulated expression of ACLY might be responsible for the development and progression of oral cancer.

2.3.4. Expression of ACLY and phospho-ACLY in the various developmental stages of oral cancer

Studies have shown that the development of OSCC is considered as an intricate multistep process such as precancerous lesions, invasion and metastasis [Rivera and Venegas, 2014]. The increase in growth and proliferation of the cancer cells required a higher amount of fatty acid synthesis and glucose. Here, a study was done in order to investigate the expression of the proteins ACLY and p-ACLY involved in fatty acid synthesis in different developmental stages of oral cancer.

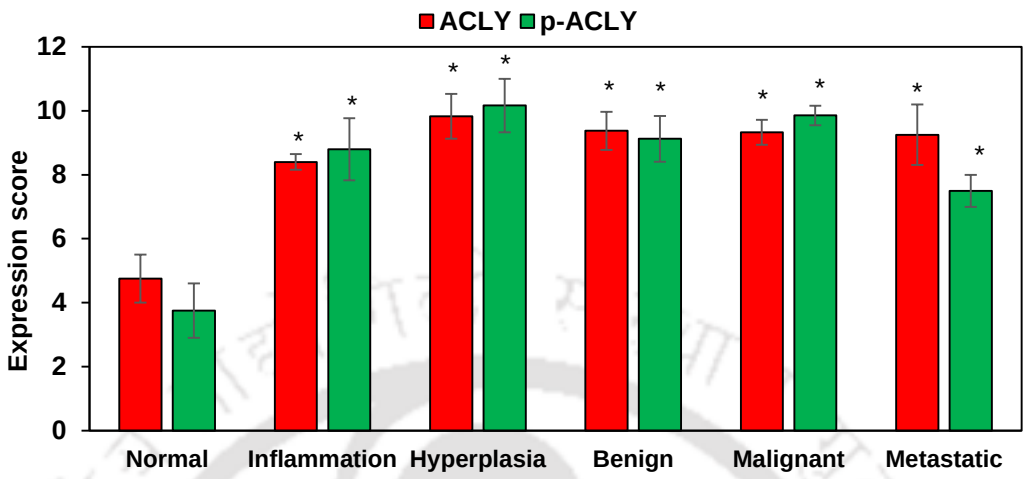


Figure 2.6. Expression of ACLY and p-ACLY in normal tissues versus oral cancer tissue of different development stages. Graphical representation of the quantification of the expression ACLY in normal versus oral tumor tissues of different development stages. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

We analyzed the expression of total ACLY and p-ACLY in different developmental stages of oral cancer such as inflammation, hyperplasia, benign, malignant and metastasis in comparison to the normal tissues of the oral cavity and found that the expression of ACLY escalates with the progression of tumor from normal to metastatic (figure 2.6). In line with this, Migita et al., have shown that the increased levels of p-ACLY observed in 162 tumor tissues were associated with stage, differentiation and poorer prognosis [Migita et al., 2008]. The highest expression in both forms of ACLY and p-ACLY in the inflammation and hyperplasia oral cancer tissues suggest that the expression of ACLY is very much important for the initial development that leads to the formation of tumor in the oral cavity. The remarkable increase in the expression of both ACLY and p-ACLY in benign and malignant also showed the importance of the ACLY protein in the uncontrollably abnormal growth of the cells that leads to the formation of cancerous tissue i.e. malignant. This malignant tumor can easily spread

and invade other tissues. In addition, the increased expression of the ACLY protein in the metastatic stage suggesting its role in the transformation of cancer cells break free from a malignant tumor and migrate to the distant tissues and organs. Several studies have also shown the association of increase upregulation in the expression of ACLY and tumor cell migration as well as metastasis. Therefore, the increase in the expression of ACLY might be responsible for the tumor initiation and progression.

2.3.5. Analysis in the expression of ACLY and phospho-ACLY in patients of different age groups (Gender wise)

Aforementioned, the mean age of people with oral cancer in India is 36 years and peak at 55–60 years. [Shukla et al., 2012]. Moreover, GLOBOCAN 2018 has reported that oral cancer in man is the second highest cancer in the world and in female it is fourth highest [GLOBOCAN]. Further, epidemiological studies have also found that oral cancer is two to three times more predominant in men than women in most ethnic groups [Rivera et al., 2015]. The higher consumption of smoking as well as smokeless tobacco by the male population might be the reason for the increased rate of oral cancer in male as compared to female. Therefore, investigation was done to determine the expression of the ACLY and p-ACLY based on the gender.

The expression analysis of the protein ACLY was also done in different age groups of male as well as female patients of oral cancer (figure 2.7 A-B). In male, a higher expression of ACLY and p-ACLY was observed in 26-50 years and 51-75years of age groups. However, in female, higher expression was observed in 0-25years age group. Moreover, the expression of both ACLY and p-ACLY was comparatively higher in male as compared to female. This may be due to the increase consumption of tobacco particularly in the male population.

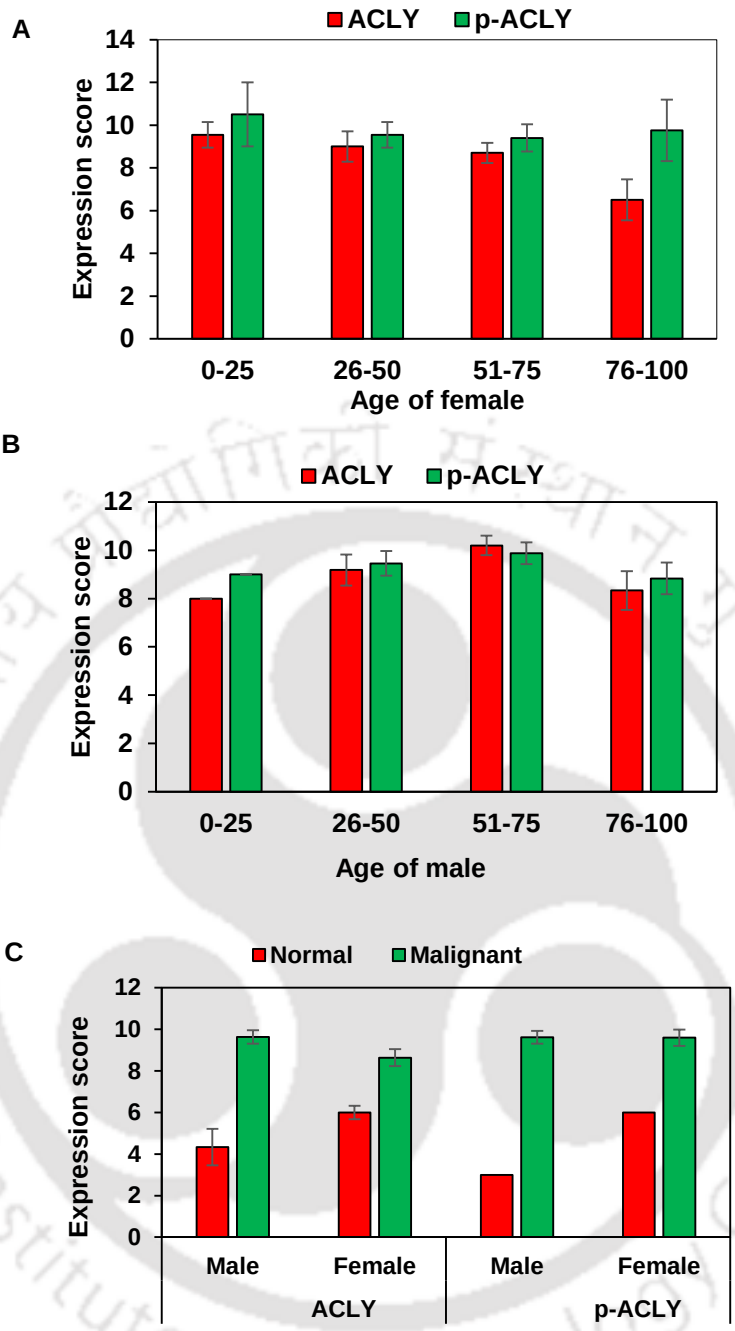


Figure 2.7. Expression of ACLY and p-ACLY in different age groups of male and female. A. Graphical representation of the quantification of the expression ACLY in normal versus oral tumor tissues of different age groups of male. B. Graphical representation of the quantification of the expression ACLY in normal versus oral tumor tissues of different age groups of female. C. Graphical representation of the quantification of the expression ACLY in male versus female tumor tissues of different age groups. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

2.3.6. Analysis in the expression of ACLY and phospho-ACLY in different grades of oral cancer:

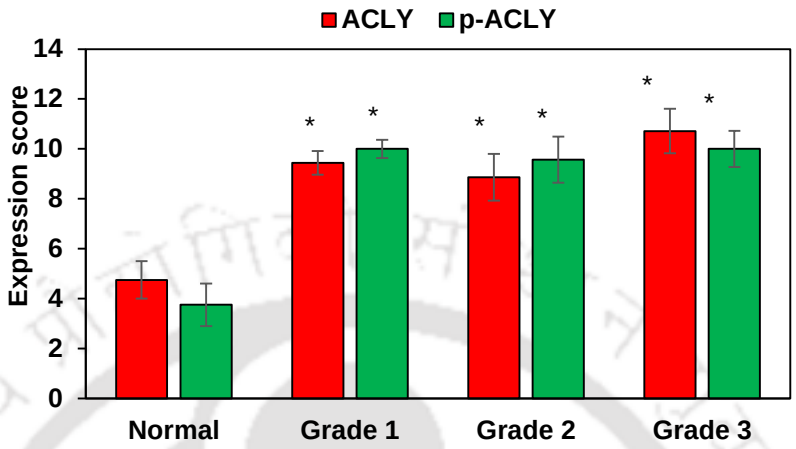


Figure 2.8. Expression of ACLY and p-ACLY in grades of oral cancer tissue. Graphical representation of the quantification of the expression ACLY in normal versus oral tumor tissues of different age groups of female. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

We have also determined the expression of ACLY and p-ACLY in different grades of oral cancer. We observed a significantly higher expression of both ACLY and p-ACLY with the increase in grades of tumor such as grade 1 (well-differentiated), grade 2 (moderately-differentiated) and grade 3 (poorly-differentiated) (figure 2.8). This indicates that increased expression of the proteins is associated with the decrease in the differentiation status of tumor tissues of the oral cavity. In support to this, Hanai et al. has reported that suppression of ACLY limits tumor cell proliferation and survival and promotes cell differentiation in lung cancer cells *in vitro*. Further, inhibition of ACLY also resulted in decreased tumor growth leading to cytostatic effect and differentiation [Hanai et al., 2012].

2.3.7. Analysis in the expression of ACLY and phospho-ACLY in different stages of the oral cancer:

The TMA slide contains stage I, II, III and IV oral cancer tissues. The stage wise difference in the expression of ACLY and p-ACLY proteins was calculated and plotted against the expression in normal tissues.

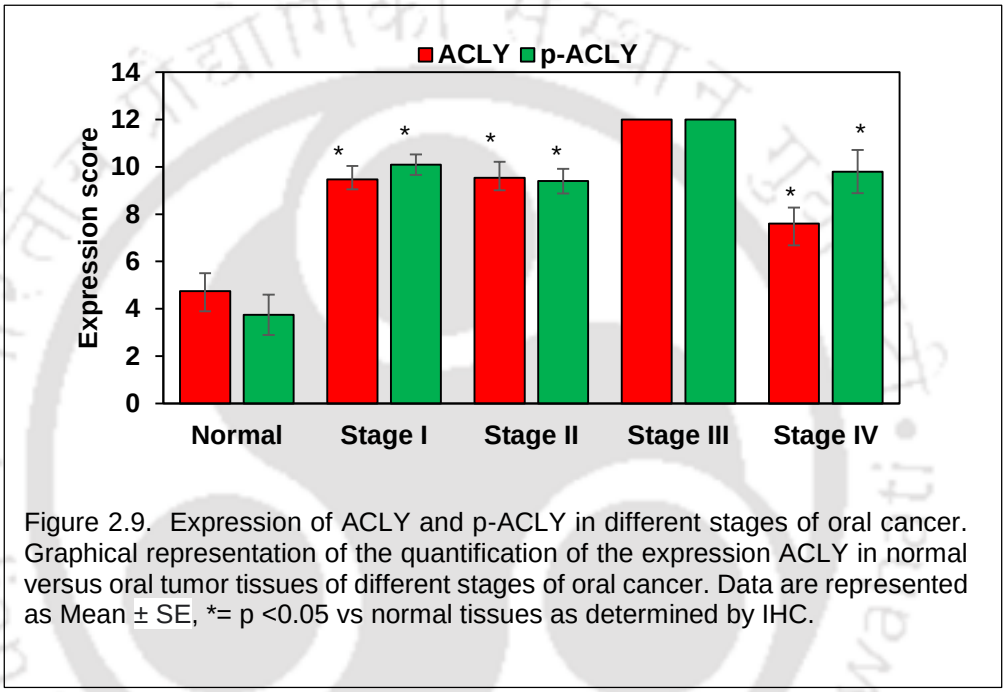


Figure 2.9. Expression of ACLY and p-ACLY in different stages of oral cancer. Graphical representation of the quantification of the expression ACLY in normal versus oral tumor tissues of different stages of oral cancer. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

Analysis of the ACLY expression showed a significant increase in the protein levels of ACLY as well as p- ACLY in stage I (small tumor in an area), stage II (larger tumor and grown in nearby tissues and lymph nodes) and stage IV (metastatic cancer) as compared to normal tissues (figure 2.9). It indicated that upregulation of the lipogenic enzyme might be responsible for the abnormal development and progression of oral cancer.

2.3.8. Analysis in the expression of ACLY and phospho-ACLY in different TNM stages of oral cancer:

The TNM staging system (tumor-lymph node-metastasis) is based on the best estimate of the extent of disease before treatment [Rivera et al., 2015]. The staging of oral cancer is highly essential for proper prognosis and therapy. It is performed mostly by using the TNM technique, where T - size of the primary tumor, N - status of the regional lymph nodes, and M - presence or absence of distant metastases [Radhakrishnan et al., 2012].

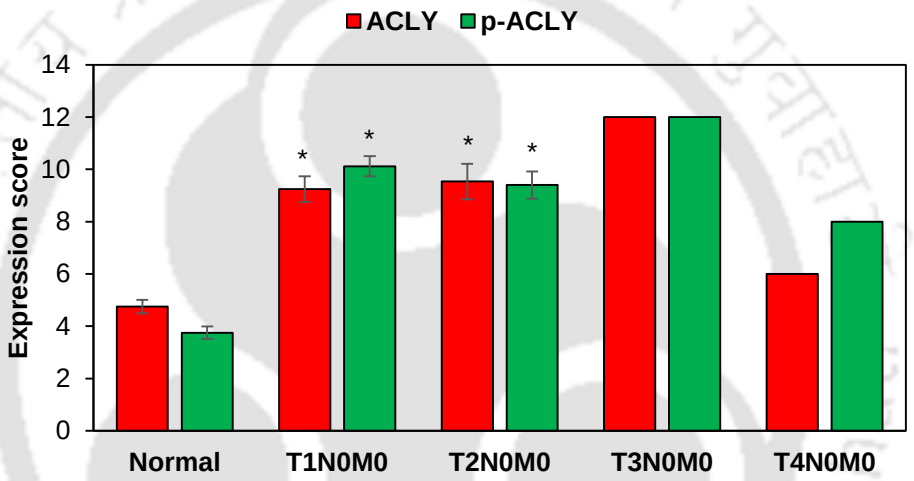


Figure 2.10. Expression of ACLY and p-ACLY in different TNM stages of oral cancer. Graphical representation of the quantification of the expression ACLY in normal versus oral tumor tissues of different stages of oral cancer. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

The analysis of the IHC results also indicated an increase in the expression of ACLY and p-ACLY in the different TNM (tumor-node metastasis) stages of oral cancer compared to normal. Significantly higher expression of ACLY and p-ACLY was observed in oral cancer tissues of T1N0M0 and T2N0M0 as compared to normal tissue of oral cavity (figure 2.10).

2.3.9. Analysis in the expression of ACLY and phospho-ACLY in tumors from the different organs of the oral cavity:

Cancer of the oral cavity involves tumor growth in the lips, cheeks, hard palate, soft palate, uvula, floor of the mouth (area under the tongue), gingiva, gums, teeth, tongue, retromolar trigone and tonsils [Jemal et al.,2008; Radhakrishnan et al., 2012; Rivera et al., 2015]. Here analysis of the expression of the ACLY and p-ACLY was done in tumors from the different parts of the oral cavity (figure 2.11).

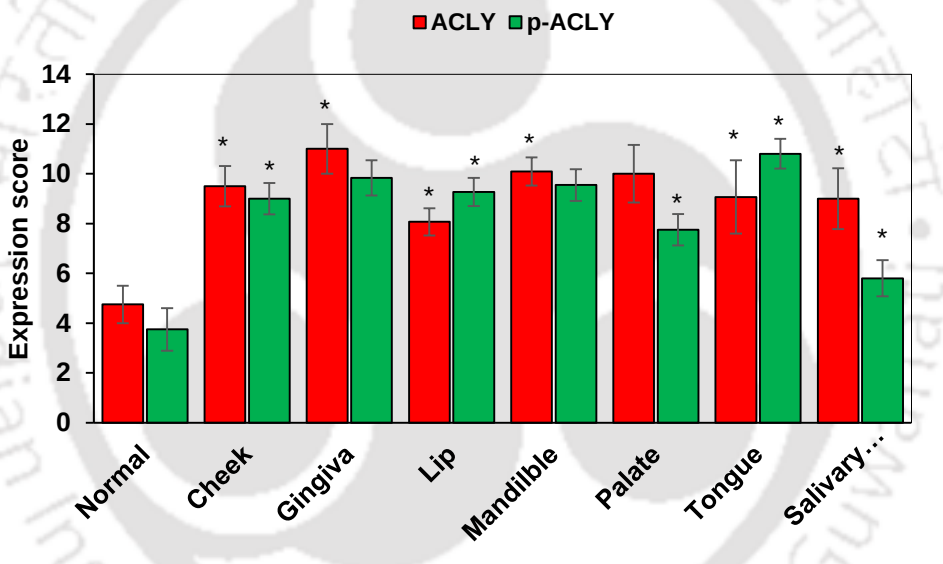


Figure 2.11. Expression of ACLY and phospho-ACLY in tumors from the different organs of the oral cavity. Graphical representation of the quantification of the expression ACLY in normal versus tumors from different tissues of the mouth. Data are represented as Mean ± SE, *= p <0.05 vs normal tissues as determined by IHC.

The expression of ACLY and p-ACLY was found to be highly upregulated in the different cancers of the mouth cavity such as cheek, gingiva, lip, mandible, palate, tongue and salivary gland as compared to normal or non-cancerous tissues of the oral cavity. Higher level of the protein was observed in cheek, gingiva and tongue cancers of the mouth or oral cavity in comparison to different organs of the oral cavity. Our

results suggested that the level of expression of both ACLY and p-ACLY are higher in all tumor parts of the oral cavity as compared to the normal tissues.

2.4. CONCLUSION

Here, we explored the differential expression of ACLY and phosphorylated ACLY in normal human tissues and compared it with the preneoplastic and neoplastic tissues from oral cancer patients using immunostaining of TMA. Our results showed a significant increase in the expression of ACLY and p-ACLY in oral tumor tissues as compared to normal (non-cancerous) tissues. In addition, the tissue micro array results showed an increase expression of total ACLY and p-ACLY protein with different oral cancer types, stages, and grades. This is the first report that shows the upregulated expression of ACLY and p-ACLY in oral cancer. The upregulation in the expression of ACLY might play an essential role in the development and progression of the oral cancer.

Chapter 3

Effect of pure tobacco extract and other tobacco related carcinogens on the expression of ACLY and p-ACLY in Human OSCC cell lines

Chapter 3

3.1. Introduction

Consumption of tobacco is one of the major risk factor for malignant lesions of the mouth and oral cancer [Warnakulasuriya et al., 2005, Xue et al., 2014]. Tobacco is consumed either as smokeless tobacco or smoking. Tobacco consumption produced many toxins like nicotine known to be responsible for tumor initiation and progression [Delitto et al., 2016]. Studies have shown nicotine and its metabolites are specific markers of exposure to smoke released from cigarettes in smokers and non-smokers unprotected to second hand smoke (SHS) [Pérez-Ortuño et al., 2016]. The Global Adult Tobacco Survey (GATS) India Report (2009-10) has reported that the number of people consuming smokeless tobacco (SLT) were found to be more than double than that of smokers [Arora & Madhu, 2012]. Interestingly, South Asia has more than 90% of the global smokeless tobacco users [Xue et al., 2014]. It has been evidenced that the risk of oral cancer was higher among those who used paan (betel leaf and betel nut quid) with tobacco than those who used it without tobacco. Further, the consumption of paan raised the oral cancer risk to about seven fold, directly and mediated by OSMF, even when it was consumed without tobacco [Merchant et al., 2014]. The use of smokeless tobacco is widespread globally and is known to cause OSMF which are likely to be malignant [Niaz et al., 2017]. Boffetta et al. has reported the presence of over 30 carcinogens in smokeless tobacco that consists of volatile and tobacco-specific nitrosamines, nitrosamino acids, metals, aldehydes, polycyclic aromatic hydrocarbons [Boffetta et al., 2006]. In the previous chapter, we have found that expression of ACLY proteins is upregulated in OSCC. To further confirm our hypothesis, we endeavoured to examine the consequence of the causative agents of oral cancer on the mRNA expression of ACLY and other related genes. Here, we examined the effect of tobacco extract and its carcinogens such as tobacco specific N'-nitrosamines (TSNAs) including N-

Nitrosonornicotine (NNN) and 4 [methylnitrosoamino]-1-[3-pyridyl]-1-butanone (NNK) on the expression of ACLY. NNK and NNN induce carcinogenesis by triggering DNA adductions and mutations as well as promoting tumor growth *via* receptor-mediated effects [Xue et al., 2014]. Moreover, nicotine is the principally present in all types of tobacco products and smoke. Nicotine is addictive and non-carcinogenic; however, it is indispensable for the activation of several signalling pathways linked with tumorigenesis [Xue et al., 2014]. Tuibur, a product of tobacco which is prepared locally by infusion of tobacco smoke in water until the color changes to cognac and gives a pungent smell [Phukan et al., 2005]. Tuibur has been noted as the major factor for the increasing incidences of cancer cases in Mizoram [Madathil et al., 2018]. Therefore, several studies have shown that tobacco and its components played an important role in the tumorigenesis of oral cancer. In this chapter we determined the effect of tobacco extract, tuibur, and the three potent carcinogens of oral cancer cells to have a clear understanding about the involvement of ACLY in OSCC.

3.2. Materials and Methods

3.2.1. Cell lines and cell culture

Human OSCC cell lines such as SAS and HSC-3 was used for the study. More details of the cells lines and their culture media have been stated in chapter 2, section 2.2.2, Page no.49.

3.2.2. Preparation of tobacco extract

Tobacco extract (TE) was prepared from the tobacco leaves purchased from the Guwahati local vendors. The leaves were powdered and 4g of tobacco powder was dissolve in 100mL of distilled water with the help of magnetic stirrer for 24 hr at room temperature (RT). Then, the extract was filtered and the filtrate was lyophilized for

preparation of stock concentration. Lastly, a working stock concentration of 50mg/mL was prepared in sterile distilled water using the lyophilized powder of (tobacco extract (TE)) and was stored in -20°C for further experimental studies.

3.2.3. Preparation of tuibur

Aforementioned, tuibur is a water-soluble tobacco smoke infused water often consumed in the North eastern states of India especially Mizoram. Tuibur was procured from a local market of Aizawl, Mizoram, India and was filtered to remove any insoluble units present in the liquid. The filtrate acquired was lyophilized and a 100 mg/mL working stock concentration was prepared in sterile distilled water and stored in -20°C for further experimental studies.

3.2.4. Tobacco components

The tobacco components such as nicotine (Cat. No. N3876), NNK (Cat No. 78013), NNN (Cat No. 75285) were purchased from Sigma- Aldrich, Missouri, USA. Tobacco was purchased from the local market of Guwahati, Assam India. Tuibur was purchased from the local market of Aizawl, Mizoram, India.

3.2.5. MTT assay

The non-cytotoxic concentration of tobacco extract, tuibur, and other tobacco components was determined by using MTT Assay. The cytotoxicity of the tobacco components mentioned above was determined by measuring the conversion of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich)) to formazan. Briefly, 2 $\times 10^3$ SAS cells per 100µl were seeded into a 96 well plate and incubated overnight. SAS cells was then treated with tobacco extract (µg/mL), tuibur (µg/mL), nicotine (µM), NNK (nM), and NNN (nM) for 0 and 24 h. Then, 10ul of the MTT (MTT; Sigma-Aldrich) in phosphate buffered saline (PBS) (5mg/ml) was added to each well. After incubation for 2 hr at 37°C, the supernatant was discarded and the

precipitate was dissolved with 100µl of dimethyl sulfoxide (DMSO; Emplura® Merk Life Sc. Private Limited, Mumbai, India).

3.2.6. RNA preparation and reverse transcription-PCR

The mRNA expression of ACLY on treatment with tobacco extract, tuibur and other tobacco components was studied using Reverse transcription-polymerase chain reaction (RT-PCR). The cell lines in logarithmic phase of growth were collected for RNA extraction. SAS cells were seeded in culture plates with a density of $5x10^5$ cells per well and was treated with different concentrations of tobacco components for 24hr. RNA isolation was done by using TRI Reagent® (SIGMA® Life Science) according to manufacturer's protocol. The total RNA was applied for first-strand cDNA synthesis with a high-capacity cDNA Reverse Transcriptase kit (Cat No. 4368814, Applied Biosystems™, USA) and 2x Hot Start Taq Master Mix (Cat No. M0496L) from New England Biolabs® (NEB, USA) (Table 1). The gene expression of ACLY were then amplified by 35 cycles of PCR using specific primers and 1µL of cDNA as template. The amplicons thus obtained were determined in 1% agarose gel electrophoresis and the band intensity was examined using Image lab software. 100 bp DNA Ladder (Cat No. N3231S, NEB, USA) was run as a standard. The housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal gene control. Gene-specific primer was obtained from Integrated DNA Technologies (IDT ®) (Table 2). PCR was carried out in 96-well plates using the Veriti (Applied Bioscience).

3.2.7. Expression of ACLY and related genes in SAS (an OSCC cell line)

cDNA was prepared from the total RNA isolated from the SAS as well as HaCat cells. The annealing temperature of the different primers was also optimized for further studies. Semi-quantitative reverse transcriptase PCR was performed with the

synthesized cDNA in order to analyse the expression of ACLY and the different genes by agarose gel electrophoresis. The ACLY gene was found to be expressed in SAS cells.

Table 3.1. Primer sequence for reverse transcriptase PCR

Primer	Sequence	Product size	Annealing temp
ACLY-F	5′ -TACGGGTGATGGGAGAAGTC- 3′	413bp	54^0C
ACLY-R	5′ -CCCAGTGAAAGGGTAGACCA- 3′		
GLUT1-F	5′ -CACTGTCGTGTCGCTGTTTG- 3	416bp	59^0C
GLUT1-R	5′ -TCCGGCCTTTAGTCTCAGGA- 3		
IGF1R-F	5′ -TCCACATCCTGCTCATCTCC- 3′	669bp	60^0C
IGF1R-R	5′ -AGAAGTCACGGTCCACACAG- 3′		
HIF1A-f	5′ -GACAAGCCACCTGAGGAGAG- 3′	348bp	57^0C
HIF1A-r	5′ -GTGGCAACTGATGAGCAAGC- 3′		
ACACA-F	5′ -CTCTCACGCTCAAGTCACCA- 3′	502bp	52^0C
ACACA-R	5′ -CGTAGGGATGTTCCCTCTGTT- 3′		
FAS-F	5′ -TCATCAAGTGGGACCACAGC- 3′	365bp	52^0C
FAS-R	5′ -CACTACCAGGTTGCCGTTCT- 3′		
REDD-1 –F	5′ -CTGATGCCTAGCCAGTTGGT- 3′	233bp	55^0C

REDD-1 –R	5′ -GAGCTAAACAGCCCCTGGAT- 3′		
GSK3-F	5′ -GGCTCATTTGGGGTCGTGTA- 3′	433bp	55^0C
GSK3-R	5′ -GGACCAACTGCTTTGCACTG- 3′		
STK11-F	5′ -TCAAAATCTCCGACCTGGGC- 3′	570bp	55^0C
STK11-R	5′ -TGTGACTGGCCTCCTCTTCT- 3′		

3.2.8. Statistical analysis:

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

3.3. Results and Discussion

3.3.1. Effect of tobacco extract on the expression of ACLY and related genes

In the previous chapter 2, we have found increased expression of ACLY both in the mRNA and protein level in the oral cancer cells as compared to normal cells respectively. Moreover, we also found increased ACLY expression with the different developmental stages of oral tumor tissues as compared to normal tissues. Aforementioned smokeless tobacco is associated with oral cancer since decades [Rodu et al., 2004]. It is well known that the consumption of smokeless tobacco usually comprises of non-occupational exposure to cancer-causing nitrosamines which are 100–1000 times grander than the exposure from foods and beverages. In addition, every gram of regularly used smokeless tobacco contains 1–5 μg of the TSNS such as NNK and NNN [Boffetta et al., 2006]. The constant mastication of paan and consumption of gutkha initiates progressive fibrosis in submucosal tissue and OSMF [Niaz et al,

2017]. The chemical products such as 3-(methylnitrosamino)-proprionitrile, nitrosamines, and nicotine are formed in the oral cavity that leads to the production of ROS. It then, finally causes formation of fibroblast, DNA, and RNA damage thereby inducing tumorigenic effect [Niaz et al, 2017]. Moreover, the formation of N-nitrosonornicotine via cytochrome P450 enzymes indicated of genotoxicity. It also leads to DNA damage and, finally caused oral cancer [Niaz et al., 2017]. Exposure to chewing tobacco was found to upregulate the expression of EGFR and its downstream proteins such as FAS and Replication factor C subunit 3 (RFC3) [Rajagopalan, 2018]. In addition, the chronic exposure to chewing of tobacco highly upregulated the expression of non-SMC condensin I complex subunit G (NCAPG) crucial for chromosome stability and mitosis and CCNB1 that controls the cell cycle checkpoint (G2 to M phase) [Rajagopalan, 2018]. This provides an indication that ACLY might possess certain association with tobacco which has been noted as one of the major risk factors of OSCC. Moreover, the role of tobacco and the various components of tobacco on the expression of ACLY is not studied till date. Therefore, this chapter will mainly focus on the effect of tobacco and its components on the expression of ACLY, oncogenes and tumor suppressor genes.

Further, we obtain the non-toxic concentration on oral cancer cells on treatment with the crude extract tobacco using MTT assay. In a dose response curve plotted for 24hr with respect to 0hr, the concentrations of TE ranging from 0.1 to 10µg/ml do not cause any significant reduction in rate of proliferation of SAS cells (Figure). The mRNA expression of ACLY was found to be significantly upregulated on treatment with the crude tobacco extract. It also significantly upregulated the expression of other lipogenic enzymes such as ACACA and FAS and the other oncogenes such as IGF1R, GLUT1, HIF1A, GSK3 and downregulated the expression of REDD1 which is a tumor

suppressor gene. Khalil et al., has been demonstrated that downregulation of the IGF1R reduces the proliferation and cell viability of tumor cells in OSCC [Khalil et al.,2019]. Moreover, HIF1A assists in the malignant transformation of OSMF [Pereira et al., 2020]. In addition, studies have shown that the increase expression of GLUT-1 in tumor cells point out augmentation in the proliferative activity, requirements for energy, aggressive behaviour and poor response to radiation [Azad et al., 2016].

It has been evidenced that GLUT-1 expression remarkably links with histological grade and pTNM staging of OSCC. GLUT1 is also associated with tobacco addiction [Azad et al., 2016]. Mishra et al., investigated increased expression of GSK3α/β in OSCC which was associated with progression of disease [Mishra et al., 2015]. In our study significant upregulation in the expression of ACLY and various other oncogenes was observed on treatment with tobacco extract. This supported our IHC results where the expression of ACLY was found to be increased with the progression of oral cancer. From our results we hypothesized that exposure to crude TE escalated ACLY expression in oral cancer cells which would result into the rise in expression of oncogenes that ultimately leads to the stimulation of downstream pathways and subsidizing the development of tumor and its progression.

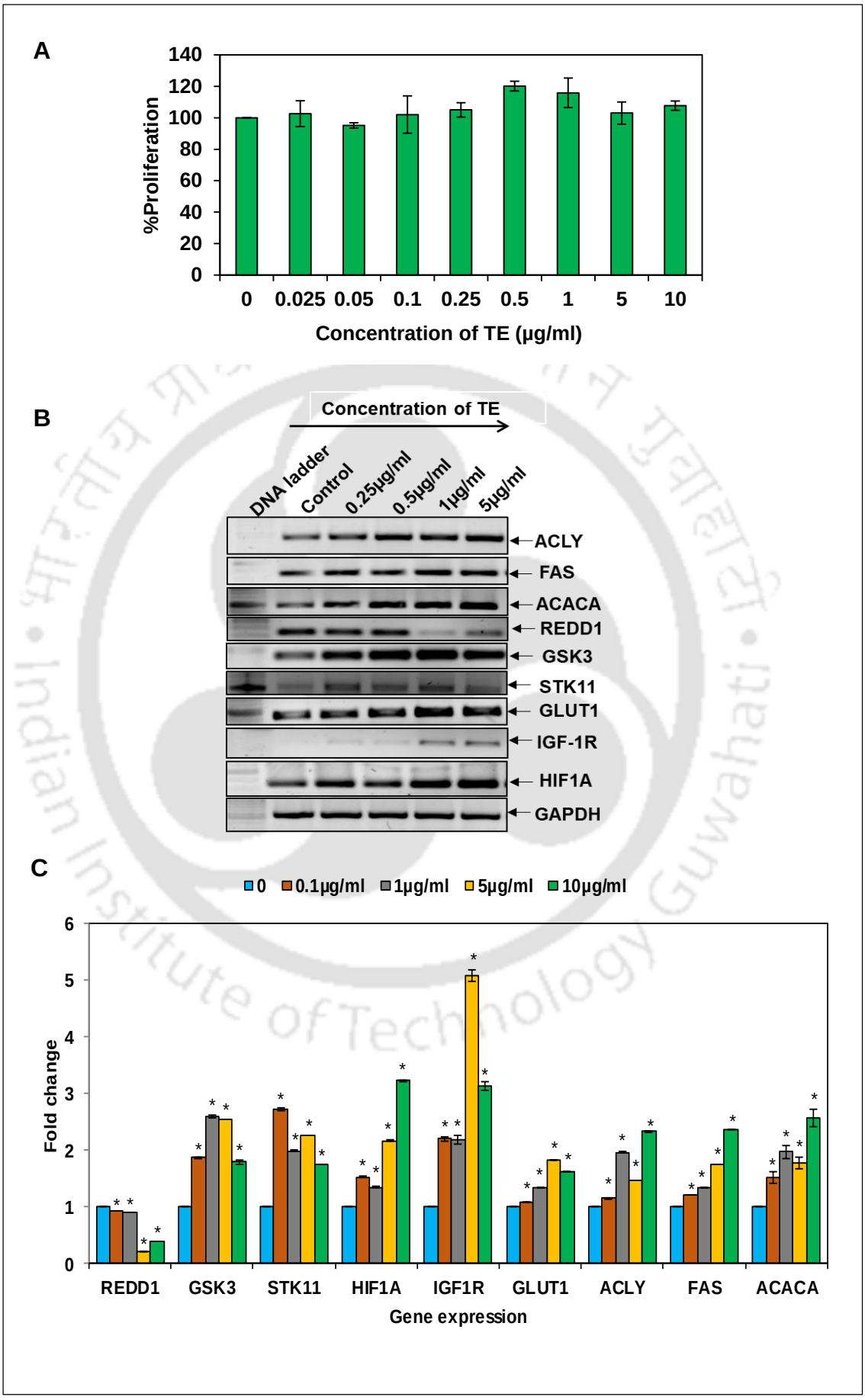


Figure 3.1. Effect of TE on the expression of ACLY and related genes in SAS cells. A. MTT assay showing the non-toxic concentrations of TE on SAS cells. B. Gene expression of ACLY and other related genes analysed using reverse transcriptase PCR. C. Quantification of the expression of ACLY, FAS, ACACA and other related genes such as REDD1, GSK3, STK11, GLUT1, HIF1A and IGF-1R in TE treated SAS cells obtained using Image Lab software. GAPDH was used as control gene. Data are represented as Mean ± SE, *= p <0.05 vs untreated cells.

3.3.2. Effect of Nicotine on the expression of ACLY and related genes

Nicotine is known to be the major addictive component of tobacco products [Sanner and Grimsrud, 2015]. Nicotine has been found to promote tumor proliferation, invasion and angiogenesis. It activates nicotinic acetylcholine receptors (nAChRs) stimulating several signalling pathways that initiates tumorigenesis [Wang et al., 2017]. Earlier studies have shown induction in the tumor growth and migration on treatment with nicotine. Moreover, nicotine instigated EGFR phosphorylation thereby, activating the downstream effectors such as PI3/AKT and p44/42 mitogen-activated protein kinases (ERK) [Nishioka et al., 2019]. In vivo experimental studies have revealed that the treatment of nicotine in xenografted tumors augmented the lymph node metastasis. Further studies have demonstrated that the application of an nAChR inhibitor suppressed lymph node metastasis and nuclear localization of p-EGFR in the xenografted tumors [Shimizu et al., 2019]. It has been found that various signaling cascades like MAPK/extracellular signal-regulated kinase pathway, PI3/AKT pathway, and JAK/STAT are induced by nicotine through nAChRs. In addition, studies have shown that nAChR initiation induces the expression of Src kinase in a β-arrestin-1-dependent manner, and inactivated the expression of Rb protein thereby instigating the expression of E2F1-regulated proliferative genes [Schaal and Chellappan, 2014]. In this study, experiments have been conducted to elucidate the effect of nicotine on the expression of ACLY and other oncogenes that would help us in the better understanding of the role of ACLY in nicotine induced oral carcinogenesis.

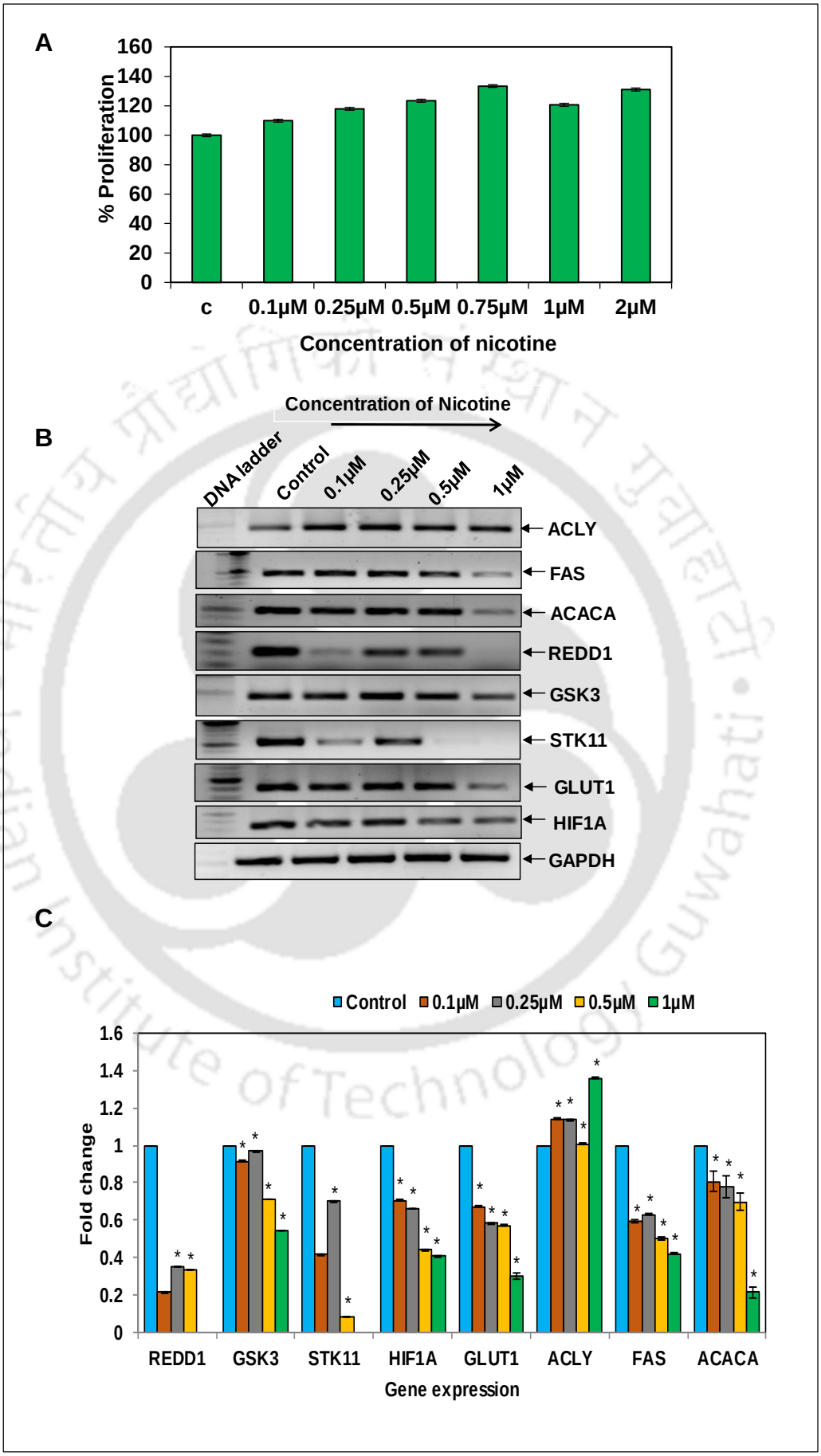


Figure 3.2. Effect of nicotine on the expression of ACLY and related genes in SAS cells. A. MTT assay showing the non-toxic concentrations of nicotine on SAS cells. B. Gene expression of ACLY and other related genes analysed using reverse transcriptase PCR. C. Quantification of the expression of ACLY, FAS, ACACA and other related genes such as REDD1, GSK3, STK11, GLUT1, HIF1A and IGF-1R in nicotine treated SAS cells obtained using Image Lab software. GAPDH was used as control gene. Data are represented as Mean ± SE, *= p <0.05 vs untreated cells.

Besides, studies have proven that nicotine at low concentrations rouse cell growth, while higher concentrations of it induced cytotoxicity in the cells [Sanner and Grimsrud, 2015]. Initially, MTT assay was performed to select the non-toxic proliferative concentrations of nicotine. SAS cells were treated with various concentrations of nicotine (100nM, 250nM, 500nM, 750nM, 1µM and 2µM) for 0h and 24h. In a dose response curve plotted for 24h with respect to 0h has showed that the concentrations of nicotine ranging from 100nM-2µM do not cause any significant reduction in the proliferation of SAS cells (figure 3.2 A). Therefore, concentration of nicotine from within this range such as 0.1µM, 0.25µM, 0.5µM, and 1µM was used for studying the mRNA expression of ACLY. SAS cells were treated with the mentioned non-toxic concentrations of nicotine for 24hr. Total RNA was isolated and semi-quantitative RT-PCR was performed to analyse the expression of ACLY and other genes. The mRNA levels of ACLY were found to be upregulated in all the concentrations of nicotine with respect to the untreated control (figure 3.2B-C). It has been demonstrated that nicotine promotes endothelial cell migration, proliferation, survival, tube formation, and nitric oxide (NO) production *in vitro* [Sanner, 2015]. In addition, nicotine induces epithelial–mesenchymal transition (EMT), which is necessary for the procurement of malignant phenotype [Sanner, 2015]. However, no significant upregulation in the expression of the other lipogenic enzymes such as FAS and ACACA and oncogenes like HIF1A, GLUT1 was observed with the increase in the concentration of nicotine. Further, downregulation in the expression of STK11 and

GSK3 was observed with the increase in the concentration of nicotine. Therefore, nicotine induced upregulation of *ACLY* and downregulation of the tumor suppressor genes that might play a critical role in the development and progression of oral cancer. However, further detailed analysis is necessary to manifest the in depth mechanisms associated.

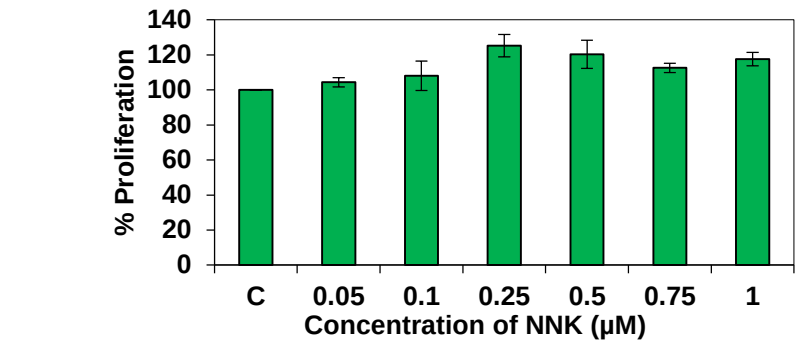
3.3.3. Effect of Tobacco-specific nitrosamines (NNK and NNN) on the expression of ACLY and related genes

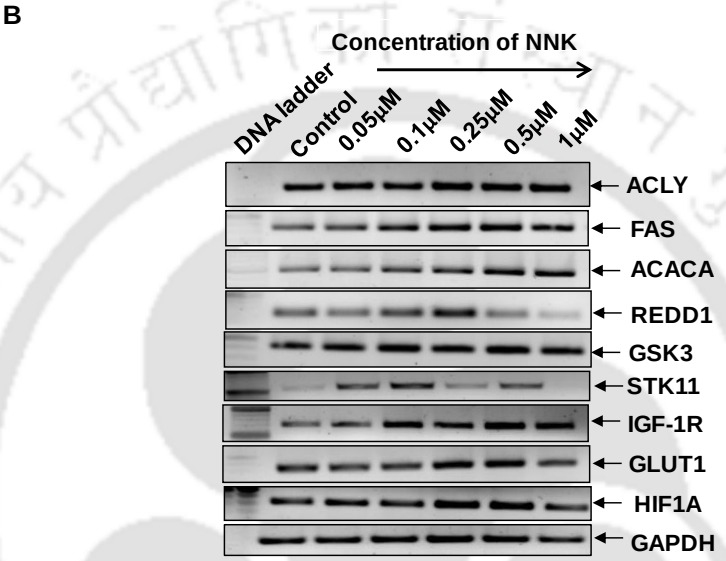
Studies have found that nicotine gets converted to carcinogenic nitrosamines like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) during the process of smoking [Xue et al., 2014, Pérez-Ortuño et al., 2016]. NNK and NNN are carcinogenic to human and promote tumorigenesis when exposed to it [Xue et al., 2014, Pérez-Ortuño et al., 2016]. It initiates development of tumor by deleterious mutations in the oncogenes and tumor suppressor genes due to formation of DNA adduct [Xue et al., 2014]. In addition, nitrosamines bind to nicotinic acetylcholine receptors and promote tumor growth [Xue et al., 2014]. Aforesaid, it is well established that TSNA contribute to tobacco related cancer. In this chapter, an attempt has been made to study the effect of TSNA on the expression of ACLY and other oncogenes associated with tumor metabolism.

A. Effect of NNK on the expression of ACLY and related genes

It has been evidenced that NNK not only instigated single-strand DNA breaks and oxidative DNA damage but also stimulates the survival and proliferation of normal lung epithelial and lung cancer cells [Deng, 2014]. We performed MTT assay to investigate the non-toxic proliferative concentration of NNK on SAS cells. Treatment of SAS cells with NNK (0.05μM, 0.1 μM, 0.25 μM, 0.5 μM and 1 μM) for 0hr and 24hr was performed to investigate the rate of proliferation of SAS cells as compared

to the control untreated group. A dose response curve was plotted for 24hr with respect to 0hr. Here, the lower concentrations of NNK ranging from 0.05μM-1μM enhances the rate of proliferation and do not cause any significant reduction in the proliferation of SAS cells (figure 3.3 A). Further, we determined the mRNA levels of ACLY of SAS cells treated with non-toxic concentrations of NNK (50nM, 100nM, 250nM, 500nM). Total RNA was isolated and mRNA expression of ACLY and other lipogenic enzymes such FAS, ACACA and also the oncogenes was studied using semi-quantitative rT-PCR. The mRNA levels of ACLY and other lipogenic enzymes such FAS, ACACA were found to be upregulated in all the treated concentrations of NNK as compared to the untreated control (figure 3.3 B-C). In addition, NNK treatment increased the mRNA expression of GLUT1, IGF1R and, HIF1A and downregulated the mRNA expression of tumor suppressor genes such as STK11, GSK3 and REDD1.





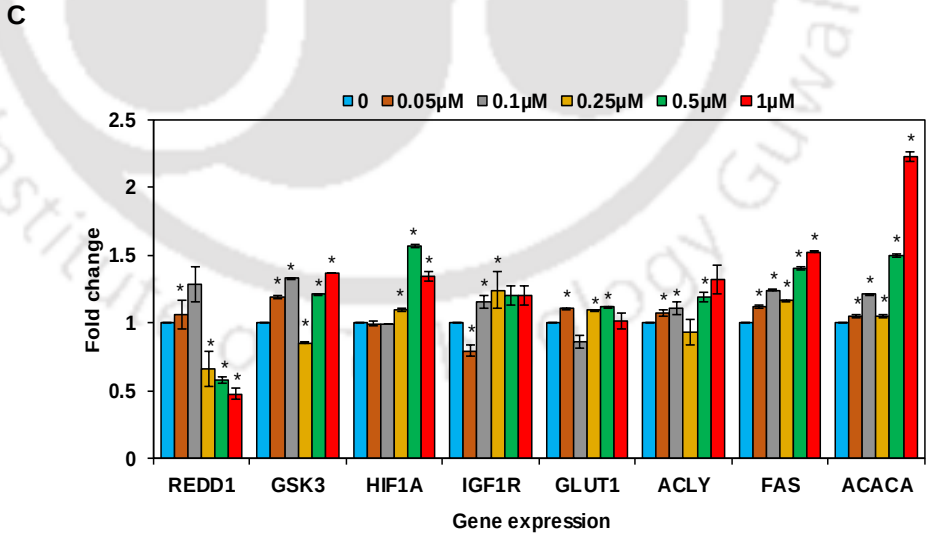
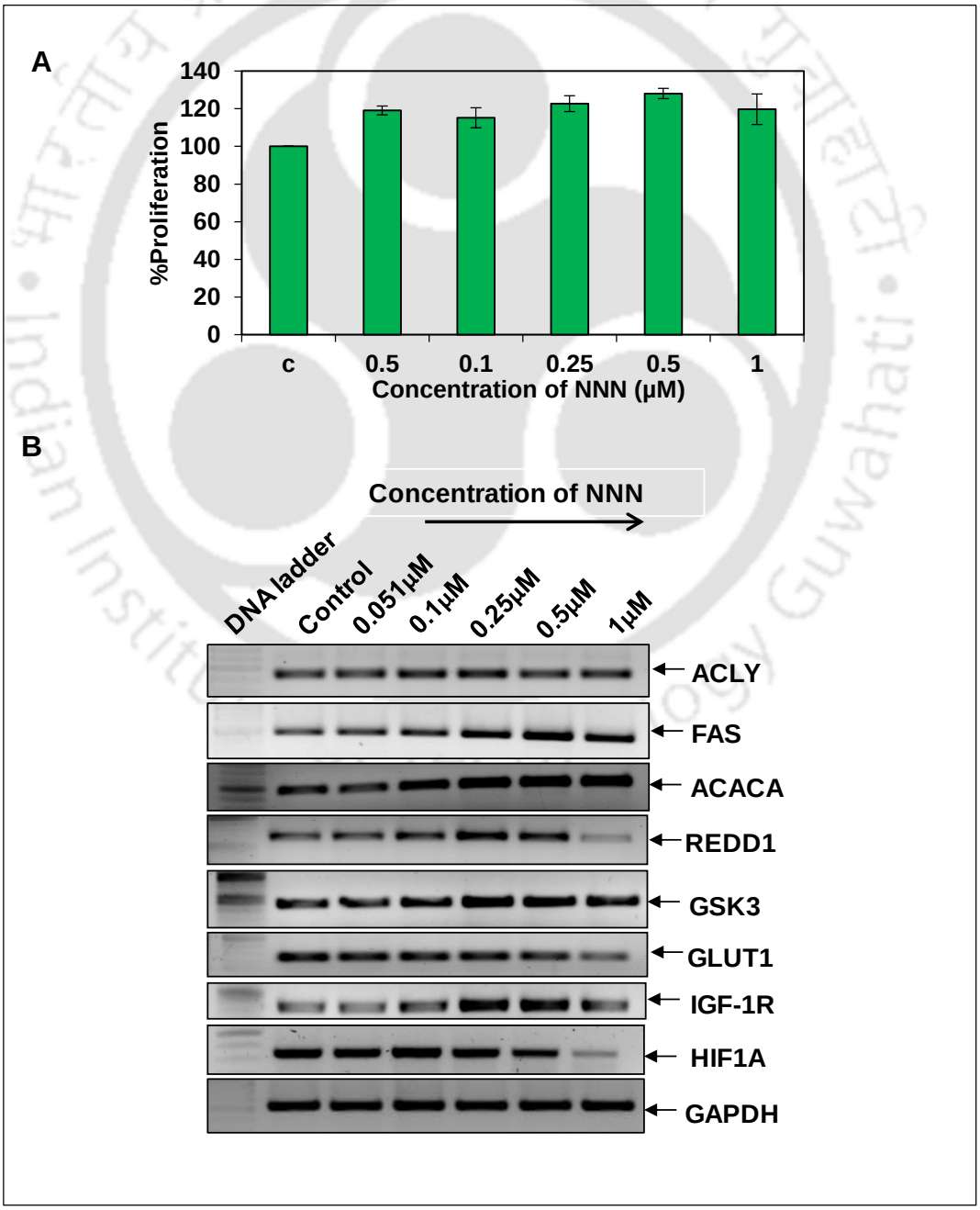


Figure 3.3. Effect of NNK on the expression of ACLY and related genes in SAS cells. A. MTT assay showing the non-toxic concentrations of NNK on SAS cells. B. Gene expression of ACLY and other related genes analysed using reverse transcriptase PCR. C. Quantification of the expression of ACLY, FAS, ACACA and other related genes such as REDD1, GSK3, STK11, GLUT1, HIF1A and IGF-1R in NNK treated SAS cells obtained using Image Lab software. GAPDH was used as control gene. Data are represented as Mean ± SE, *= p <0.05 vs untreated cells.

B. Effect of NNN on the expression of ACLY and related genes

The non-toxic proliferative concentration of NNN was obtained using MTT assay. SAS cells were treated with different concentrations of NNN such as 50nM, 100nM, 250nM, 500nM and 1000nM for 0hr and 24hr. A dose response curve was plotted for 24hr with respect to 0hr. It was observed that lower concentrations of NNN ranging from 50nM-1000nM enhances the rate of proliferation and do not cause any significant reduction in the proliferation of SAS cells (Figure 3.4A).



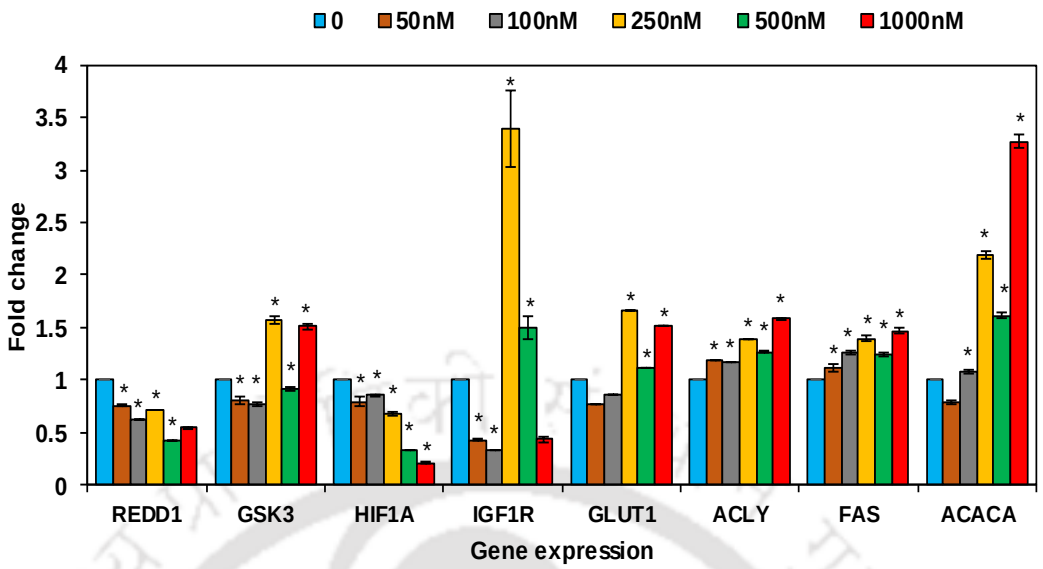


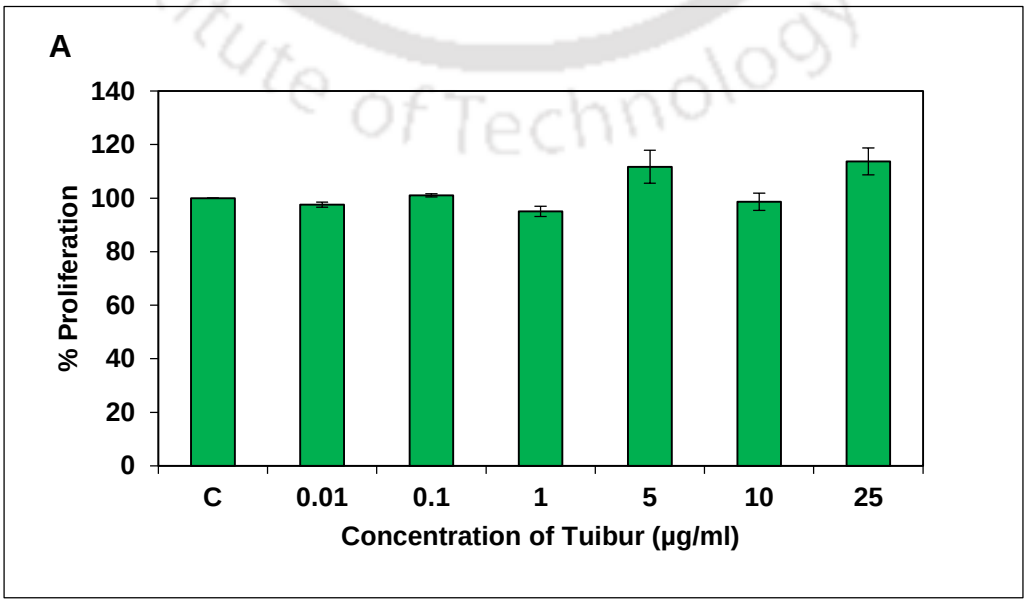
Figure 3.4. Effect of NNN on the expression of ACLY and related genes in SAS cells. A. MTT assay showing the non-toxic concentrations of NNN on SAS cells. B. Gene expression of ACLY and other related genes analysed using reverse transcriptase PCR. C. Quantification of the expression of ACLY, FAS, ACACA and other related genes such as REDD1, GSK3, STK11, GLUT1, HIF1A and IGF-1R in NNN treated SAS cells obtained using Image Lab software. GAPDH was used as control gene. Data are represented as Mean ± SE, *= p <0.05 vs untreated cells.

In addition, the total RNA from NNN (50nM, 100nM, 250Nm, 500nM and 1000nM) treated SAS cells was isolated and expression of ACLY and other genes was studied by semi-quantitative rT-PCR. The treatment with NNN upregulated the mRNA expression of ACLY in all the five concentrations of NNN as compared to the untreated control (Figure 3.4 B-C).

3.3.4. Effect of Tuibur on the expression of ACLY and related genes

Tuibur (tobacco smoke-infused water) is a watery tobacco product made by passing tobacco smoke through water [Sinha et al., 2004]. It is mainly consumed by the people in the state Mizoram, India and has been predicted to play a crucial role in increasing the risk of stomach cancer in the region [Phukan et al., 2005, Malakar et al., 2012]. An

experimental study generated by Allium test system showed that tuibur reduces the root growth in a dose-dependent manner and the effect was significant even at low concentrations [Mahanta et al., 1998]. Additionally, significant tumoral growth was also observed in the roots proximal to the tips. Tuibur promotes micronuclei formation, c-mitosis and lagging chromosomes leading to mitotic damage during the process of cell division [Mahanta et al., 1998]. Therefore, knowing the effect of tuibur on the expression of ACLY would result in new findings in tuibur induced oral carcinogenesis. Firstly, MTT assay was performed to measure the effect of tuibur on proliferation of SAS cells. After treatment with tuibur (0.01, 0.10, 1, 5, 10 and 25µg/ml) for 0hr and 24hr, the rate of proliferation of SAS cells was compared to the control group. A dose response curve was plotted for 24hr with respect to 0hr. It was observed that lower concentrations of tuibur ranging from 0.01µg/ml- 25µg/ml increases the rate of proliferation and do not cause any significant reduction in the proliferation of SAS cells (figure 3.5 A). Secondly, total RNA was isolated and expression of ACLY and other genes was studied by semi-quantitative RT-PCR. Treatment with tuibur upregulated the mRNA levels of ACLY in all the concentrations as compared to the untreated control (figure3.5 B-C).



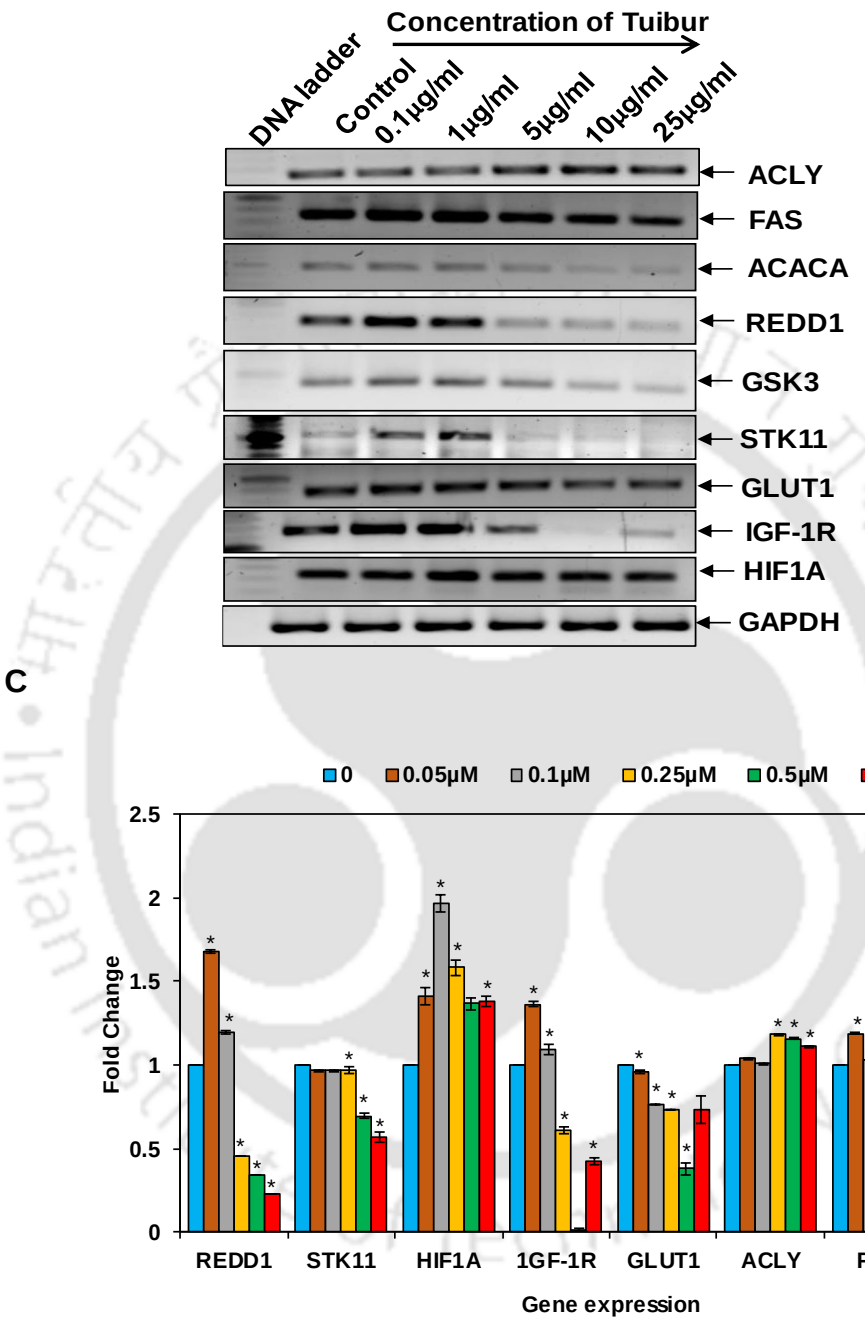


Figure 3.5. Effect of tuibur on the expression of ACLY and related genes in SAS cells. A. MTT assay showing the non-toxic concentrations of tuibur on SAS cells. B. Gene expression of ACLY and other related genes analysed using reverse transcriptase PCR. C. Quantification of the expression of ACLY, FAS, ACACA and other related genes such as REDD1, GSK3, STK11, GLUT1, HIF1A and IGF-1R in tuibur treated SAS cells obtained using Image Lab software. GAPDH was used as control gene. Data are represented as Mean ± SE, *= p <0.05 vs untreated cells.

3.4. Conclusion

It is evidenced that lipogenesis is a highly coordinated process, and perturbation at different steps of the lipogenic pathway can lead to greater modifications in the expression and activity of other metabolic genes [Migita et al., 2013]. Upregulation in the expression of ACLY have been observed in several cancers such as breast, bladder, colorectal, glioblastoma, lung cancer, ovarian and prostate cancer and inhibition of ACLY led to drastic changes in cancer metabolism that aids in the suppression of tumor growth and proliferation [Khwairakpam et al 2015]. In this chapter, we investigated the effects of tobacco and its components in OSCC on the expression of different genes in order to elucidate the role of ACLY in the several pathways. This is the first study reporting the role of ACLY in OSCC. SAS cells treatment with TE, NNK and NNN was found to upregulate the expression of ACLY, FAS and ACACA. ACACA and FAS is the lipogenic enzyme that takes part in fatty acid synthesis which is known to be upregulated in many cancer types [Migita et al., 2013]. We also observed the increase expression of IGF1-R, GLUT1 and HIF-1A on tobacco treated SAS cells and that increase expression of it initiates tumor proliferation and progression.

Moreover, we also observed the significant increase in the expression of GSK3 in SAS cells on treatment with tobacco. Further, we also found that the treatment of tobacco decreases the expression of LKBI. Studies have revealed that AMPK is the best recognized LKB1 downstream target [Miller et al., 2016]. We also observed reduction in the expression of REDD1 with the increase concentration of tobacco. REDD1 and its expression have been found to be downregulated in human cancers is a negative regulator of mTOR [Sahra et al., 2011]. Moreover, in our study, nicotine and tuibur has been found to increase the expression of ACLY, however reduce expression of genes such as ACACA, FAS, HIF1A, GSK3, GLUT1, LKB1 was observed. Our study

indicates that ACLY may affect several oncogenes and consequently its suppression may influence multiple downstream pathways. It is therefore important to study the mechanistic role of ACLY in tobacco induced OSCC.

Chapter 4

Role of ACLY proteins in the Development and Progression of OSCC

Chapter 4

4.1. Introduction

In our previous chapter, we found elevated expression of ACLY both in the levels of mRNA and protein in oral cancer cells. Significant upregulation in the expression of ACLY was observed in the oral cancer tissues as compared to the normal oral tissues. The expression of ACLY was increased with the different stages, grades and different TNM stages as compared to the normal tissues. Moreover, crude tobacco extract, tuibur (a product of tobacco) and tobacco carcinogens such as nicotine, NNK, and NNN noted to be the major risk factors of oral cancer was also found to instigate increased expression of ACLY and other lipogenic enzymes such as ACACA and FAS. Not only this, dysregulation in the expression of various oncogenes and tumor suppressor occurred on treatment with tobacco extract, tuibur and other tobacco carcinogens. Altogether, these studies provide us a clear idea that ACLY is involved in the positive regulation of oral tumorigenesis. Therefore, taken together, results of our preliminary studies proposed that deregulation of ACLY have a vital role in the initiation and progression of oral cancer. Furthermore, in order to elucidate the role of ACLY particularly and their downstream targets, it is crucial to silence the expression of ACLY protein. It is known that CRISPR-Cas9 system is an effective alternative approach to study the loss-of-function phenotypes thereby, manipulating the gene expression and function [Peretz et al., 2018]. Moreover, no study is reported so far on the role of ACLY in oral cancer. Therefore, in this chapter we have knockout ACLY in HSC-3 cells, a human tongue carcinoma cell line using CRISPR (Clustered regularly interspaced short palindromic repeats) /Cas9-method of gene editing and determined the effect of gene knockout on different hallmarks of cancer. Additionally, we also examined the downstream targets which are involved in oral tumorigenesis.

4.2. Materials and Method:

4.2.1. Chemicals

Fetal bovine serum (FBS; Gibco, Life Technologies, Paisley, PA49RF, UK), trypsin-Ethylenediaminetetraacetic acid (EDTA) (1×; 0.25%; Gibco, Life Technologies, USA), penicillinstreptomycin (Penstrep; Gibco, Life Technologies, USA), Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Paisley, PA49RF, UK), minimum essential medium Eagle (MEM; Gibco, Life Technologies, Paisley, PA49RF, UK), phosphate buffer saline (PBS; Cell Clone), dimethyl sulfoxide (DMSO; Emplura® Merk Life Sc. Private Limited, Mumbai, India), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich),

4.2.2. Bacterial culture and plasmid isolation

The DH5α *E. coli* strains were used to maintain and amplify small plasmid DNA. The competent DH5α was prepared in the laboratory using $CaCl_2$ and finally the plasmids were transformed into the competent DH5 α *E. coli.* The clones that were resistant to carbenicillin were cultured and the plasmids were isolated from the strain using HiPurA™ PCR Product Purification Kit, Ref. MB512-50PR from Himedia, Mumbai, India.

4.2.3. Cell culture

Aforementioned in the previous chapter 2, different human OSCC cell lines such as SAS, and HSC-3 were used for the study.

4.2.4. Gene knockout *via* CRISPR/cas9

CRISPR are principally advantageous for investigations of cell biology and mammalian genetics, as the mammalian somatic cells have generally demonstrated to be highly refractory to genetic alteration [Giuliano et al., 2019]. The expression of the ACLY

gene was disrupted using CRISPR/cas9 system of gene editing. The commercially available ACLY sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat No: K0029305) and scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human) (Cat No. K010) were purchased from Applied Biological Materials (abm), Canada. The sgRNA target sequences are given in the table 1. The SAS and HSC-3 cells were seeded in the 24-well plates at a cell density of $5x10^3$ cells/500µL. When the cells are nearly 60-70% the cells were transfected with 0.5 to 1µg of each plasmids using Lentifectin™ transfection reagent (Cat no. G074) from Applied Biological Materials (abm), Canada in incomplete opti-MEM media. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 5-8hr. Then, 10% FBS (Gibco ®, NY, USA) was added and incubated for 24h. After incubation for 24hr, the media containing plasmids were removed from the wells and fresh MEM media containing 10% FBS and 1% penstrep was added and incubated for recovery of the transfected cells. When the cells recovered, positive selection of the cells was done using 1.5µg/mL of puromycin (Cat. No. P8833). The transfected cells are resistant to puromycin, as the ACLY CRISPR/cas9 carries a puromycin resistance gene, while the non-transfected cells will be deselected. The puromycin resistant cells were cultured until it forms colonies and then single cell selection of the transfected cells was done to obtain the clones of ACLY CRISPR/cas9. Then, western blot analysis was performed to confirmed the knockout from the clones obtained after single cell selection. Those clones that showed complete inhibition of ACLY expression were considered successful knockout of the ACLY gene and they were taken and grown in the culture for further studies. Moreover, the clones were also freeze and stored in -80°C for future use.

Table 4.1. sgRNA target sequences

Gene	Target	sgRNA target sequences
ACLY	Target 1	9CAAGGCAATTTCAGAGCAGA
	Target 2	78GAATCGGTTCAAGTATGCTC
	Target 3	178GACCAGCTGATCAAACGTCG
Scrambled	-	GCACTCACATCGCTACATCA

4.2.5. Proliferation assay

The consequence of ACLY knockout on the proliferation of HSC-3 cells was investigated using MTT assay. Here, the scrambled sgRNA transfected cells (CRISPR/Cas9 scrambled) and the ACLY knockout HSC-3 cells (CRISPR/Cas9 ACLY) were seeded in 96-well plates with cell density of 2 $\times 10^3$ cells/100µL and incubated overnight. The MTT (Cat. no. M6494, Invitrogen) was performed for 0 hr, 24 hr and 48 hr. Subsequently, 10µL of MTT (5mg/mL) was added to each well and incubated for 2hr at 37°C at each time point mentioned above. Then, the supernatant was from the wells was discarded, and 100µl DMSO (Cat. No. 1.16743.0521, Merck, Darmstadt, Germany) was added to dissolved the precipitate. Lastly, the absorbance of the colored solution was taken at 570nm using the microplate reader (TECAN Infinite200 Pro multimode reader, Switzerland). The inhibition in the proliferation of HSC-3 cells due to knockout of ACLY was calculated by normalizing the absorbance value of 24h with 0h and also considered the absorbance of scrambled as 100%.

4.2.6. Colony forming assay

The outcome of ACLY knockout in the colony forming ability of HSC-3 cells was studied via colony forming assay. The CRISPR/Cas9 scramble HSC-3 cells and

CRISPR/Cas9 ACLY HSC-3 cells were seeded in a 6-well plate at a density of 500 cells/2mL. The media from the wells were replaced with fresh media after two days and cells incubated for 10-12days. Then, the cell were rinsed with 1x PBS after removing the media from the wells. It was then fixed in absolute ethanol for 1hr and stained with 0.01% (w/v) crystal violet (cat no. 548-6209, SRL Pvt. Ltd, India) for 5mins. Then, the clear image of the plate was taken and analyzed using ImageJ software. Then the survival fraction was determined using the following formula:

Plating efficiency (PE) = (No. of colonies counted/No. of cells plated) x 100

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

4.2.7. Migration assay

The migration ability of the CRISPR/Cas9 ACLY HSC-3 cells as compared to the CRISPR/Cas9 scramble HSC-3 cells was investigated using the migration or the wound healing assay. For this, CRISPR /Cas9 scramble and CRISPR/Cas9 ACLY HSC-3 cells were plated at a density of $5x10^5$ cells/2mL in a 6-well plate and were grown to confluency till the formation of monolayer. Then, for 7-8hrs the cells were serum-starved and a vertical scratch was created in the center of the culture well. The wells were washed with 1xPBS twice in order to eliminate the debris. The images were taken using at regular intervals (0hr, 24hr, and 48hr) during the migration of the cells to close the wound. Further, ImageJ software was utilized to estimate the wound area.

4.2.8. Western blot analysis

The western blot analysis was used to determine the successful gene knockout and its downstream targets of ACLY. As mentioned in chapter 2, section 2.2.4, page no.52. similar protocol for western blot analysis was performed to investigate the role of the

ACLY protein in tumorigenesis of HSC-3 cells. 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

Antibodies	Cat. No.	Company	Dilution
Anti- ACLY	13390BC	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti- p-ACLY (T447+S451)	Ab53007	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti- p-ACLY (S455)	4331BC	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti-GAPDH antibody	2118S	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti-Cox-2 antibody	12282P	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti- Jak1 antibody	3344	Cell Signalling Technologies, Danvers, MA, USA	1:1000
Anti- p-Jak1 antibody	74129	Cell Signalling Technologies, Danvers, MA, USA	1:1000
Anti-p-MAPK antibody	3424	Cell Signalling Technologies, Danvers, MA, USA	1:1000
Anti-VEGFA antibody	ab46154	abcam®, Cambridge, USA	1: 4000
Anti-MMP-9 antibody	13667P	Cell Signalling Technologies, Danvers, MA, USA	1:1000
Anti-survivin antibody	2808BC	Cell Signalling Technologies, Danvers, MA, USA	1:1000
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-Akt1	2938S	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti-p-Akt (Thr 308)	9275S	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti-p-Akt (Ser473)	4060S	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti-cyclin D1	2978BC	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti- E-cadherine	3195	Cell Signalling Technologies, Danvers, MA, USA	1:2000
Anti-EGFR	4267	Cell Signalling Technologies, Danvers, MA, USA	1:2000

4.3. Results and Discussion

The dysregulation of multiple signaling molecules and metabolic alternations are the hallmark of malignant cells which includes uncontrolled proliferation and growth, resistant to cell death, replicative immortality, angiogenesis, invasion and metastasis [Osugi et al., 2015]. Glycolysis and lipogenesis are necessary metabolic processes critical for the growth and maintenance of tumor cells [Osugi et al., 2015]. The alterations in DNL dysregulated the expression of enzymes intricated in fatty acid biogenesis [Mashima et al., 2009; Bauer et al., 2005; Khiewkamrop et al., 2018]. Interestingly, in our study we have demonstrated an association of ACLY proteins with the development and progression of oral cancer. However, to confirm our hypothesis it is crucial to established the evidence of the involvement of ACLY proteins in the modulation of cancer hallmarks. To achieve our objectives, we used CRISPR/Cas9 based gene silencing method to knockout ACLY in HSC-3 cells, a human tongue carcinoma cell line.

4.3.1. Successful knockout of ACLY as confirmed by Western blot

The application of CRISPR/Cas9-mediated gene editing enabled the successful knockout of ACLY in HSC-3 cells. The transfection with target 3 sgRNA/Cas9 plasmids generated successful ACLY knockout HSC-3 clones and a scrambled sgRNA was used to produce scrambled control cells. Further, western blot analysis was performed to confirmed the knockout of ACLY proteins in HSC-3 cells (Figure 4.1B) in comparison with SCR and these clones were multiplied in the cell culture and used for further experiments.

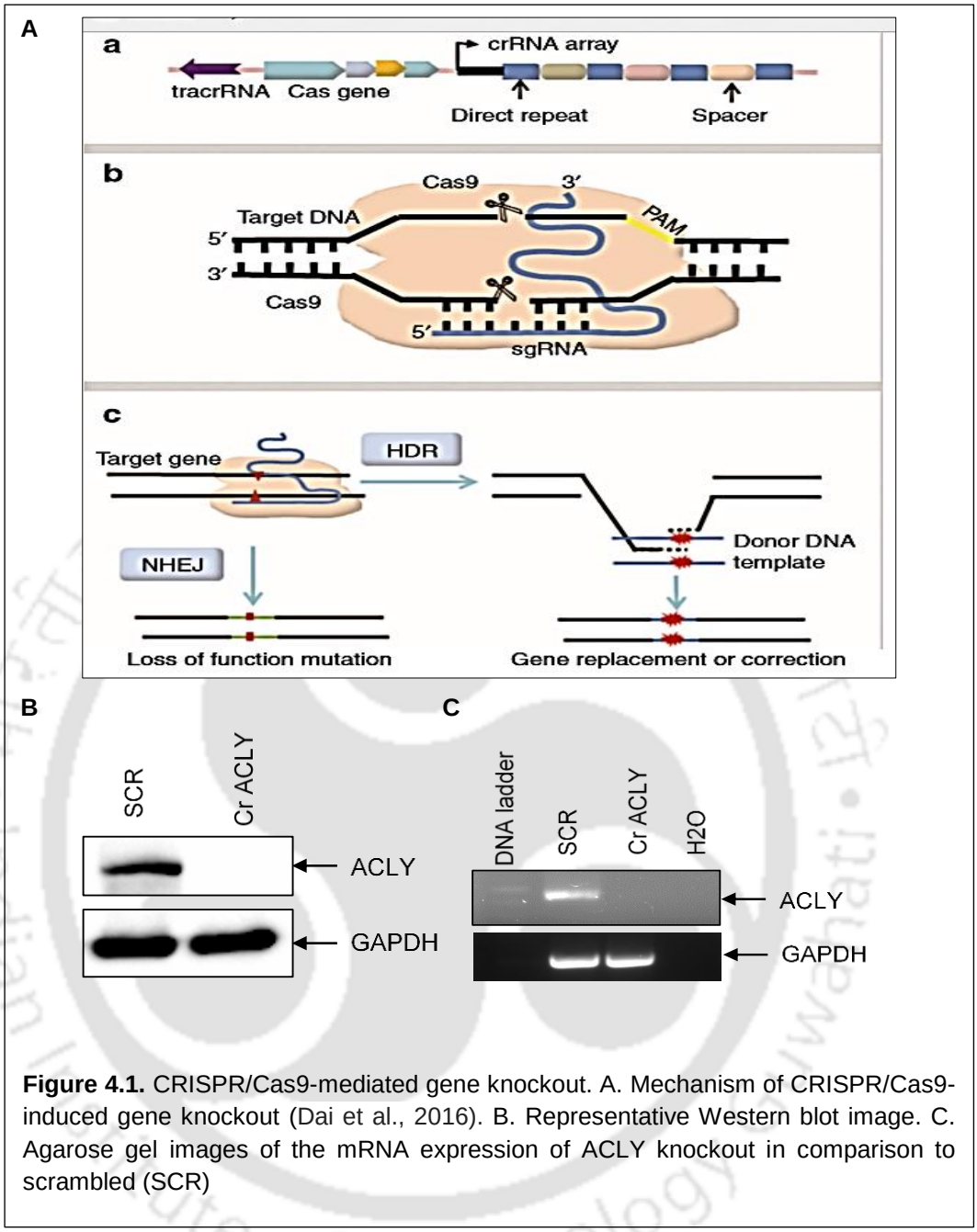


Figure 4.1. CRISPR/Cas9-mediated gene knockout. A. Mechanism of CRISPR/Cas9-induced gene knockout (Dai et al., 2016). B. Representative Western blot image. C. Agarose gel images of the mRNA expression of ACLY knockout in comparison to scrambled (SCR)

4.3.2. ACLY knockout decreased the proliferation of oral cancer cells

It has been reported that the unchecked growth and proliferation of cells are considered as the first characteristic phenotypic feature of cancerous cells [Farber, 1995]. Here, we examined the effect of ACLY knockout on the proliferation of HSC-3 cells using MTT assay. The ACLY knockout HSC-3 cells showed reduced proliferation rate of nearly 50% as compared to scrambled control (figure 4.2). There are several reports which

demonstrated that the inhibition in expression of ACLY reduces proliferation and growth of tumor cells [Khwairakpam et al., 2015]. This is the first report showing that the knockout of ACLY induced reduction in the proliferation of OSCC cells.

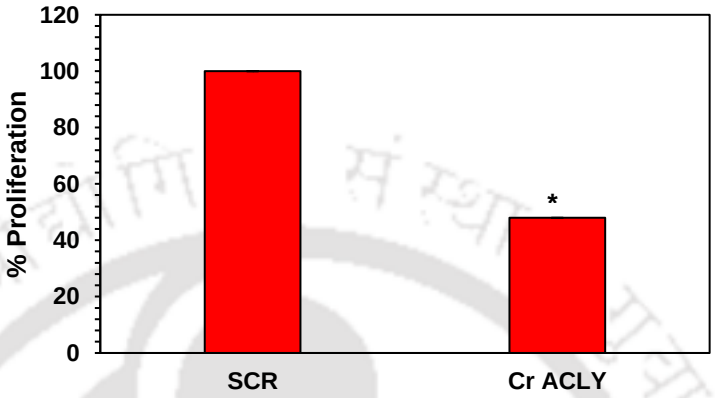


Figure 4.2. Effect of CRISPR/Cas9-mediated deletion of ACLY proteins on the proliferation of HSC-3 cells was evaluated by MTT assay and the percentage proliferation was calculated in comparison to SCR control HSC-3 cells. Data are mean ± *=p<0.05.

4.3.3. ACLY knockout inhibited the clonogenic potential of oral cancer cells

It is well known that increase in survival, uncontrolled proliferation, resistant to cell death and replicative immortality are some of the important characteristics of tumor cells. Here we studied the effect of ACLY knockout on the survival and proliferation of HSC-3 cells in comparison to SCR control cells using clonogenic assay. We observed significant reduction in the colony forming ability of the ACLY knockout cells as compared to scramble control (figure 4.3A-B). It shows that ACLY has an important role in the survival and proliferation of OSCC cells.

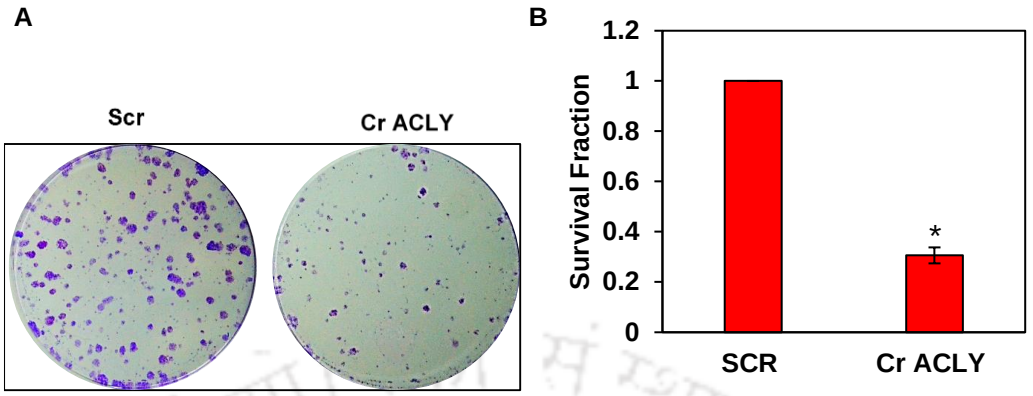


Figure 4.3. Effect of CRISPR/Cas9-mediated deletion of ACLY proteins on the survival and clonogenic potential of HSC-3 cells was estimated by colony forming assay. (A, B) Representative image and graphical illustration of the survival fraction of Cr ACLY HSC-3 cells as determined by colony forming assay. Data are mean ± *=p<0.05.

4.3.4. ACLY knockout inhibits the migration potential of oral cancer cells

It has been evidenced that the advanced stages of oral cancer are often associated with poor prognosis. It is therefore requisite to determine the effect of ACLY proteins on the invasion and migratory ability of HSC-3 cells in order to investigate their involvement in the initiation and progression of the disease. Hence, the migration potential of the knockout clones of ACLY proteins as compared to SCR control cells were investigated using in vitro wound healing assay. Interestingly, the loss of expression of ACLY protein considerably decreased the migration of oral cancer cells (figure 4.4 A-B).

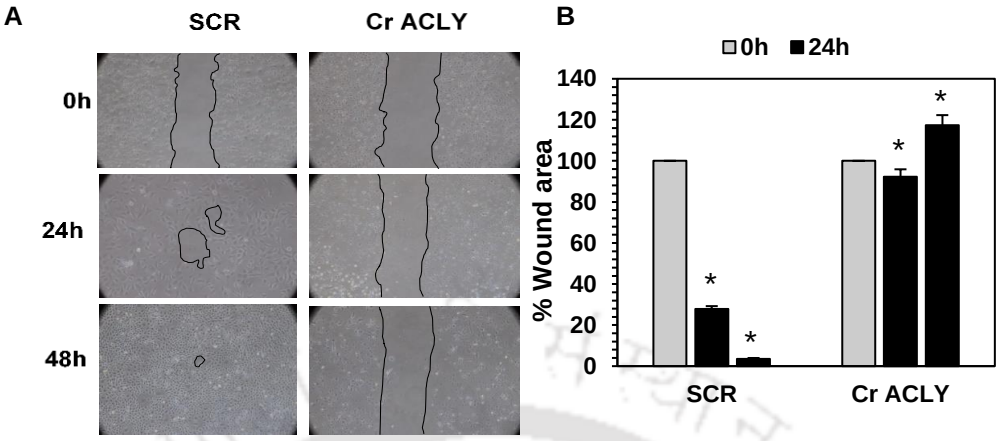


Figure 4.4. Effect of CRISPR/Cas9-mediated deletion of ACLY proteins on the migration of HSC-3 cells was measured by in vitro scratch/ wound healing assay. A, B) Representative image and graphical illustration on the effect of ACLY knockout on the migration potential of HSC-3 cells as determined by wound healing assay.

4.3.5. ACLY knockout downregulated the expression of proteins involved in the growth survival, proliferation, angiogenesis and migration of OSCC cells *via* Akt/mTOR/S6 pathway

We examined the causal mechanism upon knockout of ACLY proteins in HSC-3 cells. There are reports which states that a rise in the phosphorylation of ACLY trigger stabilization of the protein *via* kinases, such as Akt, PKA, GSK-3, and cAMP-dependent protein kinase [Pinkosky et al., 2017]. Therefore, we investigated the phosphorylation of ACLY at the serine 455 and threonine 447+ serine 451 residues after ACLY knockout. Interestingly, total inhibition in the above phosphorylation of ACLY was observed upon deletion of the ACLY protein (4.4 A-B). This shows that ACLY knockout also inhibited the phosphorylation of the protein. Moreover, western blot analysis showed that knockout of ACLY reduced the expression of key proteins involve in the tumorigenesis of OSCC such as cyclin D1, survivin, COX2 and VEGF (figure 4.6C-D).

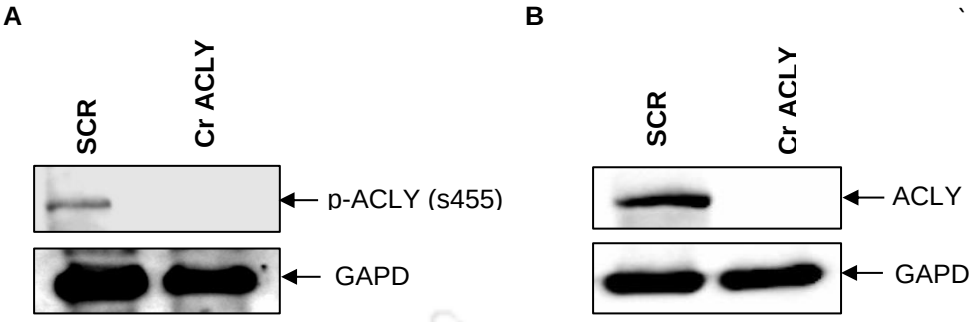


Figure 4.5. Effect of CRISPR/Cas9-mediated deletion of ACLY proteins on the phosphorylation of ACLY proteins. A. phospho-ACLY (ser455). B. phospho-ACLY (T447+S451) as determined by western blot analysis.

The survivin protein is known to be responsible for tumor cell survival; COX2 and cyclin-D1 in proliferation; VEGF-A in angiogenesis tumor cells. In addition, decreased expression of anti-apoptotic proteins such as XIAP and cIAP was found to be downregulated (figure 4.6 C). In our study, overexpression of the E-cadherin protein was observed upon knockout of ACLY in the oral tumor cells (figure 4.6 D). Besides, in the epithelial tissues, E-cadherin has been marked as remarkable molecule participating in the cell-cell adhesion [Pećina-Slaus, 2003]. The expression of E-cadherin has been involved in tumor metastasis, often as a tumor suppressor, but also as a promoter of growth and metastasis [Na, 2020]. Further, reduction in the expression of phospho-S6 was observed in the ACLY knockout HSC-3 cells. This ribosomal protein S6 (RPS6) is known to be a downstream effector of the mTOR which is stimulated in various cancers. Additionally, higher frequency of phosphorylated RPS6 has been reported in oral epithelial dysplasia tissue samples as compared to control indicating its correlation with the early events of tumor progression in OSCC [Chaisuparat et al., 2013]. We also found that the loss of ACLY protein downregulated the expression of Akt1, Akt2 and mTOR in OSCC cells [Figure 4.6 A-B]. Akt and

mTOR are decisive to many aspects of cell growth, survival, and in physiological as well as in pathological conditions like cancer [Porta et al., 2014]. However, no change in the expression of phospho- Akt (ser473) was observed. Our finding suggested that the loss of ACLY might suppressed the early development and progression of the OSCC via suppression of Akt/mTOR/S6 pathway thereby inhibiting the cell survival, proliferation, angiogenesis and migration of OSCC cells.

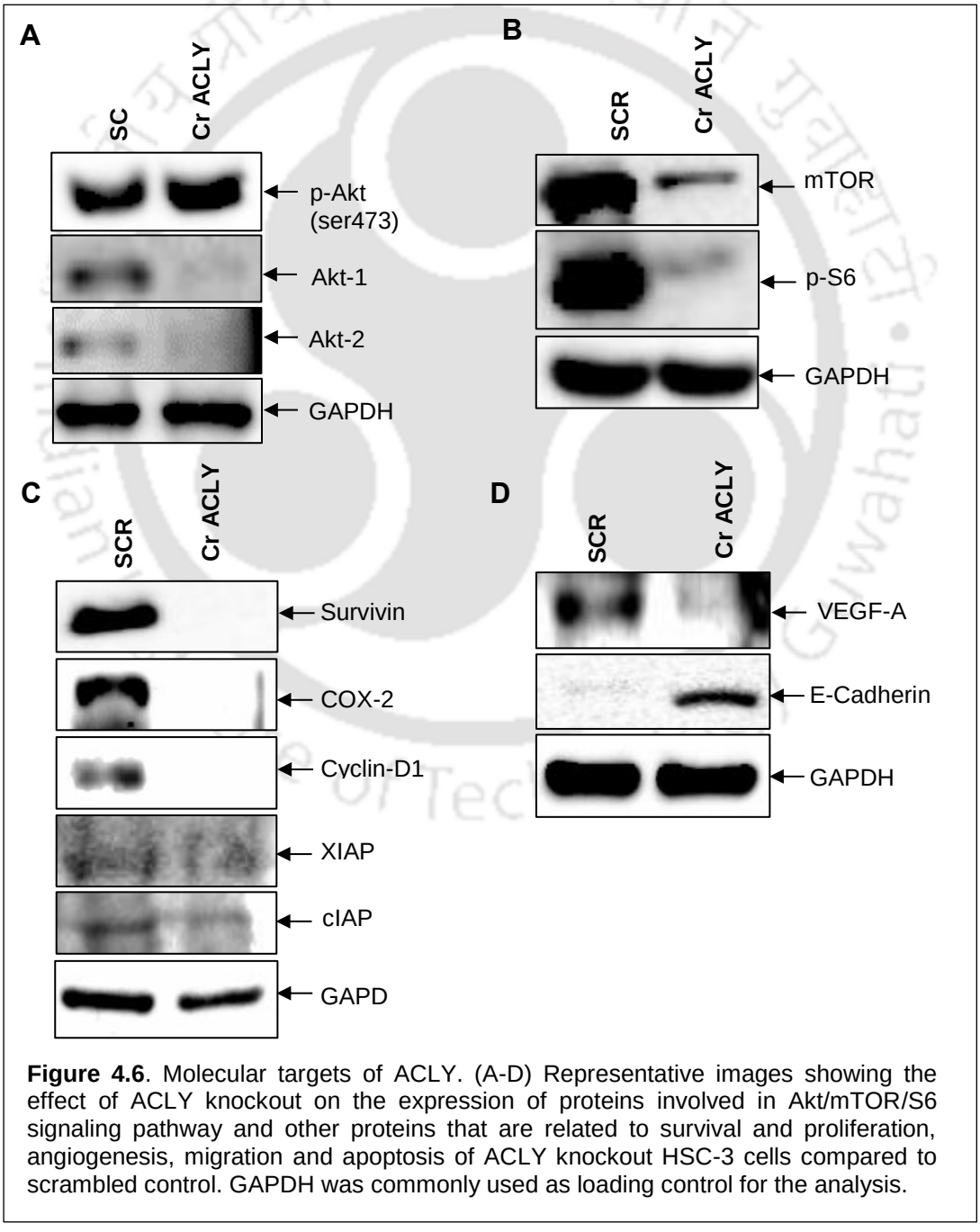


Figure 4.6. Molecular targets of ACLY. (A-D) Representative images showing the effect of ACLY knockout on the expression of proteins involved in Akt/mTOR/S6 signaling pathway and other proteins that are related to survival and proliferation, angiogenesis, migration and apoptosis of ACLY knockout HSC-3 cells compared to scrambled control. GAPDH was commonly used as loading control for the analysis.

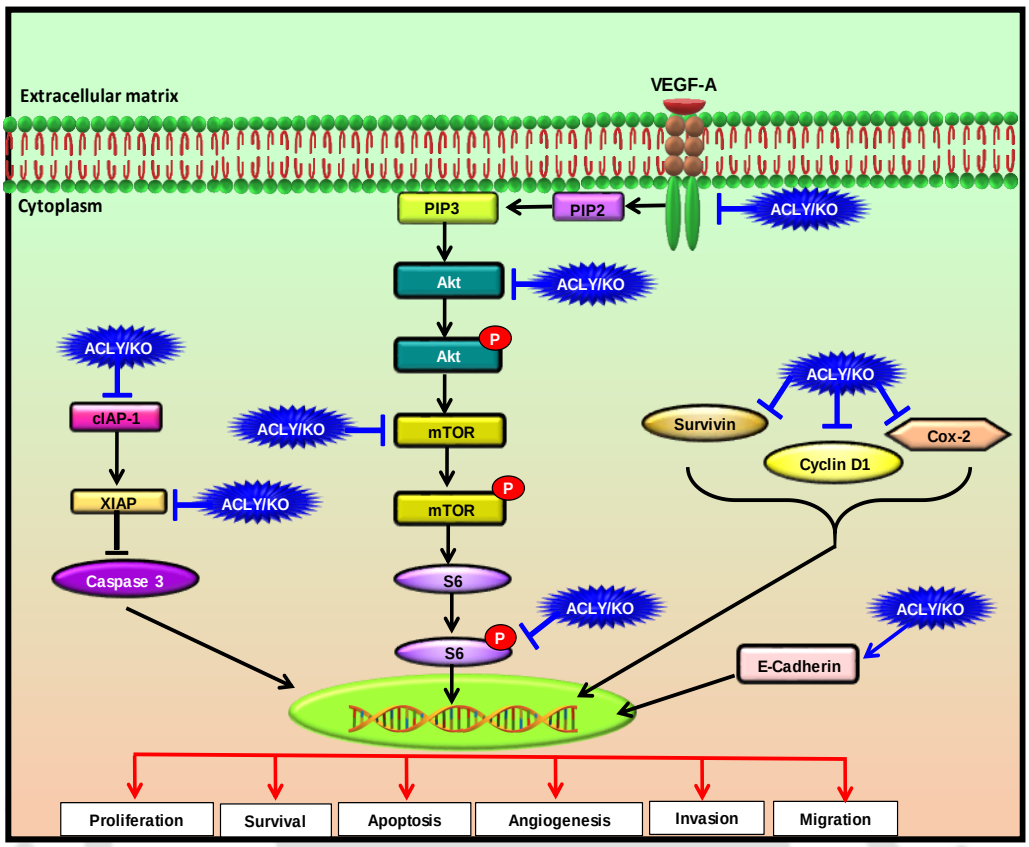


Figure 4.7. Molecular targets of ACLY. The knockout of ACLY in HSC-3 oral cancer cells induced reduction in the expression of important anti-apoptotic proteins and other proteins involved in tumor cell survival, proliferation, angiogenesis, metastasis thereby affecting the Akt/mTOR/S6 pathway

4.4. Conclusion

Oral cancer is one of the deadliest cancers of the world. Over 90% of OSCC have been found particularly in patients of middle aged to senior citizens who often smoke and drink excessive. The main etiologic factors in the carcinogenesis of oral cancer are tobacco usage and alcohol consumption. Several researchers have found that fatty acid metabolism is impaired in many human diseases including cancer. Therefore, there is an urgent need to identify an important biomarker and chemotherapeutic agent to fight against this disease. Several studies have found that the upregulated expression of ACLY is correlated with the escalation of lipid biosynthesis thereby promoting

uncontrolled growth of cancerous cells. It has been evidenced that inhibition of ACLY leads to changes in cancer cell metabolism thereby reducing the process of tumorigenesis in different cancer types. cancer. In our previous studies using tissue micro array, a highly upregulated expression of the ACLY was observed in the oral tumor tissues in comparison to the normal and non-cancerous tissues of the oral cavity signifying its crucial role in the development and progression of this deadly disease. It correlated with the results of our previous objective where we showed that treatment with tobacco and tobacco components resulted in the increased expression of ACLY in OSCC cells. i.e. an increased mRNA levels of ACLY was observed in tobacco carcinogens treated SAS cells as compared to untreated cancer cells. Additionally, we have also found that tobacco and its components induced increased expression of oncogenes and decreased expressions of the tumor suppressor genes that might be involved in the lipogenic pathway. All these results showed that tobacco and its related compounds might be responsible for the induction of tumor growth in oral cancer through the regulation of the ACLY expression. Therefore, to further uncover the exact role of ACLY in OSCC and determine its pathway in the development and progression of oral cancer we suppress the expression of ACLY using CRISPR/Cas9-mediated gene editing system. Here, we observed that the successful knockout of ACLY leads to significant reduction in the growth, survival, proliferation, angiogenesis, and migration of HSC-3 oral cancer cells. Further studies also showed that loss of ACLY inhibited the important signaling molecules such as Akt1, Akt2, mTOR, and p-S-6 thereby downregulating the expression of the important proteins such as survivin, cyclin-D1, COX-2, VEGF-A, (oncogenes) and anti-apoptotic genes such as cIAP and XIAP. Moreover, increased expression of E-cadherine (tumor suppressor, the loss of which is involved in the formation of epithelial types of cancers such as carcinomas) was observed

upon knockout of ACLY. Therefore, this studies indicated that ACLY plays important role in tumor cell growth, survival, proliferation, angiogenesis and migration of oral cancer in human.

Chapter 5

Discussion and Conclusion

Chapter 5

5.1. Discussion and Conclusion

Cancer of the buccal cavity is one of the highly prevalent cancers worldwide [Gupta et al., 2016]. Some of the characteristics of the disease includes late diagnosis, high mortality rates and morbidity [Gupta et al., 2016]. There are several risk factors that leads to the development of oral cancer such as alcohol and areca nut, excessive intake of tobacco, smoking, smokeless tobacco (snuff or chewing tobacco), reverse end smoking, HPV, and sunlight exposure [D'souza et al., 2018]. The potentially malignant lesions are also one of the important risk factors of oral cancer. These precancerous lesions are a chronic disease of oral mucosa and are accountable for nearly 20% of oral cancer [Paré et al.,2017]. Despite of the significant advances in the treatment modalities of OSCC in chemotherapy, radiotherapy, targeted therapy, and surgery, the 5-year survival rate is still <60% i.e., it has not improved over the last decades due to high recurrence rate and metastasis. It has been shown that oral cancer metastasizes to the cervical lymph nodes frequently [Sasabe et al., 2017]. Oral neoplasm is also largely attributable to late diagnosis owing to the asymptomatic nature of the ailment in the early stages [Saraswat et al., 2020]. Moreover, the poor survival rates of oral cancer patients are commonly due to susceptibility for early spread, the absence of effective tools for screening and early diagnosis and the incapability of systemic therapy for the treatment of the metastatic disease. Therefore, there is an acute need to identify and develop novel biomarkers for the proper diagnosis and prognosis which can accelerate the actual management of this aggressive disease.

Accumulating evidence have shown that the expression of ACLY are closely related to tumorigenesis and development of various cancer types such as breast cancer, bladder cancer, brain cancer, colon cancer, lung cancer, hepatocellular carcinoma,

ovarian and prostate cancer etc. [Khwairakpam et al., 2015]. Aforementioned, ACLY produces OAA and acetyl-CoA from cytoplasmic citrate, the latter is known to be the building block of fatty acid synthesis [Migita et al., 2014]. This enzyme usually forms crossroads between the fatty acid synthesis pathway and glucose metabolism [Khwairakpam et al., 2020]. In addition, the overexpression of ACLY is linked with increase in lipid biosynthesis that promotes abnormal growth of tumor cells [Granchi, 2018]. It has been evidenced that inhibition of ACLY leads to changes in cancer cell metabolism thereby, reducing the process of tumorigenesis in different cancer types [Khwairakpam et al., 2015]. Additionally, ACLY expression was augmented in melanoma and facilitated the tumor cell proliferation and growth in both the *in vitro* and *in vivo* conditions [Guo et al., 2020]. Migita et al., have demonstrated that the diminution of ACLY partly reduced the mitochondrial fatty acid transporter carnitine palmitoyltransferase 1 (CPT1A) due to reduction in the PPARα thereby, obstructing fatty acid transport in the mitochondria [Migita et al., 2014]. Further, studies have shown that depletion of ACLY in tumor cells blocks the fatty acid elongation and suppresses the tumor growth and proliferation and induced apoptosis accompanied by accumulation of triglycerides with altered fatty acid composition [Migita et al., 2014]. Therefore, we hypothesized ACLY to be involved in the initiation and progression of oral cancer. To validate our hypothesis, firstly we analyzed the expression of ACLY protein in tissue samples of human with the assistance of oral cancer TMA which comprises of tissues from different developmental stages and grades of oral cancer along with preneoplastic lesions as well as normal oral epithelial tissues. We found increased expression of ACLY protein in the oral malignant tissues as compared to the normal oral tissues. Additionally, we also observed significantly high mRNA level of ACLY in tumor cells as compared to normal cells. This is the first report that shows the

upregulated expression of ACLY and p-ACLY in oral cancer. The overexpression of ACLY have been reported in various types of human cancers such as glioblastoma, CRC, breast cancer, lung cancer, and HCC etc. which has been found to be associated with the increase in lipid synthesis and tumor progression [Khwairakpam et al., 2015, Wang et al., 2017]. This increase in the level of ACLY was found to be correlated with the development and progression of oral tumorigenesis. Further, our studies have shown increased expression of ACLY as well as p- ACLY in the different developmental stages of oral tumorigenesis such as inflammation, hyperplasia, benign, malignant and metastatic oral tumor tissues. Moreover, we also showed upregulation in the expression of ACLY and p-ACLY with the increase in stage and grade of oral cancer when compared to the normal oral tissue. In line with our findings, Wen et al. have investigated that ACLY elevated the migration and invasion potential of colon cancer *via* interacting and stabilizing the CTNNB1 protein thereby, instigating the transcriptional activity of CTNNB1 [Wen et al., 2019]. Similarly, the elevated level of ACLY in gastric adenocarcinoma was associated with lymph node metastasis and advanced stages [Wen et al., 2019]. Additionally, the p-ACLY expression levels were identified as a factor for predicting poor prognosis in NSCLC, jointly with clinical stage and tumor size [Wen et al., 2019]. Likewise, Teng et al. have observed remarkably higher protein and mRNA expression of ACLY in RCC tissues as compared to the normal adjacent tissues [Teng et al., 2018].

Secondly, consumption of tobacco and its product are important causal agents of oral cancer. In our study, we investigated whether tobacco extract, tobacco product and tobacco components or carcinogens has any effect on the expression of ACLY, tumor suppressors and oncogenes that are involved in the cancer metabolism. Our investigations have found that the mRNA expression of ACLY was upregulated

significantly on treatment with tobacco. Moreover, the expression of the oncogenes such as HIF1A, GLUT1, IGF-1R etc. was found to be upregulated on treatment with tobacco extract. Further, the expression of the tumor suppressor genes such as REDD1, GSK3 and STK11 was found to be downregulated on treatment with crude tobacco extract, tuibur, and tobacco components such as nicotine, NNK and NNN.

Interestingly, studies have found that the reduction of ACLY expression in tumor cells increases the level of intracellular reactive oxygen species (ROS) that leads to phosphorylation of AMP-activated protein kinase (p-AMPK), a significant regulator of lipid metabolism [Migita et al., 2013]. ROS is known to activate hypoxia-inducible factor-1α (HIF-1α). Semenza firstly described HIF-1α in 1992 and that its expression is tightly regulated by low oxygen tension [Ma et al., 2018]. HIF-1 is a central regulator of responses to hypoxia; it augments the metastasis-associated factors like matrix metalloproteinases and VEGF [Aga et al., 2016]. In addition, it has been reported that the increase expression of Glut-1 may be interrelated with poor prognosis in OSCC [Harshani et al., 2014]. It has been evidenced that NNK and NNN induce tumorigenesis by triggering DNA adductions and mutations [Xue et al., 2014]. NNK treatment in NSCLC cell lines have been found to instigate proliferation and suppressed apoptosis mediated by chemotherapy via activation of phosphatidylinositol 3-kinase-AKT pathway and NF-κB. Moreover, studies have shown that the treatment NNK in immortalized human bronchial epithelial cells triggers the ERK1 and ERK2 pathway which initiates the transcription of NF-κB, STAT1, and GATA binding protein 3 (GATA3), whereas NNN activates only GATA3 and STAT1 [Xue et al., 2014]. Similarly, tuibur (tobacco smoke–infused water) has an alkaline pH that maintains an unprotonated form of nicotine which can absorb effortlessly over the oral mucosa [Madathil et al., 2018]. Tuibur is mainly used in Mizoram, that has been found to

increase the risk of stomach cancer [Phukan et al., 2005]. The treatment of tuibur caused increased expression of ACLY but not FAS and ACACA. Tuibur increased the expression of HIF1A. Contrastingly, tuibur downregulated the expression of GLUT1, and IGF1R. Moreover, it downregulated the expression of REDD1, and STK11 in a dose-dependent manner. Studies have shown that the increase in expression of GLUT1 in conjunction with higher mTOR signaling has been found to stimulate the key oncogenic drivers, such as c-MYC and HIF-1α [Tan, 2019].

Altogether, our findings confirmed that ACLY is involved in the oral cancer development and progression and fortified our IHC results showing upregulation of ACLY and p-ACLY in oral tumor tissues when equated to normal tissues. In addition, increased expression of *ACLY* was observed upon exposure to oral cancer causative agents such as tobacco, tobacco product and its carcinogens. Although our studies have evidenced the association of ACLY proteins in oral cancer, there is a need to investigate for more validation with this preliminary idea to obtain the signaling pathways intricated in the ACLY mediated oral tumorigenesis and their implication in the modulation of various hallmarks of cancer. To accomplish our studies, we produced knockout clones for ACLY gene using CRISPR/Cas9-mediated genome editing in HSC-3 cell line. In support to our earlier hypothesis, knockout of ACLY significantly reduced the proliferation, survival and migration of oral tumor cells. The knockout of ACLY resulted in significant reduction in the proliferation of HSC-3 cells as compared to the scrambled control (SCR). In line with our results, cell proliferation was reduced considerably in lung cancer cells treated with LY294002 or ACLY siRNA compared with negative control siRNA [Migita et al., 2008]. It has been revealed that elevated expression of ACLY is a prognostic factor for worse overall survival in a cohort of HNSCC patients [Göttgens et al., 2019]. In addition, the suppression of ACLY

particularly prevented the proliferation and growth of NSCLC tumor cells and also induced growth arrest due to intracellular accumulation of lipid [Migita et al., 2008]. The depletion of ACLY also suppressed the tumor growth in breast cancer cells [Wang et al., 2017]. Further, silencing of ACLY has been found to inhibit proliferation, thereby promoting tumor cell differentiation and senescence in colon tumor cells [Wen et al., 2019]. In our study, the survival of HSC-3 cells after knockout of ACLY was determined using colony formation assay. There was remarkable reduction in the clonogenic potential of HSC-3 as compared to the scramble control indicating that ACLY played a crucial role in increasing the survival fraction of HSC-3 cells *in vitro*. Metastasis is a multistep process that includes migration and invasion of cancer cells which are the hallmarks of malignancy. Moreover, cell migration in itself is an extremely integrated process which includes increase of cytoplasmic protrusions, attachment, and traction [Tahtamouni et al., 2019]. In our study, we analyzed whether ACLY knockout has any effect on the migration of the HSC-3 cells using wound healing assay. Here, we found that loss of ACLY suppressed the migration ability of HSC-3 cells as compared to the scramble control. Similar to our finding, studies have shown that the treatment of MCF-7 cells with ACLY siRNA significantly enhanced the apoptosis of the tumor cells. Further experiments also indicated that ACLY deletion also reduces the cell viability [Wang et al., 2017]. Taken together, ACLY is found to be involved in proliferation, survival and migration as well, showing their critical role in the initiation and progression of oral cancer. Markedly, there are various molecules or pathways associated with the cancer metabolism. Here, we investigated the different target proteins that are involved in the proliferation, survival and migration with the help of western blot analysis. Knockout of ACLY has been found to greatly reduced the expression of the anti-apoptotic proteins such as survivin, cIAP, and XIAP. These

proteins belong to Inhibitors of apoptosis (IAP) family of proteins. Any dysregulation in IAP function has an obvious association with cancer development, induction of oncogenesis or drug resistance [Garg et al., 2016]. The absence of ACLY also reduced the expression of COX-2 and cyclin- D1 that are involved in proliferation of tumor cells [Khwairakpam et al., 2019]. Cyclin D1 gene overexpression and amplification have been shown to play a role as prognostic factors in many human cancers [Lim et al., 2003]. COX-2 has a significant role in the promotion of carcinogenesis, tumor invasion and angiogenesis [Lim et al., 2003]. Higher expression of COX-2 is found to be correlated with an augmented production of prostaglandins (PGE2) which modulates tumor cell proliferation, cell death, and tumor invasion in various types of cancer [Sobolewski et al., 2010]. Moreover, inhibition of COX-2 in tumor cells caused G2/M arrest and upregulated the expression of p53 [Sobolewski et al., 2010]. Vascular endothelial growth factor (VEGF) is a proangiogenic factor that is up-regulated in various tumors [Lim et al., 2003]. The elevated COX-2 or VEGF expression or cyclin D1 overexpression were found to be more common in breast cancer patients with poor prognostic characteristics and partly associates with an unfavorable outcome [Lim et al., 2003]. Furthermore, in our study after ACLY knockout in HSC-3 cells we also found reduction in the expression of VEGF-A. Further, the expression of E-cadherin was found to be reduced in ACLY knockout cells.

It has been evidenced that AKT exists in three isoforms in mammals: AKT1, AKT 2, and AKT 3 [Guimarães et al., 2015]. The AKT1 isoform enable cell survival and the inhibition of apoptosis. The AKT2 isoform controls the intrinsic mitochondrial pathway of apoptosis, metabolism, cell invasion and migration. Moreover, AKT3 isoform delimited the migration in tumor cells [Harsha et al., 2020]. AKT is found to phosphorylate tuberous sclerosis complex 2 (TSC2), and suppressed the GTPase

activity of the TSC1/TSC2 complex. This in turn initiated the RAS homologue enriched in brain (RHEB) to stimulate mTOR activation [Guimarães et al., 2015]. Increased expression of the isoforms AKT1 and AKT2 has been observed in oral cancer. Further, silencing of both the isoforms resulted in decrease of tumor cell survival and induced cell cycle arrest at the G2/M phase Additionally, reduction in the expression of cyclooxygenase-2 (COX-2), cyclin D1 and survivin B-cell lymphoma 2 (Bcl-2) was observed [Harsha et al., 2020]. In several types of cancer, activation of the PI3K/AKT/ mTOR pathway is known to play a key role in various cellular functions counting the tumor proliferation, adhesion, migration, invasion, metabolism, and survival [Guimarães et al., 2015]. In oral cancer, AKT/mTOR signaling has been found to interlinked with circadian signaling, chemoresistance and radio-resistance in oral tumor cells [Harsha et al., 2020]. The stimulation of the AKT/mTOR pathway has been found to assist angiogenesis and the transfer of growth factors to tumors [Harsha et al., 2020]. Additionally, mTOR, is a serine/threonine protein kinase, a member of the PI3K-associated kinase protein family that contributes in recognizing the nutritional signals and modulation of cell growth and proliferation [Xu, 2020]. Annovazzi et al., has demonstrated that upregulation in the level of p-mTOR, p-AKT, and p-S6 expression with increasing histological grade in astrocytic and oligodendrocytic tumors [Annovazzi et al., 2009]. In addition, mTORC1 controlled the different cell processes *via* the activation of S6 [Harsha et al., 2020]. It has been found that suppression of the S6 phosphorylation of suppressed tumor growth and induced apoptosis in breast cancer [Woo, 2017]. Here, in our study we found that the expression of phospho-s6, AKT1, AKT2 and mTOR was decreased in the ACLY knockout HSC-3 cells as compared to the scrambled control. This shows that the loss of ACLY in oral cancer halts the progression of oral carcinogenesis *via* inhibition of the S6/ AKT/mTOR pathway.

Furthermore, we also found that the loss of expression of ACLY also decreased the expression of E-cadherin in ACLY knockout HSC-3 cells as compared to the scrambled HSC-3 cells.

Moreover, the loss of expression of E- cadherin (a tumor suppressor protein) is found to be linked with development of EMT during metastasis of tumor cells [Li et al, 2016, Na et al., 2020]. Besides, the activation of E-cadherin has been found to suppress metastasis at multiple stages, such as the increase of circulating tumor cells from the primary tumor and the extravasation of tumor cells from the vasculature [Na et al., 2020]. In addition, the loss of E-cadherin improved the inducible activity of Nrf2 whose role has been implicated in cancer chemoresistance. E-cadherin activity has been found to suppress the accretion of Nrf2 and Nrf2-mediated gene transcription by its interaction with Nrf2 [Kim et al., 2012]. Supporting this, remarkably increased expression of ACLY was observed in colon tumor cells that had developed resistance to SN38 [Zhou et al., 2013]. Further, studies have shown that the loss of E-cadherin expression resulted in the loss of contact inhibition and is concomitant with augmented cell motility, advance tumor stage, and poor prognosis in cancer patients [Mendonsa., 2018]. The loss of E-cadherin is known to be associated with advanced tumor stages and poor prognosis in patients with cancer [Mendonsa et al., 2018]. As such the activation of ACLY expression might have an important role in the regulating the expression of E-cadherin and further nrf2 in cancer metabolism and in rendering resistance to the chemotherapeutic drugs.

A greatly increased expression of p-ACLY was observed in 63.0% of patients with lung adenocarcinoma [Migita, et al., 2008]. Immunohistochemical analysis have confirmed

increase expression of p-ACLY in 162 tumors that rightly correlates with stage, differentiation grade, and a poorer prognosis [Migita, et al., 2008]. Moreover, Migita et al. have observed higher expression and activation of ACLY in lung adenocarcinoma patients that were identified to be statistically significant negative prognostic factor [Migita, et al., 2008]. Evidences have been found that ACLY is phosphorylated at different sites which can influence its activities [Dominguez et al., 2021]. Further, it has been observed that the phosphorylation of ACLY enhances the activity of the enzyme in the fatty acid synthesis pathway [Pinkosky et al., 2017]. For instance, protein kinase A and Akt is known to phosphorylate ACLY at ser455 [Dominguez et al., 2021]. Further, it has been evidenced that GSK-3 phosphorylates ACLY at Thr446 and Ser450 [Sánchez-Solana et al., 2013]. The significant overexpression of GSK3α/β has been found to be associated with OSCC [Mishra et al., 2015]. Moreover, GSK3 is known to be a key suppressor of the canonical Wnt signaling pathway as well as β-catenin and several transcription factors (OTFs), which are oncogenic such as NFκB, AP-1, c-Myc and p53 [Mishra et al., 2015]. In our study, we have found significant inhibition in the phosphorylation of ACLY at ser455 and thr447+ser451 residues which signifies that the knockout of ACLY disabled the phosphorylation of ACLY *via* Akt and GSK-3.

5.2. Limitations and future prospective of the study

The present study establishes a strong association between ACLY protein and tumorigenesis of oral squamous cell carcinoma. Nevertheless, there are a few limitations associated with the study and hence needs further appraisal. It is well known that India has high incidence of oral cancer cases mainly due to consumption of tobacco and tobacco products. The increasing oral cancer cases are the most chief concern for community wellbeing as it is one of the common types of cancers in India. Moreover, our north-eastern region has become a hub for oral cancer cases and death. Though

oral cancer development is a multistep process there are various other factors such as lifestyle or environmental factors, geographical and gender variations that is responsible for the various alterations that leads to the tumorigenesis of oral cancer. Our investigation on the expression of ACLY was done using TMA slides obtained from the US Biomax that lacks tissue samples of the Indian population. Therefore, investigation on the expression of this lipogenic enzyme in oral cancer tissues of patients in different age groups and types of organs of the oral cavity, stages and grades from Indian population are highly essential to strengthen our study. This study also lacks the expression analysis of ACLY in advanced stages of oral cancer as the TMA slide contains only a single tissue sample of stage III. Further, it will be interesting to decipher the involvement of ACLY in oral cancer development by including tissues from oral preneoplastic lesions such as leukoplakia, erythroplakia, OLP, OSMF and oral dysplasia. It has been proven that the use of tobacco and tobacco products is associated with the development and progression of oral cancer. Therefore, inclusion of patients who are exposed to tobacco and its carcinogens would provide us an insight of the involvement of ACLY in tobacco induced oral tumorigenesis. Moreover, a detailed clinicopathological studies of ACLY of the following parameters such as response to therapies, chemo and radio resistance, progression free survival, overall survival, and tumor recurrence will enable to identify its significance in the treatment of oral cancer. We also analysed the importance of the oral cancer risk factors on the regulation of ACLY protein by treating oral cancer cells with tobacco and various tobacco components. Here, we examined the effect of these carcinogens in established oral cancer cell lines that possess several molecular alterations. This study only provided information about the contribution of ACLY in the progression of oral tumorigenesis but not in oral cancer development. Therefore, there comes the need to

investigate the effect of these carcinogens on the expression of ACLY in normal oral epithelial cells.

We studied the role of ACLY protein in the tumorigenesis of oral cancer aftermath gene knockout using CRISPR /Cas9 i.e., the effect of ACLY proteins in the regulations of cancer hallmarks. Our study was restricted to the revelation of various downstream targets of ACLY. Determining the upstream mediators responsible for the modulation of ACLY in oral cancer, in particular tobacco induced oral cancer appears to be of vital importance. As tobacco is one of the important risk factors for the progression of oral cancer a detailed study is essential to enquire about the involvement of ACLY in tobacco induced oral carcinogenesis. Therefore, treatment of the ACLY knockout cells with tobacco and its carcinogens will give the information of the approximate mechanisms that are involved in the oral tumorigenesis. In addition, interpreting the possible molecular events linked with the treatment of carcinogen would deliver novel insights into the crosstalk amongst various signalling cascades involved in the development and maintenance of OSCC. Further, efforts should be done particularly to study the chemoresistant and chemosensitizing potential of ACLY. Apart from the cancer metabolism, the effect of ACLY knockout in fatty acid synthesis of oral cancer cells needs to be studied to crosstalk between the cancer hallmarks and the lipogenic enzymes. In our study we have established the correlation of ACLY with oral cancer using *in vitro* assays in a single cell line. Therefore, it is requisite to confirm our findings in multiple cell lines and in *in vivo* and clinical settings before proceeding for the development of novel diagnostic, therapeutic and prognostic methods.

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List of Abbreviations

4NQO: 4-nitroquinoline 1-oxide

ACC: Acetyl CoA carboxylase

ACLY: ATP citrate lyase

ACS1: Acetyl-CoA synthetase 1

AFP: Alpha-fetoprotein

AGPAT6: 1-acylglycerol-3-phosphate o-acyltransferase 6

AICAR: 5- aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

AIDS: Acquired immunodeficiency syndrome

ALA: Alpha lipoic acid

ALC: Acetyl-L-carnitine

AMPK: 5' AMP-activated protein kinase

AP-1: Activator protein 1

AR: Androgen receptor

BaP: Benzo[a]pyrene

BARD1: BRCA1-associated RING domain protein 1 (),

Bcl-2: B-cell lymphoma 2

Bcl-xL: B-cell lymphoma-extra large

BDK: Branched-chain α-keto acid dehydrogenase kinase

BRCA1: Breast cancer type1

BSA: Bovine serum albumin

CASP10: Caspase 10

CASP8: Caspase-8

CBP: Calcium-binding protein

CCND1: Cyclin D1

CDK: Cyclin dependent kinase

cIAP: Cellular inhibitor of apoptosis protein

c-Myc: Cellular myelocytomatosis

CoA: Coenzyme A

COX-2: Cyclooxygenase-2

CPT-1: carnitine palmitoyl transferase-1

CPT1A: Carnitine palmitoyl transferase 1A

Cr(VI): Chronic hexavalent chromium

CRC: Colorectal cancer

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/ CRISPR associated protein 9

CRPC: Castration-resistant prostate cancer

CSCs: Cancer stem cells

CuB: Cucurbitacin B

CUL3: Cullin3

DAB: 3,3'-diaminobenzidine

DGAT: Diacylglycerol acyltransferase

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

E2F1: E2F Transcription Factor 1

EBV: Epstein-Barr virus

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGFR/erbB: Epidermal growth factor receptor

ELOVL6: Long-chain fatty acid elongase

EMT: Epithelial-mesenchymal transition

ERK: Extracellular receptor kinase

ETV4: ETS translocation variant 4

FAK: Focal adhesion kinase

FAO: Fatty acid oxidation

FAS: Fatty acid synthase

FASN: Fatty acid synthase

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FGF-3/ int-2: Fibroblast growth factor-3

G3P: Glycerol 3-phosphate

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GATA3: GATA binding protein 3

GATS: Global Adult Tobacco Survey

GLUT-1: Glucose transporter 1

GPAM: Glycerol-3-phosphate acyltransferase 1

GPAT: Glycerol-3-phosphate acyltransferase

GPDH: Glycerol 3-phosphate dehydrogenase

GSK3: Glycogen synthase kinase 3

GSK3α/β: Glycogen synthase kinase 3α/β

HATs: Histone acetyltransferases

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1: Hypoxia-inducible factor 1

HIF1α: Hypoxia inducible factor 1 subunit alpha

HIV: Human immunodeficiency viruses

HK2: Hexokinase 2

HNSCC: Head and neck squamous cell carcinoma

HPV: Human papillomavirus infection

HRP: Horseradish peroxidase

HSV: Herpes Simplex viruses

IAP: Inhibitors of apoptosis

IARC: International Agency for Research on Cancer

IGF-1: Insulin-like growth factor 1

IGF-1R: Insulin-like growth factor type 1 receptor

ILKAP: ILK Associated Serine/Threonine Phosphatase

ING5: Inhibitor of growth family member 5

LDH: Lactate dehydrogenase

LDHA: lactate dehydrogenase A

let-7b: Lethal-7

LKB1: Liver kinase B1

LP: Lichen planus

LRP1B: Low-density lipoprotein receptor-related protein 1B

MAPK: Mitogen-activated protein kinase

Mcl-1: Myeloid cell leukemia-1

MEM: Minimum essential medium Eagle

MET: Mesenchymal-epithelial transition factor

MMP-2: Matrix metalloproteinase-2

MT1-MMP: Membrane type 1 metalloprotease

mTOR: Mammalian target of rapamycin

mTORC1: Mammalian target of rapamycin complex 1

mTORC2: Mammalian target of rapamycin complex 2

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MVA: Mevalonate pathway

MVD: Microvessel density

MYOD: Myoblast determination protein 1

nAChRs: Nicotinic acetylcholine receptors

NaCl: Sodium chloride

NCAPG: Non-SMC condensin I complex subunit G

NCCN: National Comprehensive Cancer Network

NF-kB: Nuclear factor kappa B

NF-κB: Nuclear factor-κB

NNK: 4[methylnitosoamino]-1-[3-6-pyridyl]-1-butanone

NNN: N-nitrosonornicotine

NOS2: Nitric oxide synthase

OAA: Oxaloacetic acid

OLP: Oral lichen planus

OSCC: Oral squamous cell carcinoma

OSMF: Oral submucous fibrosis

OTFs: Oncogenic transcription factors

p-ACLY: phospho-ACLY

PAH: Polycyclic aromatic hydrocarbon

p-Akt: Phospho Akt

p-AMPK: phosphorylation of AMP-activated protein kinase ()

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate buffer saline

PCAF: P300/CBP-associated factor

PD-ECGF: Platelet-derived endothelial cell growth factor

PDK: Pyruvate dehydrogenase kinase

PE: Plating efficiency

Penstrep: Penicillinstreptomycin

PFK1: Phosphofructokinase 1

PGE2: Prostaglandins

PGF: Placental growth factor

PI3K: Phosphatidylinositol 3-kinases

p-Jak-1: Phospho Jak-1

PKA: Protein kinase A

PKM2: Pyruvate kinase M2

p-MAPK: Phospho MAPK

PMDs: Potentially malignant disorders

PMSF: Phenylmethylsulfonyl fluoride

PPARy: peroxisome proliferator-activated receptor gamma

PPARα: Peroxisome proliferator-activated receptor alpha

PPP: pentose phosphate pathway

PPP1R7: Protein phosphatase 1 regulatory subunit 7

PRAD-1: Parathyroid adenomatosis 1

Rb: Retinoblastoma protein

RCC: Renal cell carcinoma

REDD1: Regulated in development and DNA damage responses 1

RFC3: Replication factor C subunit 3

RGCB: Rajiv Gandhi University of Biotechnology

RHEB: RAS homologue enriched in brain

RIPK3: Receptor interacting serine/threonine kinase 3

ROS: Reactive oxygen species

RT-PCR: Reverse transcription-polymerase chain reaction

SCC: Squamous cell carcinoma

SCD1: Stearoyl-CoA desaturase 1

SCR: Scrambled

SDHA: Succinate dehydrogenase complex, subunit A

SDHB: Succinate dehydrogenase complex iron sulfur subunit B

SDS: Sodium dodecyl sulfate

SF: Survival fraction

sgRNA: Single guide RNA

SHS: Second hand smoke

SLC13A5: Solute carrier family 13-member 5

SLT: Smokeless tobacco

SNP: Single nucleotide polymorphism

SREBP: Sterol regulatory element-binding proteins

STAT: Signal transducer and activator of transcription

STK11: Serine/threonine kinase 11

TCA: Tricarboxylic acid

TE: Tobacco extract

TFBS: Transcription-factor-binding site

TG: Triacylglycerol

TGF-β: Transforming growth factor beta

TKTL1: Tansketolase-like protein 1

TMA: Tissue microarray

TMJ: Temporomandibular joint

TNM: Tumour lymph node metastasis

TSC2: Tuberous sclerosis complex 2

TSNA: Tobacco-specific N-nitrosamines

VEGF: Vascular endothelial growth factor

WHO: World Health Organization

XIAP: X-linked inhibitor of apoptosis protein

α2M: α2-Macroglobulin

List of Tables

List of Figures

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1. **Khwairakpam AD,** Monisha J, Roy NK, et al. Vietnamese coriander inhibits cell proliferation, survival and migration via suppression of Akt/mTOR pathway in oral squamous cell carcinoma in "International Conference on Nutraceuticals and Chronic Diseases (INCD) 2019", held on September 23rd-25th, 2019 organized by Society for Nutraceuticals and Chronic Diseases and Indian Institute of Technology Guwahati, at IIT Guwahati, India, 2019.
2. **Amrita Devi Khwairakpam,** Ajaikumar B Kunnumakkara, A study on the role of lipogenic enzymes in the initiation and progression of OSCC at Recent Advances in Cancer Biology (RACR-2019), Indian Institute of Technology Guwahati, Guwahati, India, 28th March, 2019.
3. **Amrita Devi Khwairakpam,** Ajaikumar B Kunnumakkara, An investigation on the role of lipogenic enzymes in OSCC at Research Conclave 2019, Indian Institute of Technology Guwahati, Assam, India, March 14-17, 2019.
4. **Amrita Devi Khwairakpam,** Ajaikumar B Kunnumakkara, An Investigation on the Expression of ATP Citrate Lyase in Oral Squamous Cell Carcinoma (OSCC), Workshop on "Introduction to basic and advanced biomedical approaches for enhancing QOL in aging societies" held at Biomedical Research Institute, AIST, Tsukuba Science City, Japan, October 14-21, 2018.
5. **Amrita Devi Khwairakpam**, Javadi Monisha, Nandkishore Roy, Padmavathi G, Devivasha Bordoloi, Ajaikumar B Kunnumakkara, An investigation on the anti-cancer potential of Cambodian mint against human Oral Squamous Cell Carcinoma, INCD- 2018, 14-16 September 2018, Swami Rama Himalayan University, Rishikesh, Dehradun.

6. **Amrita Devi Khwairakpam**, Javadi Monisha, Nand Kishor Roy, Devivasha Bordoloi, Padmavathi G, Devivasha Bordoloi, Ajaikumar B Kunnumakkara, Examining the anti-cancer potential of Cambodian mint against human Oral Squamous Cell Carcinoma, National Conference on "Ethno-medicine and Traditional Health Practices in Northeast Region of India" held at NIPER-Guwahati on 25th August 2018, Assam.
7. **Amrita Devi Khwairakpam**, Nand Kishor Roy, Javadi Monisha, Ganesan Padmavathi, Bethsebie Lalduhsaki Sailo, Ajaikumar B Kunnumakkara. To Elucidate the Role of Tobacco- Mediated Lipogenesis in the Initiation and Progression of Oral Squamous Cell Carcinoma (OSCC). TCR Conference 2018, Chennai, India.
8. **Amrita Devi Khwairakpam**, Javadi Monisha, Nandkishore Roy, Devivasha Bordoloi, Ajaikumar B Kunnumakkara, The Anti-Cancerous Properties of Vietnamese coriander (Persicaria odorata) against Oral Squamous Cell Carcinoma (OSCC), International Conference on Nutraceuticals and Chronic Diseases (INCD) 2017 organized by Society for Nutraceuticals and Chronic Diseases and Indian Institute of Technology Guwahati, Goa, India.
9. **Amrita Devi Khwairakpam**, Monisha Javadi, NandKishor Roy, Devivasha Bordoloi, Ajaikumar B Kunnumakkara, Persicaria odorata as an Anticancer Agent against Oral Cancer cells, Research Conclave, Indian Institute of Technology, Guwahati, Assam, India, 2017.
10. **Amrita Devi Khwairakpam**, Harsha Choudhary, NandKishor Roy, Devivasha Bordoloi, Ajaikumar B Kunnumakkara. Anticancer Properties of Persicaria odorata on Oral Cancer Cells, Translational Cancer Research-2016(TCR-2016), February 4th-7th, 2016 Ahemdabad, India.

11. **Amrita Devi Khwairakpam**, Javadi Monisha, Nandkishore Roy, Devivasha Bordoloi, Ajaikumar B Kunnumakkara, The Anti-cancerous Properties of Vietnamese coriander (Persicaria odorata) against Oral Squamous Cell Carcinoma (OSCC), International Conference on Nutraceuticals and Chronic Diseases (INCD) 2017 organized by Society for Nutraceuticals and Chronic Diseases and Indian Institute of Technology Guwahati, Goa, India.
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13. **Amrita Devi Khwairakpam**, Harsha Choudhary, NandKishor Roy, Devivasha Bordoloi, Ajaikumar B Kunnumakkara. Anticancer Properties of Persicaria odorata on Oral Cancer Cells, Translational Cancer Research-2016(TCR-2016), Ahemdabad, India, 2016.

Awards

1. Received '**Best Oral Presentation award'** for the paper entitled "A study on the role of lipogenic enzymes in the initiation and progression of OSCC" in **"**Recent Advances in Cancer Biology (RACR-2019)", Indian Institute of Technology Guwahati, Guwahati, India, 28th March, 2019.
2. Selected for the course of Japan-Asia Youth Exchange Program in Science (Sakura Exchange Program in Science) administered by Japan Science and Technology Agency, National Institute of Advanced Industrial Science and technology, Japan, October 14-21, 2018.
3. Received '**Best Oral Presentation award**' for the paper entitled "An Investigation on the Expression of ATP Citrate Lyase in Oral Squamous Cell Carcinoma (OSCC)" at Workshop on "Introduction to basic and advanced biomedical approaches for enhancing QOL in aging societies". October 14-21, 2018, held at Biomedical Research Institute, AIST, Tsukuba Science City, Japan.
4. Received '**Second Best poster presentation award**' for the paper entitled "An investigation on the anti-cancer potential of Cambodian mint against human Oral Squamous Cell Carcinoma" at International Conference on Nutraceuticals and Chronic Diseases (INCD) 2018, September14-16, 2018, Swami Rama Himalayan University, Rishikesh, Dehradun.
5. Received '**Best Poster presentation Award'** for the paper entitled "The Anti-cancerous Properties of Vietnamese coriander (*Persicaria odorata*) against Oral Squamous Cell Carcinoma (OSCC)" at the Second International conference on Nutraceuticals and chronic diseases 2017 (INCD-2017), Goa, India, 2017.

Workshops and trainings:

1. Attended "Recent Advances in Biomedical Research (RABR-2019)", jointly organized by Indian Institute of Technology Guwahati, Guwahati, India, Biomedical Research Institute (BMRI), and AIST, Japan, 26th- 27th March, 2019.
2. Attended the workshop "Recent Advances in Cancer Biology (RACR-2019)", Indian Institute of Technology Guwahati, Guwahati, India, 28th March, 2019.
3. Attended the conference "9th Head and Neck Update" held on 1st December 2018, at North East Cancer Hospital & Research Institute, Guwahati Assam.
4. Attended the Workshop on "Introduction to basic and advanced biomedical approaches for enhancing QOL in aging societies" held at Biomedical Research Institute, AIST, Tsukuba Science City, Japan, October 14-21, 2018.
5. Participated in National conference on "Recent Developments in Medical Biomedical Biotechnology and Structure Based Drug Designing [RDMBSBDD- 2015]" held on December 6-7, 2015 organized by the Department of Biotechnology, Indian Institute of Technology Guwahati.
6. Participated in the National course on "Theoretical and Practical Aspects of Cancer Research" conducted by the Technical Educational Quality Improvement Programme sponsored by the Ministry of Human Resource Development, Government of India held on February 4th – 8th, 2015 at Indian Institute of Technology Guwahati.
7. Participated in "Flow Cytometry Data Analysis" conducted by Department of Biotechnology, Indian Institute of Technology Guwahati on January 23th - 24th, 2015.

8. Participated in the National Conference on "Recent Advances in Cancer Biology and Therapeutics- 2014 (RACBT- 2014)" organized by the Department of Biotechnology, Indian Institute of Technology Guwahati on December 5th, 2014.
9. Participated in "International Conference on Disease Biology and Therapeutics (ICDBT 2014)" held from 3rd – 5th December 2014, organized by Institute of Advance study in Science and technology, Guwahati.
10. Participated in "Next Generation Sequencing and Data Analysis" Workshop organized by Biotech Hub, Centre for the Environment, Indian Institute of Technology Guwahati held during May 14th – 17th, 2014.