STUDIES ON THE ROLE OF NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN (NGAL) IN ORAL SQUAMOUS CELL CARCINOMA

A thesis submitted for the degree of

Doctor of Philosophy

To

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

By

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Dedicated to

My loving parents

For being a great source of inspiration and support

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 **"Studies on the role of neutrophil gelatinase-associated lipocalin (NGAL) in oral squamous cell carcinoma"**, is a presentation of my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Ajaikumar B. Kunnumakkara.

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

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CERTIFICATE

This is to certify that the work described in the thesis titled, **"Studies on the role of neutrophil gelatinase-associated lipocalin (NGAL) in oral squamous cell carcinoma",** submitted by Javadi Monisha (Roll No. 126106034) 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, India.

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

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Introduction And Review of literature

1. Introduction

Despite the significant advancements in therapeutic strategies, oral cancer is a major health burden worldwide with approximately causing 128,000 deaths (Ferlay *et al*., 2012; Jemal *et al*., 2011). It is predominant in the regions of Melanesia, South-Central Asia, and Central and Eastern Europe in both the sexes (Jemal *et al*., 2011). In India, oral cancer is a serious public issue and ranks one among the top three cancers accounting to over 30% of all cancers reported in the country (Coelho, 2012). Approximately, 80,000 people are diagnosed with oral cancer annually with greater incidences in males than females (Varshitha, 2015). 'The international agency for research on cancer' envisages that the overall incidence of oral cancer in India would drastically escalate from 1 million in 2012 to more than 1.7 million in 2035, which is anticipated to increase the death rates to 1.2 million (Bray, 2008). The five year survival rate of oral cancer is 62.1% (2003-2009), however, the survival rates deteriorate with advancement in clinical stages (SEER 2003-2009) (Figure 1.1). Regardless the unquestionable benefits from the available therapeutic modalities, drug resistance and recurrence are the major hurdles that decrease the quality of life of the patients. This necessitates the need for developing novel biomarkers for the early diagnosis and novel targets for discovering more promising chemotherapeutic agents for this disease

Over the past two decades, neutrophil gelatinase associated lipocalin (NGAL) has received colossal attention in clinic, as a biomarker during kidney injury, cardiovascular injuries, and also in cancer (van Wolfswinkel *et al*., 2016; Devarajan 2010; Cruz *et al*., 2012; Roli *et al*., 2017; Wang and Zeng, 2013). NGAL belongs to Lipocalin family, which is involved in transporting small hydrophobic molecules such as steroids, bilins, retinoids, and lipids (Monisha *et al*., 2014).

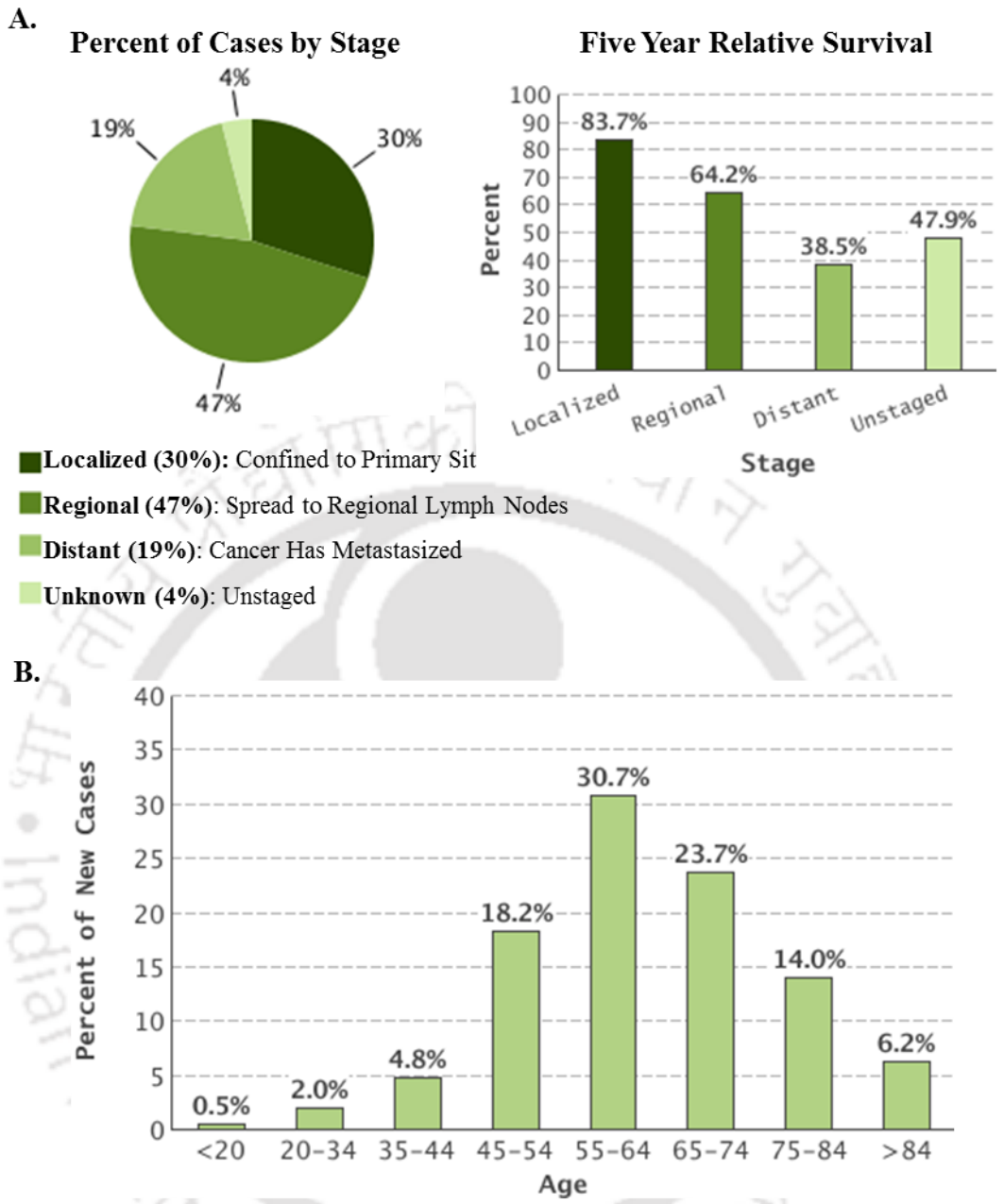

Figure 1.1 : Oral cancer statastics by SEER. A) Percent of cases & five year relative survival by stage at diagnosis: oral cavity and pharynx cancer. SEER 18 2007-2013, All races, both sexes by SEER summary stage 2000. B) Percent of new cases by age group: oral cavity and pharynx cancer. SEER 18 2010-2014, All Races, Both Sexes.

Mounting evidences suggest that NGAL is overexpressed in many cancers like breast cancers, adenocarcinomas of the lung, colon, liver, esophageal and ovarian cancers (Candido *et al.*, 2014). But the role of NGAL in oral cancer is poorly

understood. Therefore, the present study focused on the expression and role of NGAL in oral cancer.

1.1. Multistep process of development of oral cancer

The theory of multi-stage carcinogenesis was first proposed in the year 1948 by Berenblum and Schubik. The development of oral cancer is a multi-stage process that begins with preneoplastic lesions like leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis and actinic cheilitis and they metastasize to other organs like nasopharynx, bone, lymph node, and lung and form secondary tumours (Figure 1.2). These tumours arise from lips, cheeks, roof of the mouth (hard palate), back of the mouth (soft palate and uvula), floor of the mouth (area under the tongue), gums, teeth, tongue, and tonsils.

1.1.1. Preneoplastic lesions

Leukoplakia: As defined by the World Health Organization, leukoplakia is "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease" (Kramer *et al*., 1978). Early lesions appear to be whitish grey plaques but as it progresses it becomes thicker, whiter and leathery with surface fissures. These lesions are usually found near buccal mucosa, alveolar mucosa, floor of mouth, lateral tongue, and lower lip (Neville, 2002). ***Dysplasia****:* Dysplasia is a premalignant condition affecting the epithelial cells. Basal and parabasal epithelial cells are usually affected. In dysplasia the cells and the nuclei resemble the basal cells with enlarged nuclei (nuclear hyperplasia), exhibit hyperchromatism, enlarged eosinophilic nucleoli (prominent nucleoli) with increased nuclear-to-cytoplasmic ratio. Based on the cells affected it is classified to mild, moderate and severe dysplasia. Mild dysplasia is seen in basal and

ORAL NEOPLASIA

- **Epithelial precursor lesions**
- **Benign epithelial tumours**
  - **Papillomas**
    - Squamous cell papilloma and verruca vulgaris
    - Condyloma acuminatum
    - Focal epithelial hyperplasia
  - **Granular cell tumour**
  - **Keratoacanthoma**
- **Malignant epithelial tumours**
  - **Squamous cell carcinoma**
    - Verrucous carcinoma
    - Basaloid squamous cell carcinoma
    - Papillary squamous cell carcinoma
    - Spindle cell carcinoma
    - Acantholytic squamous cell carcinoma
    - Adenosquamous carcinoma
    - Carcinoma cuniculatum
  - **Lymphoepithelial carcinoma**
- **Salivary gland tumours**
  - **Salivary gland carcinomas**
    - Acinic cell carcinoma
    - Mucoepidermoid carcinoma
    - Adenoid cystic carcinoma
    - Polymorphous low-grade adenocarcinoma
    - Basal cell adenocarcinoma
    - Epithelial-myoepithelial carcinoma
    - Clear cell carcinoma, not otherwise specified
    - Cystadenocarcinoma
    - Mucinous adenocarcinoma
    - Oncocytic carcinoma
    - Salivary duct carcinoma
    - Myoepithelial carcinoma
    - Carcinoma ex pleomorphic adenoma
  - **Salivary gland adenomas**
    - Pleomorphic adenoma
    - Myoepithelioma
    - Basal cell adenoma
    - Canalicular adenoma
    - Duct papilloma
    - Cystadenoma
- **Soft tissue tumours**
  - **Kaposi sarcoma**
  - **Lymphangioma**
  - **Ectomesenchymal chondromyxoid tumour**
  - **Focal oral mucinosis**
  - **Congenital granular cell epulis**
- **Haematolymphoid tumours**
- **Mucosal malignant melanoma**
- **Secondary tumours**

Figure 1.2: WHO histological classification of tumours of the oral cavity and oropharynx

parabasal layers, moderate dysplasia damage basal layer to the middle of granular layer and severe dysplasia affects basal layer to upper and middle layer of epithelium (Shirani *et al*., 2014). ***Erythroplakia*:** In erythroplakia, the oral lesions appear to be red, with soft velvety texture. These occur mainly at the floor of mouth, lateral tongue, retromolar pad, and soft palate.

These lesions are well demarcated, and in some cases, they are intermixed with white patches (erythroleukoplakia). The lesions are found to be flat, macular, velvety appearance and most often speckled with white spots representing foci of keratosis. Erythroplakia is not common as leukoplakia, and most often they lead to oral squamous cell carcinoma (Neville, 2002; Oral cancer foundation, 2017a).

Oral lichen planus (OLP): It is a chronic inflammatory condition affecting the mucous membrane of the oral cavity. It gives reticular, papular, plaque-like, erosive, atrophic or bullous appearance and the basal keratinocytes are degenerated forming colloid or Civatte bodies that resemble apoptotic karatinocytes due to DNA damage. Moreover, epithelial basement membrane and basal membrane disrupt causing a microscopic cleft between epithelium and the connective tissue. The buccal mucosa, tongue and the gingiva are the regions which are affected the most (Sugerman *et al*., 2000). ***Oral submucous fibrosis (OSMF)*:** OSMF is a premalignant condition characterized by inflammation and progressive fibrosis of submucosal tissues. Oral cavity, pharynx and upper third of the esophagus are the most commonly affected areas. It is more prevalent in India, Bangladesh, Sri Lanka, and other South Asian countries. Areca nut chewing in Indian continent and HPV infection in western countries are the major cause of OSMF (Tilakaratne *et al*., 2006). Diffuse fibrosis in the submucosa with chronic inflammatory infiltrate is the main feature of OSMF. The disease starts initially with formation of small vesicles, leading to mucosal ulceration, which gets replaced by

granulation tissue. However, clear histologic features of OMSF are not reported in the literature (Isaac *et al*., 2008).

1.2. Individual cancers of oral cavity

1.2.1. Lip cancer:

It is the most common malignancy of oral cancer and predominant in men. Lip cancers are of two types namely basal cell carcinoma and squamous cell carcinoma. 90% of lip cancers arise from the squamous cells lining of the lip and oral cavity. Lip carcinomas frequently appear as a result of radiodermitis, chronic chelitis and xeroderma pigmentosum. Pipe-smoking, tobacco-chewing, chronic alcohol consumption and exposure to UV are the causative factors for lip cancers. In the initial phase, the tumour appears as a papule or a plate which eventually advance to vegetative or ulcerative form. By the time of diagnosis, lymph node metastases are observed in 8% of the patients. Ultrasonography (US), computed tomography (CT) scan and/or magnetic resonance (MR) confirm the presence of cancer and also its spread to regional lymph nodes. Usually, surgery and radiotherapy is the preferred treatment strategy. Early stage tumours have good prognosis compared to the treatment of advanced lesions, which usually alters the appearance and functionality of the lip (Moretti *et al*., 2011).

1.2.2. Floor of the mouth cancer:

Floor of the mouth is a horseshoe-shaped area under the tongue, between the lower jaw bones (the mandible) and when the malignant tumor grows in this area it is called floor of the mouth cancer (Cedars-Sinai, 2017). These tumors account for 28 – 35% of all mouth cancers. Typically these tumors begin as a small asymptomatic nodular or ulcerative lesion in the hidden areas, which is most of the time ignored. Men are diagnosed three to four times more often than women. Smokeless tobacco (including

snuff and betel nut) is the major cause for this cancer than cigarettes since the plug of tobacco is pressed against the skin in the mouth (Cedars-Sinai, 2017). These cancers are usually diagnosed in advanced stages and are seen in older people. Surgery and radiotherapy are the preferred treatment modalities (Medscape, 2017).

1.2.3. Buccal mucosa cancer:

The buccal mucosa is the lining of the cheeks and the back of the lips, inside the mouth where they touch the teeth and the tumours arising in this regions are known as buccal mucosa cancer (Cedars-Sinai, 2017). These cancers occur in the thin, flat cells that line the buccal mucosa and other parts of the mouth. Most of the buccal cancers are squamous cell carcinomas in pathology. Tobacco (cigarettes or smokeless) and alcohol, and particularly women who use snuff are at utmost risk of developing this cancer (Cedars-Sinai, 2017). Beetle nut chewing is another cause which tends to develop these cancers at a younger age, however, it is more prevalent between the ages forty to seventy. In India, 90% of patients with buccal cancers possess the history of beetle nut chewing (Klem, 2017). In early stages, these cancers are treated with surgery, but in advanced stages combination of surgery and chemotherapy are given.

1.2.4. Tongue cancer:

Tongue cancers usually start in the flat thin cells that cover the surface of the tongue. It is mainly of two types; cancer of the oral tongue (occurs in the front two-third region of the tongue) and cancer of the base of tongue (occurs in the base one-third of the tongue). Tongue cancers are the most common cancers of oral cavity and is more prevalent in Indian populations. Tobacco, alcohol, and HPV infections are the common risk factors for tongue cancers. The five-year disease-free survival rate is 70% in early stage, which tends to fall to less than 30% in advanced stages (MyVMC, 2017). Surgery

is the preferred treatment for the smaller tumours, however, radiation therapy and chemotherapy is given in case of large tumours and metastatic tumours.

1.2.5. Hard palate cancer:

These tumours arise from the bony part of the roof of the mouth. In the advanced stages these tumours spread to nasal cavity. Half of all hard palate cancers are squamous cell carcinomas (SCCs), and non-squamous cell cancers include minor salivary gland cancers, sarcomas, and melanomas. Histologically hard palate cancers comprise 33% of SCC, 15% of adenoid cystic carcinoma, 10% of mucoepidermoid carcinoma, and 4% of anaplastic carcinoma. Cigarette smoking particularly reverse smoking is the main cause for this cancer. Surgery is preferred for patients suffering from this cancer. Radiation and chemotherapy are given when the patients are in their advanced stages (Prakash, 2011; Sadeghi and Sebeih, 2017)

1.2.6. Salivary gland tumours:

Salivary glands are exocrine organs that are responsible for the production and secretion of saliva. They are comprised of parotid, sub-mandibular, sublingual, and many other minor glands and the tumours arising in these glands are known as salivary gland tumours.

Viruses, radiation, lifestyle and nutrition are the major risk factors for salivary gland tumours. Histologically, salivary gland tumours are classified into following types:

***Mucoepidermoid carcinoma*:** These are the most common type and account to 2.8-15.5% of salivary gland tumours. These tumors majorly occur in parotid glands (43%), submandibular glands (7%) and sublingual glands (1%). Histologically these tumors comprise of mucus secreting cells (muco-), squamous cells (-epidermoid) and lymphoid infiltrating cells. Initially, the tumours start as fixed painless swellings, which may

worsen to pain, otorrhoea, paraesthesia, facial nerve palsy, dysphagia, bleeding and trismus. These tumours usually spread to lymph nodes, submandibular region, upper jugular lymphatic chain, respiratory tract etc. The survival rates in low grade tumours is 98% with low local recurrence, which tends to fall down to 35% in high grade tumours with high recurrence. Surgery with/without preservation of facial nerve and adjuvant radiotherapy are the most commonly preferred (Goode and El-Naggar, 2005).

Adenoid cystic carcinoma (ACC): Adenoid cystic carcinomas encompass almost 10% of all epithelial salivary neoplasms and most commonly involve the parotid, submandibular and minor salivary glands. Epithelial minor salivary gland tumours affect maximum the palate, followed by the tongue, buccal mucosa, lip and floor of the mouth. These tumours mostly arise from ductal and myoepithelial cells and most often seen in middle-aged and older patients. ACC tends to spread along nerves, known as a perineural invasion, or through the bloodstream and metastasize to lungs, lymph, bone, brain, and liver. Lymph node metastases is seen in only about 5-10% of cases. The five-year survival rate is approximately 35% and 80-90% of the patients die within 10-15 years of the disease diagnosis. The recurrence rate for these cancers is high and only 20% of patients with distant metastases survive five years. Surgery and postoperative radiotherapy are given to the patients. Till now there is no effective chemotherapy against metastatic and/or unresectable ACC (Eveson et *al.*, 2005; Oral cancer foundation, 2017b).

***Adenocarcinomas*:** Adenocarcinoma is a collective term that describes the tumours of glandular origin. There are many different types of adenocarcinomas: ***Acinic cell carcinoma*:** The neoplastic cells exhibit serous acinar cell differentiation, characterized by cytoplasmic zymogen secretory granules. 80% of acinic cell carcinomas occur in the parotid gland, about 17% in the intraoral minor salivary glands, 4% develop in the

submandibular gland, and less than 1% arise in the sub-lingual gland. They usually metastasize to cervical lymph nodes and lung (Ellis and Simpson, 2005). ***Polymorphous low-grade adenocarcinoma (PLGA):*** These tumours are characterized by cytologic uniformity, morphologic diversity, and infiltrative growth pattern with low metastatic potential. It is the second most common intraoral malignant salivary gland tumour, accounting for 26% of all carcinomas. These tumours occur in the palate, buccal mucosa, retromolar region, upper lip, and the base of the tongue (Luna and Wenig, 2005). ***Adenocarcinoma, not otherwise specified (NOS):*** These tumours possess ductal differentiation but they lack histomorphologic features that characterize the other defined types of salivary carcinoma. The term "not otherwise specified" is included as most of the epithelial salivary gland tumours are adenocarcinomas. 60% of these tumours occur in major glands and about 40% in minor glands and usually affect parotid glands, hard palate, buccal mucosa, and lips (Auclair and van der Wal, 2005). ***Rare adenocarcinomas:*** Some of the adenomas are rare tumours and are categorized to low grade and high grade tumours. Basal cell adenocarcinoma, clear cell carcinoma, cystadenocarcinoma, sebaceous adenocarcinoma, sebaceous lymphadenocarcinoma, mucinous adenocarcinoma are low grade tumours associated with good outcome. Oncocytic carcinoma and salivary duct carcinoma are high grade tumours and are associated with poor outcome (American cancer society, 2017).

1.3. TNM (Tumour lymph node metastasis) staging

TNM staging for oral cancer was first reported by Pierre Denoix in 1940 (Denoix PF, 1944). TNM stands for Tumour Node Metastasis, and the staging is made mainly based on the anatomical features as well as involvement of regional lymph nodes and distant metastasis.

Table 1.1: TNM staging of oral cancer.

T — Primary Tumour	**N — Regional Lymph Nodes**	**M — Distant Metastasis**
TX Primary tumour cannot be assessed	**NX** Regional lymph nodes cannot be assessed	**MX** Distant metastasis cannot be assessed
T0 No evidence of primary tumour	**N0** No regional lymph node metastasis	**M0** No distant metastasis
Tis Carcinoma in situ	**N1** Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension	**M1** Distant metastasis
T1 Tumour ≤ 2 cm	**N2** Metastasis	
T2 Tumour >2 cm ≤ 4 cm	**N2a** Metastasis in a single ipsilateral lymph node, > 3 cm ≤ 6 cm	
T3 Tumour > 4 cm	**N2b** Metastasis in multiple ipsilateral lymph nodes, ≤ 6 cm	
T4a(lip) Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin(chin/nose)	**N2c** Metastasis in bilateral or contralateral lymph nodes, ≤6 cm	
T4a(oral cavity) Tumour invades through cortical bone, into deep/extrinsic muscle of tongue, maxillary sinus, or skin of face	**N3** Metastasis in lymph node >6 cm	
T4b (lip and oral cavity) Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery		

1.4. Etiology of oral cancer

The development of oral cancer shows a multifactorial etiology – both endogenous (genetic) and exogenous (environmental and behavioral) factors are involved in this process (Figure 1.3). Tobacco and alcohol are the major risk factors for oral cancer. Approximately, 57% of men and 11% of women between 15–49 years are exposed to tobacco either by consumption or from the environment (Coelho, 2012). Reports suggest a twenty fold increased risk of oral cancer in heavy smokers and a five-fold increase in heavy drinkers of age below 46 while the combination of heavy smoking and drinking increased the risk to fifty fold (Rodriguez *et al.,* 2004). Other factors contributing to the development of oral cancers are age, gender, viral infections like human papilloma virus (HPV), air pollution and immune deficiency syndromes (Kumar *et al*., 2016; Oral cancer foundation, 2017c). These factors increase the difficulty in managing this disease.

1.4.1. Tobacco:

Increasing lines of evidences advocate that tobacco is the major risk factor for this disease. Tobacco is consumed either as smokeless tobacco or smoking. It is used in various forms like chewing tobacco, oral use of snuff, smoking of cigars, cigarettes, bidis, pipes etc. (Radhakrishnan *et al*., 2012). Numerous carcinogens have been isolated from tobacco which includes nicotine, polycyclic aromatic hydrocarbons (PAH), such as benzo[a]pyrene, tobacco specific N'- nitrosamines (TSNAs) such as N-nitrosonornicotine (NNN) and 4 [methylnitrosoamino]-1-[3- pyridyl]-1-butanone (NNK) etc. These carcinogens form DNA adducts mainly at the region of 06-methylguanine, induce mutations and interferes with replication of the DNA (Johnson *et al*., 2001). These tobacco specific nitrosamines are also known to generate free

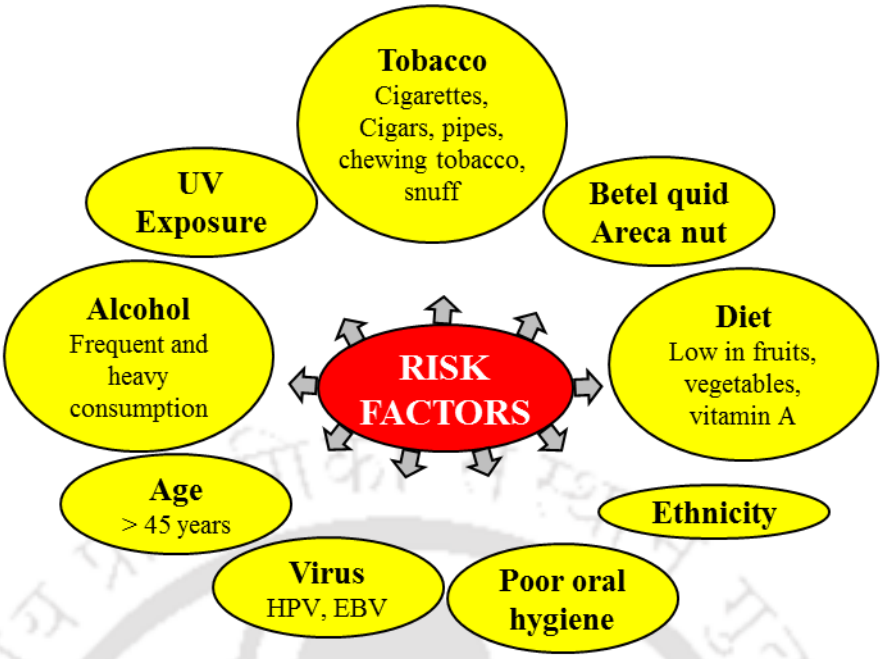

Figure 1.3: Risk factors that attribute to the development of oral cancer.

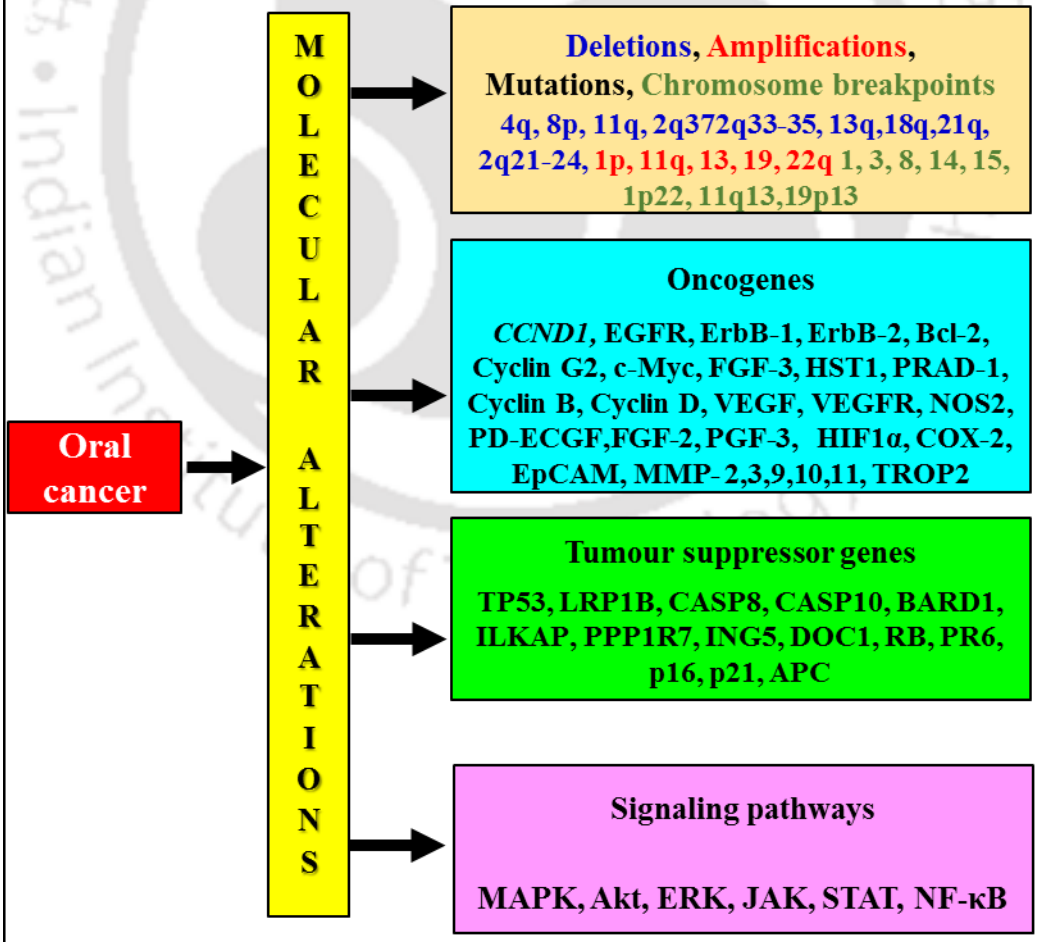

Figure 1.4: Molecular mediators associated with the development of oral cancer.

radicals and induce mutations and DNA damage, which results in oral cancer (Bartsch *et al*., 1999). Nicotine, the major constituent of tobacco smoke promotes cancer by activating signaling pathways involved in cancer cell growth, angiogenesis, migration, and invasion (Xue *et al*., 2014). Nicotine is converted to reactive metabolites during smoking. Majorly nicotine is converted to continine or nicotine-*N*-oxide (NNO) by cytochrome P450 (CYP) 2A6, CYP2B6, and aldehyde oxidase. Nicotine and nitrosamines (*i.e*., NNK, NNN,) can also aid in tumor promotion by activating nicotinic acetylcholine receptors (nAChRs) and β-adrenergic receptors (β-AdrRs), leading to downstream activation of signal transduction pathways (Xue *et al*., 2014).

1.4.2. Alcohol:

Numerous convincing evidences suggest that, alcohol is the main risk factor for oral cancer and in combination with tobacco shows synergistic effect (Radhakrishnan *et al*., 2012). Typically, oral mucosal cells are permeable to particles of size 0.02μm which helps in phagocytosis and pinocytosis, eventually the permeability is augmented due to consumption of alcohol (Ogden, 2005). Studies have also shown that polyploidy is very common in oral cancer patients who consume alcohol (Ogden *et al*., 2009). Apart from these, alcohol damages the liver, due to which the body fails to detoxify harmful carcinogens that can induce oral cancer (Radhakrishnan *et al*., 2012).

1.4.3. Virus infection:

Approximately 42% of oral cancer patients show positive for human papillomavirus (HPV) infection. 77 types of HPV have been identified, of which types 2, 6, 11, 16, 18, 31, 33, 35 are detected in oral epithelial dysplasia while HPV-16 and 18 are seen in OSCC. These viruses incorporate two genes, E6 and E7, to the host genome and persistent expression is maintained throughout the lifetime. E6 /high-risk HPVs form

complexes with cell cycle regulators that bind to p53 and degrades it. E7/low risk HPV's interacts with pRb and blocks its downstream activity (Krishna *et al*., 2015). Apart from HPV, epstein – barr virus (EBV), herpes simplex viruses (HSV), and retroviruses are also known to cause oral cancer (Radhakrishnan *et al*., 2012; Kumar *et al*., 2016).

Other factors: In addition, many other factors are also known to cause oral cancer. These include age, immunosuppression by chemotherapeutic drugs, graft-versus-host disease (GVHD) during stem- cell transplantation, irritation from dentures etc.

1.5. Molecular alterations in oral cancer

As alluded above, development of oral cancer is a multi-step process and entails different molecular alterations in each stage for its progression. These alterations result in remarkable changes in cell survival, cell proliferation, morphology, angiogenesis, longevity and other properties, which are characteristics of cancer cells.

Multiple genetic alterations have been reported in the development of oral cancer (Figure 1.4). The genetic alterations during oral carcinogenesis comprise of point mutations, amplifications, rearrangements, and deletions. Point mutations either activate or inactivate the genes and are usually reported in *K-RAS* and *TP53* genes. Loss of heterozygosity of the *TP53* gene has been reported in 20% of OSCCs, and 22% of premalignant oral lesions (Mehrotra and Yadav, 2006). Frequent deletions at the regions of 2q21-24, 2q33-35, and 2q37 affecting tumour suppressor genes- *LRP1B, CASP8, CASP10, BARD1, ILKAP, PPP1R7*, and *ING5* are seen in OSCC (Yanamoto *et al*., 2007). Deletions in chromosome 3p, 5q, and 9p and gain in 3q are seen in well differentiated tumours, while in poorly differentiated tumours deletions of 4q, 8p, 11q, 13q, 18q, and 21q and gains in 1p, 11q, 13, 19, and 22q are reported implicating their

association with tumour progression (Bockmühl *et al*., 1988). Allelic loss in the region 9p21, coding for cyclin dependent kinase inhibitors p16 and p14 and abnormalities in the regions of 5q21-22, 22q13, 4q, 11q, 18q, and 21q are also observed in different stages of this disease (Ohta *et al*., 2009; Moles *et al*., 2008). Allelic loss at the regions 3p and 9p and other regions encompassing tumour suppressor genes have been reported in precursor lesions of OSCC with varying degrees of dysplasia compared with normal epithelium (Califano *et al*., 1996). Allelic loss or imbalance of TP53 and loss of heterozygosity in DCC (deleted in colon carcinoma), and alterations in chromosomal regions 3p21.3-22.1 and 3p12.1-13 were reported in areas of dysplasia adjacent to infiltrating carcinoma (Emilion *et al*., 1996; Williams, 2000). The allelic loss in premalignant lesions are 77% similar to oral cancer and the premalignant patients possess 73% chances of developing oral cancer within five years (Partridge M *et al*., 1998). Chromosome breakpoints are frequently seen in centromeric regions of chromosomes 1, 3, 8, 14, 15, 1p22, 11q13, and 19p13. Bcl-1, INT-2, and HST-1 located on 11q13 and N-RAS on 11q13, are activated as a result of chromosome breakpoint alterations (Todd *et al*., 1997; Field, 1992; Williams, 2000).

Several oncogenes have been implicated in oral carcinogenesis. Aberrant expression of the proto-oncogenes, *CCND1*, epidermal growth factor receptor (EGFR)/ErbB-1, members of the Ras family, as well as c-Myc, INT-2, HST-1, PRAD-1, and Bcl-2, are associated with the development of oral cancer (Moles *et al*., 2008). Overexpression of cyclin B, cyclin D, ErbB-1 and ErbB-2 are also reported in oral cancer tissues (Tsantoulis *et al*., 2007). It has been shown that dysregulation of human cyclin G2 (hCG2) leads to malignant transformation of oral epithelial cells and elevated levels of DICER leads to the downregulation of let-7b that results in the cell proliferation, that is seen in the early stages of oral cancer (Kim *et al*., 2002; Jakymiw *et al*., 2010). It has

been reported that VEGF promotes the progression of OSCC by upregulating MVD (microvessel density) (Tsantoulis *et al*., 2007). The proangiogenic factors VEGF-R, NOS2, PD-ECGF, FGF-2, PGF-3, HIF-1α and COX-2 play a key role in the angiogenesis of oral cancer (Schliephake, 2005; Wakulich *et al*., 2002; Sudbø, 2004). Flt-4, a member of VEGF is involved in lymph node metastasis of oral cancer while gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), collagenases (MMP-1 and -13) and membrane-bound MMPs are involved in the progression of this disease (Thomas *et al*., 1999). Aberrant activation of transforming growth factor α (TGF-α) is reported in hyperplastic epithelium, and also in carcinoma within the inflammatory cell infiltrate, particularly in the eosinophils, surrounding the infiltrating epithelium. TGF-α induces cell proliferation by binding to EGFR in autocrine and paracrine fashion, and also induces angiogenesis (Derynck, 1992; Grandis and Tweardy, 1993; Todd *et al*., 1997; Williams, 2000). Upregulation of EGFR is often accompanied by the increased production of its ligands, TGF-α and EGF. Increase in number of EGFR receptors in oral cancer is strongly associated with the degree of differentiation of the tumours (Grandis and Tweardy, 1993; Christensen *et al*., 1993; Williams, 2000).

Tumour suppressor genes, cellular negative regulators are also involved in the development of oral cancer. Inactivation of the tumour suppressor genes by point mutations, deletions, and rearrangements in the both gene copies is a major event in the development of oral cancer (Williams, 2000). One of the most important tumour suppressor gene is *TP53*, and gain of function mutation in this gene is implicated in various cancers. The *TP53* gene causes cell cycle arrest at G1 to S phase, DNA repair, and induces apoptosis. Point mutations in this gene structurally alters the tumour suppressing activity while deletions results in loss of expression of *TP53* (Williams, 2000). Approximately in 70% of solid tumours *TP53* is found to be mutated (Hollstein

et al., 1991). Inactivation of p53 is also reported in oral cancer, and restoration of p53 in *in vitro* and *in vivo* settings reversed malignant phenotypes (Schantz, 1995). Smoking and tobacco are well known to cause mutations in *TP53* gene (Langdon *et al*., 1992; Brennan *et al*., 1995; Williams, 2000). Alterations in p21 have also been reported as an early event in oral carcinogenesis (Agarwal *et al*., 1998). Other tumor suppressor genes include *DOC-1, RB, pR6, p16,* and *APC*. Mutations in *DOC-1* has been reported in malignant oral keratinocytes resulting in decrease in expression and loss of function (Todd *et al*., 1995). Loss of expression of pR6 is reported in 66% of oral squamous carcinomas and 64% of premalignant lesions. Similarly, absence of the p16 is seen in 63% of oral squamous carcinomas and in 59% of premalignant lesions. Aberrant expression of pRb/p16 is associated with heavy consumption of betel and tobacco, implicating its role as an early event in the malignant transformation (Pande *et al*., 1998; Williams, 2000).

Apart from these, other molecules involved in oral pathogenesis include increased levels of COX-2, human trophoblast cell-surface antigen (TROP2), epithelial adhesion molecule (EpCAM), which are strongly associated with shorter survival and known to trigger tumor size, lymph node metastasis, histologic differentiation, and invasiveness (Fong *et al*., 2008; Yanamoto *et al*., 2007; Pérez-Sayáns *et al*., 2009). Overexpression of MMP-2 and MMP-9 are well known to induce invasive potential to the tumors. Studies suggest that alcohol induce the overexpression of MMP's and induce oral carcinogenesis (Moles *et al*., 2008; Pérez-Sayáns *et al*., 2009).

From these studies it is very clear that, oral carcinogenesis is a multistep process developed as a result of that genetic processes that can alter the function of oncogenes, tumor suppressor genes, and other related molecules.

1.6. Therapies available

1.6.1. Surgery: In surgery, the tumour and a thin lining of healthy tissue surrounding the tumour are removed. Surgery mainly involves removal of a part or all of the jaw, maxillectomy, removal of lymph nodes and other tissue in the neck, tracheotomy or placing a hole in the windpipe to assist in breathing for patients with large tumours or after surgical removal of the tumour and dental surgery to remove teeth or assist with reconstruction.

1.6.2. Radiation therapy [external beam radiotherapy (EBRT)]: Radiation therapy is the most common in oral and oropharyngeal cancers and sometimes it is given after surgery to destroy small areas of cancer that could not be removed surgically. EBRT is usually employed as an adjuvant to primary surgery or in patients intolerable or unsuited for surgery or as a salvage treatment (American Society of Clinical Oncology, 2017; Huang and O'Sullivan, 2013).

1.6.3. Chemotherapy:

Chemotherapy in oral cancer is mostly given before surgery or concurrently with irradiation. Adjuvant chemoradiotherapy is now serving as a gold standard for treating advanced oral cancers. The first line treatment strategy includes cisplatin at a dose of 100 mg/m^2 on days 1, 22 and 43. Daily low-dose and weekly intermediate dose of cisplatin in oral cancer patients provided survival benefits in oral cancer patients. Apart from cisplatin, other chemotherapeutic agents currently being used are carboplatin, 5-fluorouracil (5-FU), paclitaxel (Taxol®), docetaxel (Taxotere®), and less often methotrexate, ifosfamide (Ifex®) and bleomycin. The main advantage of chemotherapy is that it can be used in any stage of the tumour progression. Apart from these, proton therapy, targeted drug therapy, immunologic response modifiers like alpha interferon

and interleukin have been used in combination with other therapies to boost the patient's immune response against oral carcinoma (American cancer society, 2017; Deng *et al*., 2011).

1.7 Problems associated with therapies:

1.7.1. Chemoresistance: The multidrug resistance proteins ABCB1 (also known as MDR1 or P-gp), ABCC1 (also known as MRP1), ABCG2 (also known as BCRP or MXR) are known to be involved in the development of chemoresistance in oral cancer (Wang C *et al*., 2016). Overexpression of P-gp has been reported in oral cancer and is found to be induced by chemotherapeutic drugs and radiation therapy in oral cancer cells (Nakamura *et al*., 2005; Ng *et al*., 1998). MRP1, was also found to be upregulated in oral cancer compared to normal tissues and was associated with chemoresistance and poor survival of oral cancer patients (Zhang *et al*., 2012). Increased expression of Bcl-2 and Bcl-xL (anti-apoptotic proteins) resulting in dysfunction of apoptosis also lead to drug resistance in oral cancer cells (Camisasca *et al*., 2009; Coutinho-Camillo *et al*., 2010). Upregulation of survivin, increased levels of heat shock proteins 70 (HSP70), mutations in *TP53,* also contribute to chemoresistance *in vitro* (Henriksson *et al*., 2006; Temam *et al*., 2000; Cabelguenne *et al*., 2000; Perrone *et al*., 2010). Wang *et al*., 2012 reported that the development of cisplatin chemoresistance in oral cancer is due to defective DNA damage signaling (Wang *et al*., 2012). Tongue cancer chemotherapy resistant-associated protein 1 (TCRP1) is also involved in developing resistance against cisplatin and reduces apoptosis in Tca8113 cells (Gu *et al*., 2011). Copper-transporting P-type adenosine triphosphate (ATP7B) has also been reported to be associated with cisplatin resistance *in vitro* (Komatsu *et al*., 2000). These evidences suggests that chemoresistance is a major hurdle in treatment of oral cancer.

1.7.2. Tumour recurrence: Local and regional recurrence is one of the reasons for poor prognosis of oral cancer. The reported reasons for tumour recurrence are tumour thickness and FOXP3 subcellular localization (Pinto *et al*., 2003; Weed *et al*., 2013). Overexpression of P-gp was observed in OSCC compared to normal mucosa with oral lesions and was found to be involved in the recurrence of oral cancer (Jain *et al*., 1977). Similarly, upregulation of ABCG2 also induced local recurrence in OSCC patients treated with neoadjuvant chemotherapy (Yanamoto, 2014).

1.8. Neutrophil Gelatinase Associated Lipocalin (NGAL)

NGAL, also known as Lipocalin-2 (LCN2), is a 24 kDa glycoprotein in humans encoded by *LCN2* gene located on chromosome 9 at the locus 3p11. It was initially purified from a culture of mouse kidney cells infected with simian virus 40 (SV-40) and in humans it was isolated from supernatant of human neutrophils (Kjeldsen *et al*., 1993). It is majorly involved in innate immune response and protects the body against bacterial infection, by sequestering iron, thus limiting the bacterial growth (Yang *et al*., 2002). In addition, NGAL is also annotated to be involved in protein-protein interaction, and hence sometimes appears to be complexed with MMP-9. Based on whether NGAL is free or bound to a ligand, it is termed as "apo" or "holo" NGAL respectively. It is expressed in neutrophils, kidney, prostate, and epithelia of the respiratory and alimentary tracts, and is majorly used as a biomarker in kidney injury (Cowland and Borregaard, 1997). In recent years, it has emerged as a biomarker for several benign and malignant diseases.

1.8.1. Structure of NGAL:

Coles *et al*., 1999 elucidated the three dimensional structure of NGAL with the help of NMR. Human NGAL is a 198 amino acid residue with 20-amino acid signal peptide at

the N- terminal end followed by 'lipocalin' domain. The lipocalin domain contains eight antiparallel beta strands forming a barrel. Three bulges are found in this barrel which helps in ligand binding (1 in 1β and 2 in 6β). These beta strands are connected to alpha helix, which in turn is connected to the C- terminal beta sheet. One end of the barrel is open, providing access to the binding site within the barrel cavity, while the other is closed by a short 3_{10}-helix. Hydrophobic residues (tryptophan, valine and phenylalanine) present at the base of the barrel are responsible for direct binding to the ligand. The positively charged amino acid residues (lysine and arginine) present near the mouth of the barrel and projecting into the open end of the molecule is also involved in binding to the ligands (Figure 1.5) (Chakraborty *et al*., 2012).

1.8.2. Function of NGAL:

NGAL is synthesized as a component of the late granules of neutrophils. Primarily NGAL was found in the azurophilic [or myloperoxidase peroxidase (MPO) positive] neutrophil granules co-localized with MPO (Chakraborty *et al*., 2012). Later voluminous studies reported the expression of NGAL in various organs. NGAL is expressed in several adult normal tissues like breast ducts, kidney, liver, lungs, trachea, small intestine, bone marrow, thymus, prostate, adipose tissue and macrophages. A weak to no expression of NGAL is observed in endometrial glands, thymus and peripheral blood leucocytes. Nevertheless, NGAL is completely absent in brain, heart, skeletal muscle, spleen, testes (Chakraborty *et al*., 2012).

The levels of free iron accessible in human body is extremely low as the iron binding proteins like transferrin, ferritin and lactoferrin form complexes with the free iron molecules. During bacterial infections, in order to survive poor iron environment, these

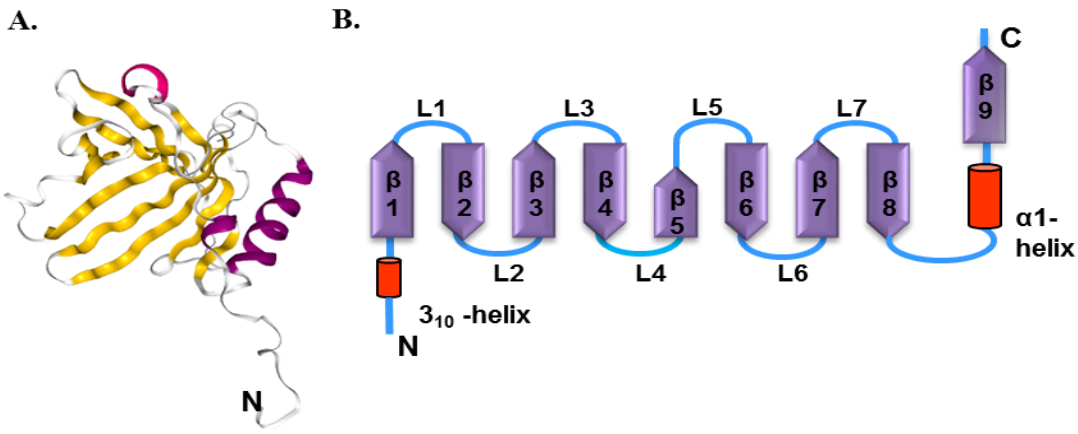

Figure 1.5: Structure of NGAL. A) The structure wad generated using RCSB PDB database (http://www.rcsb.org) (PDB ID: 1NGL). B) Topology diagram of structure of NGAL. Structure elucidation by Coles *et al.*,1999 was used as reference.

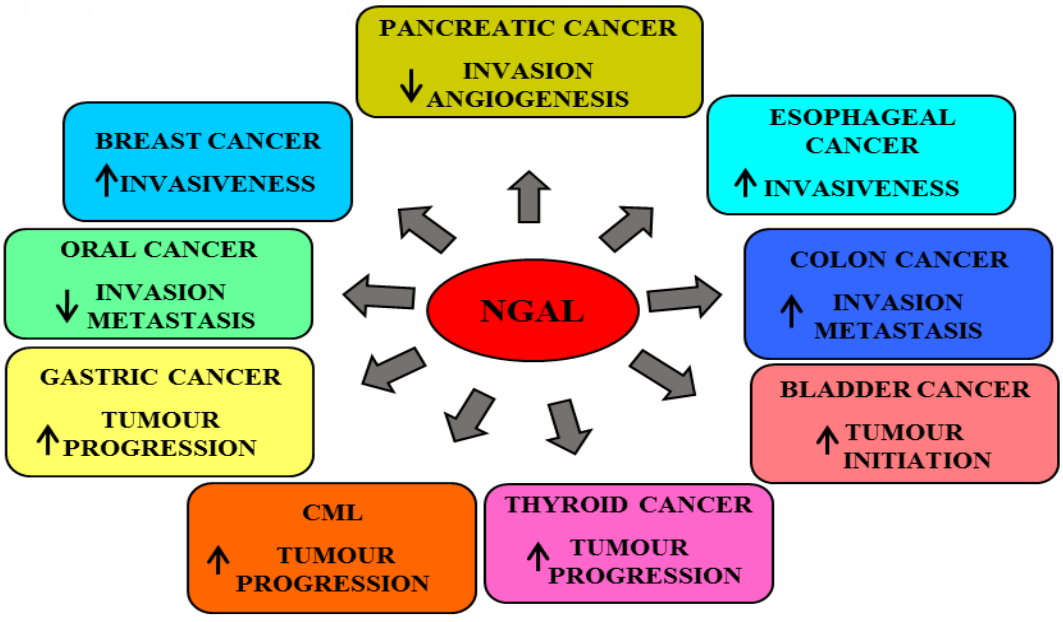

Figure 1.6: Differential expression and role of NGAL in various cancers; CML: Chronic myeloid leukemia; Adapted from Monisha et al., 2014.

bacteria have developed special proteins known as siderophores. These siderophores, possess high affinity towards the iron molecules than the endogenous iron chelators.

This property of siderophores facilitates them not only to bind to free iron molecules, but also to mine the iron from iron binding molecules. At the time of infections, the bacteria produce these siderophores, which in turn bind to iron forming iron-siderophores complex, which is then transported to bacteria, and helps in the proliferation. Studies have showed that NGAL has high affinity towards these siderophores. NGAL can bind to these siderophores both in their holo or apo form, and transport them through its receptor NGALR. As iron is crucial for the growth of bacteria, by exhausting iron stores, NGAL inhibits bacterial growth and exerts its bacteriostatic activity (Chakraborty *et al*., 2012).

1.8.3. NGAL in Malignancy:

Although NGAL is indispensable for its role during innate immune responses, it is also referred as protumorigenic protein and is responsible for progression of various tumours (Figure 1.6) (Moniaux *et al*., 2008; Leung *et al*., 2012). The expression of NGAL was first studied by Nielssen and group in colon cancer in the year 1996. Later the expression and role of NGAL in different cancers have been studied by different groups. It has been well documented that NGAL increases the invasiveness and metastases of certain cancer types and also shown to induce drug resistance (Fernández *et al*., 2012; Tong *et al*., 2008; Leung *et al*., 2012).

1.8.4. Expression of NGAL in different cancers:

Differential expression of NGAL has been evinced in different malignancies. The expression of NGAL was upregulated in breast, colon, bladder, lung, cervical, esophageal, ovarian, liver cancers and chronic myeloid leukemia, while its expression was downregulated in pancreatic and oral cancers (Wenners *et al*., 2012; Provatopoulou *et al*., 2009; Dokun *et al*., 2008; Candido *et al*., 2014; Li *et al*., 2003; Wu *et al*., 2014;

Wang *et al*., 2010; Du *et al*., 2011a; Syrjänen *et al*., 2010; Chung *et al*., 2016; Lim *et al*., 2007; Cho *et al*., 2009; Wang *et al*., 2011; Zhang *et al*., 2012; Wang *et al*., 2013; Lee *et al*., 2011; Alonci *et al*., 2012; Tang *et al*., 2015; Song *et al*., 2015; Ruiz-Morales *et al*., 2015; Tong *et al*., 2008; Tong *et al*., 2011; Hiromoto *et al*., 2011; Lin *et al*., 2016). NGAL was found to upregulated in the tumour tissues of breast cancer patients compared to normal tissues and strongly correlated with negative steroid receptor status, HER-2/neu status, poor histologic grade, lymph node metastases, high proliferative index and poor prognosis (Bauer M, 2008). NGAL was also detected in the urine and serum of breast cancer patients either in free form or bound to MMP-9 and can be used to monitor breast cancer progression non-invasively (Wenners *et al*., 2012; Provatopoulou *et al*., 2009; Fernández *et al*., 2005). The binding of MMP-9 to NGAL, prevents the degradation of MMP-9 and drives tumour progression (Fernández *et al*., 2005; Provatopoulou *et al*., 2009). In bladder cancer both mRNA and protein levels of NGAL were upregulated compared to normal counter parts (Dokun *et al*., 2008; Candido *et al*., 2014). High levels of NGAL was seen in the urine of bladder cancer patients and positively correlated with the invasiveness of the tumour (Candido *et al*., 2014). Moreover, overexpression of NGAL was independent of LCN2 promoter methylation in bladder cancer (Dokun *et al*., 2008). In colorectal cancer patients, overexpression of NGAL was observed in sequential development of colorectal adenoma-carcinoma, in colonic epithelium in areas of inflammation, premalignant and malignant neoplastic lesions and was concomitant with tumour progression, overall survival and led to poor prognosis (Barresi *et al*., 2011a & 2011b; Hu *et al*., 2009; Sun *et al*., 2011; Maier *et al*., 2014; Catalán *et al*., 2011; Nielsen *et al*., 1996). High levels of NGAL was also detected in the plasma of tumor patients and was associated with higher neoplastic tissue volume, invasion, and recurrence (Duvillard *et al*., 2014; Martí

et al., 2013; Ding *et al*., 2014). Studies revealed that elevated expression of NGAL indicated poor prognosis and can serve as a potent diagnostic and prognostic biomarker of colorectal cancer (Wang and Zeng, 2014). Likewise, overexpression of NGAL was observed in ESCC compared to normal tissues and was strongly correlated with malignant transformation, cell differentiation and poor survival (Li *et al*., 2003; Wu *et al*., 2014; Du *et al*., 2011a). Moreover, enzymatic activity of NGAL/MMP-9 complex was higher compared to normal mucosa and was strongly associated with tumor invasion in ESCC (Zhang *et al*., 2007; Li *et al*., 2003). Higher expression of NGAL was pragmatic in gastric cancer tissues and correlated with size of tumor, Lauren's classification (gastric cancer is classified into two types; intestinal type and diffuse type adenocarcinoma), lymph node metastasis, vascular invasion, distant metastasis, and TNM stage (Wang *et al*., 2010; Du *et al*., 2011b). Similar to ESCC, the association of NGAL/MMP-9 complex was significantly high in gastric cancer (Kubben *et al*., 2007). 12-O-tetradecanoylphorbol-13-acetate (TPA) and *Helicobacter pylori* infection was found to enhance the expression of NGAL in gastric cancer (Du *et al*., 2011b). These studies suggest that NGAL can serve as a potent diagnostic and prognostic biomarker for gastric cancer. In line with this, NGAL was also found in the serum and urine of gastric cancer patients, and can be used as a potent biomarker (Wang *et al*., 2010; Shimura *et al*., 2015). Upregulation of NGAL was also observed in ovarian and cervical cancers and was found to correlate with grade, EMT, migration and invasion (Syrjänen *et al*., 2010; Chung *et al*., 2016; Lim *et al*., 2007; Cho *et al*., 2009; Wang *et al*., 2011). Augmented levels of NGAL was also detected in serum of ovarian and cervical cancer patients compared to normal healthy individuals (Lim *et al*., 2007; Cho *et al*., 2009).

High expression of NGAL and its receptor NGALR was observed in HCC and was strongly associated with vascular invasion, TNM stage, tumor recurrence, shorter survival of HCC (Zhang *et al*., 2012; Wang *et al*., 2013; Lee *et al*., 2011). As many reports advocate that NGAL is detected in tissues and secreted in serum, Lee and his group developed aptamer for early diagnosis of HCC. The preliminary results of this study revealed that the aptamer could detect dynamic range of NGAL in serum of the patients, indicating that NGAL holds immense potential in diagnosis of HCC (Lee *et al*., 2015). Further, significantly high expression of NGAL was seen in CML patients which paralleled the expression of BCR-ABL at early stages of CML (Villalva *et al*., 2008). The levels of NGAL detected in the serum of CML patients was high compared to healthy controls while in patients after imatinib therapy the levels of NGAL was decreased (Alonci *et al*., 2012). Furthermore, preclinical and clinical studies in lung cancer showed that NGAL was overexpressed in lung cancer and directly correlated with lung tumorigenesis and poor prognosis (Tang *et al*., 2015; Song *et al*., 2015; Ruiz-Morales *et al*., 2015). Unlike the above mentioned cancers, downregulation of NGAL expression was observed in pancreatic cancer tissues compared to normal tissues and was found to be correlated with poor prognosis, EMT, tumor invasion and migration (Xu *et al*., 2012; Xu *et al*., 2013). In pancreatic cancer cell lines, well to moderately differentiated cells presented increased expression of NGAL, and in moderate to poorly differentiated cells the expression was weak (Tong *et al*., 2008). In this cancer, MUC4 was shown to regulate the expression of NGAL by stabilizing HER2 and activating downstream PI3K/Akt/NF-κB pathway (Kaur *et al*., 2014). The expression of NGAL was also downregulated in AML patients compared to normal healthy controls (Frankfurt and Platanias, 2013). In the same line, the expression of NGAL was downregulated in oral cancer compared to normal tissues and was associated with the

degree of differentiation (Hiromoto *et al*., 2011). Expression of NGAL was high in weakly invasive, weak in mildly invasive (HSC-3, OSC-19, and SCC-25) and negative in highly invasive (HOC-313 and TSU) oral squamous cell carcinoma cell lines and was found to be inversely correlated with E-cadherin and MMP-9 expression (Hiromoto *et al*., 2011).

1.8.5. Role of NGAL in cancer cell survival and proliferation:

There are only couple of reports available about the role of NGAL in cancer cell survival and proliferation. Overexpression of NGAL augmented the proliferation of cervical cancer cells through downregulation of p53 and p21 (Wang *et al*., 2011). Similarly, in lung cancer, overexpression of NGAL increased cell proliferation, while its silencing reduced cell survival, proliferation and induced ER stress via Nrf2/HO-1 signaling (Song *et al*., 2015). However, stable expression of NGAL in some cancers have been shown to inhibit JNK, PI3K/Akt signaling and induced caspase dependent apoptosis and inhibited tumor growth (Xu *et al*., 2012).

1.8.6. Role of NGAL in EMT, invasion and metastases:

Increasing lines of evidence suggest that NGAL is a crucial regulator of EMT, invasion and metastases in different cancers. The association of NGAL with MMP-9, one of the key proteins involved in invasion and metastases, was first reported by Yan L and group in 2001, where they have shown that NGAL binds to MMP-9 and prevents its degradation (Yan *et al*, 2001). Later, it was shown that overexpression of NGAL persuaded the expression of mesenchymal markers, and augmented cell motility and invasiveness, while silencing of NGAL repressed the expression of ER-α and cell migration *in vitro* and mammary tumor formation and metastasis *in vivo* (Leng *et al* 2009; Yang *et al*., 2009; Yang *et al*., 2013; Ören 2016). Gomez-Chou and group,

reported that silencing of NGAL reduced extracellular matrix deposition, immune cell infiltration, PanIN formation, tumor growth and increased the survival *in vivo* (Gomez-Chou 2017). Upregulation of NGAL was found to be STAT3 and CEBPβ dependent and activates endogenous MMP-9 activity, by increased activation of MEK/ERK pathway and promotes migration and invasion by altering F-actin rearrangements (Jung *et al*., 2012; Li *et al*., 2003). Overexpression of NGAL was well known to induce EMT via activation of snail, twist, N-cadherin, fibronectin, MMP-9, and NF-κB, resulting in upregulation of genes associated with stemness, adhesion, motility and drug efflux (Chung *et al*., 2016; Mongre *et al*., 2016; Leung *et al*., 2012). In line with this, silencing of NGAL has been shown to reduce migration and invasion via downregulation of vimentin, MMP-2, MMP-9 and increased the expression of E-cadherin (Tang *et al*., 2015).

However, a handful of studies suggest that NGAL inhibits EMT, invasion and metastases. For example, decreased expression of NGAL was observed in pancreatic cancer tissues compared to normal tissues and was found to be correlated with EMT, tumor invasion and migration (Xu *et al*., 2012; Xu *et al*., 2013). Stable overexpression of NGAL in cancer cells was shown to inhibit cell adhesion, invasion, and angiogenesis along with the decrease in expression of focal adhesion kinase (FAK) tyrosine-397 phosphorylation, VEGF, MMP-2, N-cadherin, vimentin, alpha-smooth muscle actin, fibronectin and reduced tumour volume, local and distant metastases *in vivo* (Tong *et al*., 2008; Xu *et al*., 2012; Wang *et al*., 2013; Lee *et al*., 2011). In PDAC cells, EGF impedes the expression of NGAL, via EGFR-driven ERK pathway and prevents the NF-κB mediated transactivation of NGAL. Additionally, E-cadherin also regulated the expression of NGAL, where upregulation of E-cadherin induced NGAL and vice-versa (Tong *et al*., 2011). Unlike tissues samples, in a highly metastatic human colon cancer

cell line, KM12SM, decreased expression of NGAL was observed, while ectopically expressing, it suppressed invasion *in vitro* and liver metastasis *in vivo* (Lee *et al*., 2009). In oral cancer, ectopic expression of NGAL abridged migration, invasion and metastasis, while silencing of NGAL enhanced cell motility by inhibiting carbonic anhydrase IX (CAIX) via HIF-1α and miR-4505 (Lin et *al*., 2016).

1.8.7. Role of NGAL in chemoresistance:

Recent reports suggest that upregulation of NGAL induce resistance against tyrosine kinase inhibitors and its downregulation was shown to sensitize NSCLC to erlotinib *in vitro* and *in vivo* by altering proapoptotic protein BIM. Moreover, NSCLC patients with lower levels of plasma NGAL were sensitive to erlotinib therapy (Krysan *et al*., 2013). These studies suggest that NGAL can serve as a potent therapeutic target in lung cancer. Likewise, downregulation of NGAL also sensitized pancreatic ductal adenocarcinoma to gemcitabine both *in vitro* and *in vivo (*Leung *et al*., 2012).

1.9. Importance of the study

The survival rate of oral cancer has shown no improvement over the decades. Early diagnosis of this disease is difficult due to lack of biomarkers. In addition, chemoresistance and tumor recurrence also contribute to the poor prognosis of oral cancer patients. It has been well established that molecular alterations in many proteins are also involved in oral cancer progression. Previous studies have shown that NGAL is downregulated in oral cancer, however, its expression and role in different types and different process of oral cancer development has not been studied thoroughly. Therefore, studying the expression of NGAL in different process of development of oral cancer would help us to identify promising biomarker for oral cancer. As it is well known that tobacco is main risk factor for oral cancer, understanding the role of tobacco

components, in altering the expression of NGAL, would provide us new insights on the development of this disease. Deciphering the role of this protein in different hallmarks of oral squamous cell carcinoma would help us to develop novel highly efficacious therapies for this dreadful disease.

1.10 The main objectives of this study are:

1. Determine the expression of NGAL in human normal oral epithelium and different stages of oral squamous cell carcinoma (along with preneoplastic lesions).
2. Examine the effect of various tobacco components (carcinogens) on the expression of NGAL in oral cancer cell lines.
3. Determine the role of NGAL in different process of development of oral squamous cell carcinoma.

2 CHAPTER

Expression Of NGAL In Human Oral Cancer

2. Introduction

As detailed in the previous chapter, oral cancer is the most predominant cancer in India with limited effective therapies till date. Therefore, identification of novel biomarkers for early diagnosis and therapeutic targets to develop effective therapies for this disease has become imperative. The literature review in the preceding chapter suggests that NGAL is altered in different benign and malignant tumors and can be employed as a biomarker and therapeutic target for cancer drug discovery as this protein was shown to induce invasion, metastases and chemoresistance in different malignancies. Hence, the present study was intended to evaluate the expression and role of NGAL in oral cancer. Although, limited studies have evaluated the expression of NGAL in oral cancer tissues, the expression of this protein in different stages, grades, cytological types and organs of oral cancer has remained unexplored. The development of this cancer is a multi-stage process which starts with preneoplastic lesions such as leukoplakia, erythroplakia, oral lichen planus, sub mucous fibrosis, hyperplasia, dysplasia and finally leads to well differentiated, moderately differentiated and poorly differentiated carcinoma. Oral cancer affects mostly all the anatomical sites of the oral cavity such as tongue, cheeks, palate, gingiva, lips etc. and are of different cytological types such as adenoid cystic cell carcinoma, basal cell carcinoma, squamous cell carcinoma and metastasize to mainly lymph nodes, lungs and bone. Hence, better understanding of the expression of this protein in different premalignant lesions, stages and grades of oral cancer is highly indispensable to study the role of this protein in diverse process of oral carcinogenesis. Thus, the main objective of this chapter is to ascertain the expression of NGAL in the above-mentioned stages, grades and cytological types of oral cancer in patient samples. This would help us to comprehend whether NGAL can be used as a supporting diagnostic and prognostic biomarker for

oral cancer and as a therapeutic target to discover novel, highly potent therapies for this disease.

2.1. Materials and Methods

2.1.1. Tissue microarray:

Tissue microarray slides for head and neck squamous cell carcinoma (Cat no: HN803b) and oral squamous cell carcinoma (Cat no: OR802) were purchased from US Biomax, Derwood, USA. Head and neck squamous cell carcinoma tissue microarray contained tissues from eighty different patients which contains sixty cases of squamous cell carcinoma, one case of carcinoma sarcomatodes, eight cases of metastatic carcinoma, nine cases of normal tissue and two cases of adjacent normal tissues. The tissue microarray slide from OSCC contained tissues from seventy nine different patients with twenty eight cases of squamous cell carcinoma, four cases of adenocarcinoma, eight cases of mucoepidermoid carcinoma, two cases of basal cell carcinoma, four cases of metastatic carcinoma, eight cases of adamantinoma, six cases of hyperplasia, five each of adjacent tissue, inflammation, adjacent normal tissue and normal tissue.

2.1.2. Tissue microarray details:

Multiple head and neck cancer tissue array with normal tissue, including TNM, clinical stage and pathology grade, 80 cases/ 80 cores

Name: HN803b

Description: Multiple head and neck cancer tissue array with normal tissue, including

Panel: Multiple head and neck carcinoma tissue microarray

Cases: 80

Cores: 80

Diameter: 1.5mm

Rows: 8

Columns: 10

Table 2.1: Head and neck cancer tissue array details (HN803b).

Position	Age	Sex	Organ	Pathology diagnosis	TNM	Grade	Stage
A1[a]	61	F	Tongue	Squamous cell carcinoma of palate	T1N0M0	1	I
A2[a]	61	M	Tongue	Squamous cell carcinoma of lower lip	T1N0M0	1	I
A3[a]	77	M	Tongue	Squamous cell carcinoma of lower lip	T1N0M0	1	I
A4[a]	75	F	Tongue	Squamous cell carcinoma of upper jaw (chronic inflammation of fibrous tissue and blood vessel)	T1N0M0	-	I
A5[a]	55	M	Tongue	Squamous cell carcinoma of tongue	T1N0M0	1	I
A6[a]	67	M	Tongue	Squamous cell carcinoma of right palate	T2N0M0	1	II
A7[a]	67	M	Larynx	Squamous cell carcinoma of larynx	T2N0M0	1	II
A8[a]	70	M	Nose	Squamous cell carcinoma of right maxillary sinus (fibrous tissue and blood vessel)	T2N0M0	-	II
A9[a]	40	M	Larynx	Squamous cell carcinoma of larynx	T2N1M1	2	IV
A10[a]	55	M	Larynx	Squamous cell carcinoma of epiglottis	T4N0M0	2	IV
B1[a]	57	M	Tongue	Squamous cell carcinoma of pharynx	T4N0M0	2	IV
B2[a]	49	M	Tongue	Squamous cell carcinoma of left gingiva	T4N0M0	1	IV
B3[a]	50	M	Tongue	Squamous cell carcinoma of root of tongue	T3N0M0	2	III
B4[a]	54	M	Tongue	Squamous cell carcinoma of left upper jaw	T3N0M0	2	III
B5[a]	72	M	Larynx	Squamous cell carcinoma of larynx	T2N0M0	2	II
B6[a]	50	M	Tongue	Squamous cell carcinoma of tongue (sublingual gland tissue)	T2N0M0	-	II
B7[a]	74	M	Nose	Squamous cell carcinoma of nasal root	T2N0M0	2	II
B8[a]	49	M	Larynx	Squamous cell carcinoma of larynx	T4N1M0	2	IV
B9[a]	50	M	Larynx	Squamous cell carcinoma of larynx	T2N1M0	2	III
B10[a]	90	M	Tongue	Squamous cell carcinoma of cheek	T2N0M0	2	II
C1[a]	67	M	Larynx	Squamous cell carcinoma of larynx	T3N1M0	2	III
C2[a]	56	M	Larynx	Squamous cell carcinoma of epiglottis	T4N0M0	2	IV
C3[a]	66	M	Larynx	Squamous cell carcinoma of left laryngeal pharynx (sparse)	T3N2M0	2	IV
C4[a]	58	M	Larynx	Squamous cell carcinoma of larynx	T3N1M1	2	IV
C5[a]	60	M	Tongue	Squamous cell carcinoma of tongue	T2N0M0	3	II
C6[a]	67	M	Larynx	Squamous cell carcinoma of sinus piriformis	T2N0M0	3	II
C7[a]	56	F	Nose	Squamous cell carcinoma of nasopharynx	T2N0M0	3	II
C8[a]	48	M	Tongue	Squamous cell carcinoma of left submaxilla	T2N0M0	1	II
C9[a]	56	M	Larynx	Squamous cell carcinoma of laryngeal pharynx	T2N0M0	2	II
C10[a]	43	M	Tongue	Squamous cell carcinoma of right submaxilla	T2N0M0	2	II
D1[a]	70	M	Tongue	Squamous cell carcinoma of gingiva of right submaxilla	T2N0M0	2	II
D2[a]	61	F	Tongue	Squamous cell carcinoma of left submaxilla	T2N0M0	2	II
D3[a]	56	F	Tongue	Squamous cell carcinoma of mouth floor	T2N0M0	2	II
D4[a]	55	M	Larynx	Squamous cell carcinoma of larynx	T3N1M0	2	III
D5[a]	50	M	Larynx	Squamous cell carcinoma of larynx	T3N1M0	2	III
D6[a]	49	M	Larynx	Squamous cell carcinoma of larynx	T2N1M0	3	III
D7[a]	71	M	Larynx	Squamous cell carcinoma of epiglottis	T3N1M0	2	III
D8[a]	50	F	Larynx	Squamous cell carcinoma of laryngeal pharynx	T2N1M0	2	III
D9[a]	58	M	Larynx	Squamous cell carcinoma of sinus piriformis	T3N0M0	2	III
D10[a]	71	M	Larynx	Squamous cell carcinoma of laryngeal pharynx	T2N1M0	2	III
E1[a]	61	M	Larynx	Squamous cell carcinoma of epiglottis	T2N1M0	2	III

E2[a]	44	M	Nose	Squamous cell carcinoma of laryngeal pharynx	T2N1M0	2	III
E3[a]	61	M	Larynx	Squamous cell carcinoma of larynx	T3N0M0	2	III
E4[a]	45	M	Tongue	Squamous cell carcinoma of right cheek	T2N0M0	3	II
E5[a]	47	M	Larynx	Squamous cell carcinoma of larynx	T2N0M0	2	II
E6[a]	63	M	Larynx	Squamous cell carcinoma of epiglottis	T3N1M0	2	III
E7[a]	61	M	Larynx	Squamous cell carcinoma of epiglottis	T2N0M0	3	II
E8[a]	75	M	Tongue	Squamous cell carcinoma of maxillary sinus	T4N0M0	3	IV
E9[a]	50	M	Nose	Squamous cell carcinoma of sinus piriformis	T2N0M0	3	II
E10[a]	64	M	Larynx	Squamous cell carcinoma of larynx	T2N0M0	3	II
F1[a]	38	F	Tongue	Squamous cell carcinoma of lower lip	T2N0M0	3	II
F2[a]	56	F	Tongue	Squamous cell carcinoma of cheek	T3N0M0	3	III
F3[a]	42	F	Nose	Squamous cell carcinoma of nasal sinus	T3N0M0	3	III
F4[a]	47	M	Larynx	Squamous cell carcinoma of larynx (mixed gland tissue)	T2N1M0	-	III
F5[a]	51	M	Larynx	Squamous cell carcinoma of larynx (sparse)	T2N1M0	2	III
F6[a]	58	M	Larynx	Squamous cell carcinoma of sinus piriformis with necrosis	T2N2M0	3	IV
F7[a]	74	M	Larynx	Squamous cell carcinoma of laryngeal pharynx with necrosis	T4N0M0	3	IV
F8[a]	48	M	Larynx	Squamous cell carcinoma of laryngeal pharynx	T4N0M0	2	IV
F9[a]	65	M	Larynx	Squamous cell carcinoma of larynx	T3N2M0	3	IV
F10[a]	75	M	Nose	Squamous cell carcinoma of nasal sinus	T4N0M0	3	IV
G1[a]	32	F	Tongue	Carcinoma sarcomatodes of maxillary sinus	T2N0M0	-	II
G2[b]	40	F	Tongue	Metastatic mucoepidermoid carcinoma of neck	-	3	-
G3[b]	67	M	Tongue	Metastatic mucoepidermoid carcinoma of base of tongue	-	3	-
G4[b]	55	M	Tongue	Metastatic squamous cell carcinoma of left upper gingiva	-	2	-
G5[b]	47	M	Tongue	Metastatic squamous cell carcinoma of oral cavity	-	1	-
G6[b]	49	M	Tongue	Metastatic squamous cell carcinoma of hypoglottis	-	2	-
G7[b]	53	M	Tongue	Metastatic squamous cell carcinoma of laryngeal pharynx	-	3	-
G8[b]	43	M	Tongue	Metastatic acinic cell carcinoma of neck	-	3	-
G9[b]	52	F	Tongue	Metastatic acinic cell carcinoma of neck	-	1	-
G10[d]	38	M	Tongue	Cancer adjacent normal tongue tissue	-	-	-
H1[c]	28	M	Tongue	Normal tongue tissue	-	-	-
H2[c]	27	F	Tongue	Normal tongue tissue	-	-	-
H3[c]	48	M	Tongue	Normal tongue tissue	-	-	-
H4[d]	42	F	Tongue	Cancer adjacent normal tongue tissue	-	-	-
H5[c]	15	F	Tongue	Normal tongue tissue	-	-	-
H6[c]	19	F	Tongue	Normal tongue tissue	-	-	-
H7[c]	35	M	Tongue	Normal tongue tissue	-	-	-
H8[c]	18	F	Tongue	Normal tongue tissue	-	-	-
H9[c]	19	F	Tongue	Normal tongue tissue	-	-	-
H10[c]	28	M	Tongue	Normal pharynx tissue	-	-	-

a: malignant tissues, b: metastatic tissues, c: Normal tissues, d: Normal adjacent tissue

Oral cavity disease spectrum (oral cavity cancer progression) tissue array, 79 cases/80 cores

Name: OR802

Panel: Oral cavity disease spectrum (oral cavity carcinoma progression) tissue microarray

Cases: 79

Cores: 80

Diameter: 1.5mm

Rows: 8

Columns: 10

Table 2.2: Oral cavity tissue array details (OR802).

Position	Age	Sex	Organ	Pathology diagnosis	TNM	Grade	Stage
A1[a]	40	M	Gingiva	Squamous cell carcinoma	T4N0M0	1	IV
A2[a]	47	F	Tongue	Squamous cell carcinoma	T1N0M0	1	I
A3[a]	81	M	Lip	Squamous cell carcinoma	T2N0M0	1	II
A4[a]	57	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I
A5[a]	52	F	Lip	Squamous cell carcinoma	T1N0M0	2	I
A6[a]	53	M	Cheek	Squamous cell carcinoma	T2N0M0	1	II
A7[a]	62	F	Cheek	Squamous cell carcinoma	T1N0M0	1	I
A8[a]	48	M	Base of tongue	Squamous cell carcinoma	T2N0M0	-	II
A9[a]	68	M	Right palate	Squamous cell carcinoma	T2N0M0	1	II
A10[a]	56	F	Cheek	Squamous cell carcinoma	T2N0M0	1	II
B1[a]	79	M	Cheek	Squamous cell carcinoma	T2N0M0	1	II
B2[a]	60	M	Gingiva	Squamous cell carcinoma	T1N0M0	1	I
B3[a]	55	M	Cheek	Squamous cell carcinoma	T1N0M0	1	I
B4[a]	66	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I
B5[a]	46	F	Tongue	Squamous cell carcinoma	T1N0M1	1	IV
B6[a]	39	F	Tongue	Squamous cell carcinoma	T1N0M0	1	I
B7[a]	78	M	Tongue	Squamous cell carcinoma	T2N0M0	1	II
B8[a]	78	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV
B9[a]	54	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV
B10[a]	75	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV
C1[a]	60	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I
C2[a]	73	M	Lip	Squamous cell carcinoma	T1N0M0	1	I
C3[a]	60	M	Gingiva	Squamous cell carcinoma	T1N0M0	2	I
C4[a]	78	M	Lip	Squamous cell carcinoma	T1N0M0	2	I
C5[a]	55	M	Gingiva	Squamous cell carcinoma	T1N0M0	2--3	I
C6[a]	47	M	Lower mandible	Squamous cell carcinoma	T2N0M0	3	II
C7[a]	41	M	Dental alveoli	Squamous cell carcinoma	T1N0M0	3	I
C8[a]	60	M	Tongue	Squamous cell carcinoma	T2N0M0	3	II
C9[a]	40	F	Palate	Adenoid cystic carcinoma	T1N0M0	-	I
C10[a]	45	M	Left lower mandible	Adenoid cystic carcinoma (sparse)	T1N0M0	-	I
D1[a]	64	M	Palate	Adenoid cystic carcinoma	T2N0M0	-	II
D2[a]	66	M	Parotid gland	Acinic cell carcinoma	T2N0M0	-	II

D3[a]	71	M	Mouth floor	Mucoepidermoid carcinoma	T1N0M0	1	I
D4[a]	57	M	Palate	Mucoepidermoid carcinoma	T2N0M0	1	II
D5[a]	50	F	Cheek	Mucoepidermoid carcinoma	T2N0M0	1	II
D6[a]	57	M	Upper lip	Mucoepidermoid carcinoma (skeletal muscle and blood vessel)	T1N0M0	-	I
D7[a]	48	F	Right lower mandible	Mucoepidermoid carcinoma	T1N0M0	2	I
D8[a]	55	M	Gingiva	Mucoepidermoid carcinoma	T1N0M0	3	I
D9[a]	60	M	Right lower mandible	Mucoepidermoid carcinoma	T3N0M0	3	III
D10[a]	50	M	Root of tongue	Mucoepidermoid carcinoma (sparse)	T1N0M0	-	I
E1[a]	79	F	Lip	Basal cell carcinoma (sparse)	T2N0M0	-	II
E2[a]	48	F	Lip	Basal cell carcinoma	T2N0M0	-	II
E3[b]	70	F	Lymph node	Metastatic squamous cell carcinoma of neck from cheek	-	2	-
E4[b]	79	M	Lymph node	Metastatic squamous cell carcinoma of neck from tongue	-	1	-
E5[b]	59	F	Lymph node	Metastatic squamous cell carcinoma of neck from mandible	-	2	-
E6[b]	40	F	Lymph node	Metastatic mucoepidermoid carcinoma of neck from mandible	-	3	-
E7[e]	11	M	Mandible	Adamantinoma	-	-	-
E8[e]	28	M	Left mandible	Adamantinoma	-	-	-
E9[e]	51	M	Right mandible	Adamantinoma	-	-	-
E10[e]	64	M	Mandible	Adamantinoma (fibrous tissue and blood vessel)	-	-	-
F1[e]	37	F	Mandible	Adamantinoma	-	-	-
F2[e]	40	M	Lower mandible	Adamantinoma	-	-	-
F3[e]	47	F	Mandible	Adamantinoma	-	-	-
F4[e]	70	F	Right jaw bones	Adamantinoma	-	-	-
F5[f]	67	M	Lip	Hyperplasia of squamous epithelium	-	-	-
F6[f]	40	M	Lip	Mild atypical hyperplasia of squamous epithelium	-	-	-
F7[f]	82	M	Lip	Hyperplasia of squamous epithelium	-	-	-
F8[f]	46	M	Tongue	Hyperplasia of squamous epithelium (skeletal muscle and blood vessel)	-	-	-
F9[f]	60	F	Tongue	Hyperplasia of squamous epithelium	-	-	-
F10[f]	3	F	Tongue	Hyperplasia of squamous epithelium	-	-	-
G1[g]	68	M	Parotid gland	Cancer adjacent tissue (with squamous cell carcinoma sparse)	-	-	-
G2[g]	53	F	Tongue	Cancer adjacent tissue	-	-	-

G3[g]	53	M	Tongue	Cancer adjacent tissue (hyperplasia of squamous epithelium)	-	-	-
G4[g]	70	M	Parotid gland	Cancer adjacent tissue (with mucoepidermoid carcinoma)	-	2	-
G5[g]	63	M	Lip	Cancer adjacent tissue (chronic inflammation of fibrous tissue and blood vessel)	-	-	-
G6[h]	43	M	Sub maxillary gland	Chronic submaxillaritis	-	-	-
G7[h]	23	F	Parotid gland	Chronic parotitis	-	-	-
G8[h]	66	F	Parotid gland	Chronic parotitis	-	-	-
G9[h]	40	F	Right cheek	Chronic inflammation of mucosa	-	-	-
G10[h]	75	F	Lower lip	Chronic inflammation of mucosa of No. 20	-	-	-
H1[d]	48	F	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H2[d]	48	F	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H3[d]	37	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H4[d]	63	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H5[d]	56	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H6[c]	42	F	Tongue	Normal tongue tissue	-	-	-
H7[c]	38	F	Salivary gland	Normal salivary gland tissue	-	-	-
H8[c]	48	M	Tongue	Normal tongue tissue	-	-	-
H9[c]	50	M	Salivary gland	Normal salivary gland tissue	-	-	-
H10[c]	22	M	Salivary gland	Normal salivary gland tissue	-	-	-

a: malignant tissues, b: metastatic tissues, c: Normal tissues, d: Normal adjacent tissue, e: benign tissues, f: hyperplastic tissues, g: cancer adjacent tissue, h: inflammation

2.1.3. Immunohistochemistry (IHC):

Expression of NGAL was determined by immunohistochemical analysis. Histostain plus kit (Cat no: 859043, Life technologies, California, USA) was used according to manufacturer's protocol. Anti-hNGAL monoclonal antibody was purchased from (Cat no: ab23477, Abcam, Cambridge, USA). The TMA were deparaffinised and rehydrated using xylene and decreasing concentrations of EtOH. The endogenous peroxidase

activity was blocked by 3% hydrogen peroxide in methanol for 30 minutes and antigen retrieval was performed by heating in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 30min. The sections were then incubated in blocking solution for 30 min in a humidified chamber and then incubated with primary antibody (1:10 dilution) at 4 °C for overnight. The following day the sections were incubated with secondary antibody for 1 h at room temperature, stained with DAB, counter stained with haematoxylin and were mounted using DPX.

2.1.4. Scoring:

All slides were observed under Nikon Eclipse Ti-E, and the intensity of immunoreactivity for NGAL was examined. The staining intensity was graded on a scale of 0 to 3+ (0 for no staining; 1+ for weak immunoreactivity; 2+ for moderate immunoreactivity; and 3+ for strong immunoreactivity). The percentage of cells positive for NGAL were graded by the following protocol: grade 0 intensity (<10% positive cells); grade 1+ intensity (10-25% positive cells), grade 2+ intensity (25-50%), grade 3+ intensity (50-75% positive cells), and grade 4+ intensity (75-100% positive cells). The staining intensity score and the percent immunoreactivity score were then multiplied to obtain a composite score (Choi *et al*., 2008).

2.1.5. Statistical analysis:

One way ANOVA followed by Tukey test was carried out to determine the statistical significance. *p-value* < 0.05 was accepted as statistically significant.

2.2. Results and Discussion

Aforementioned, the main aim of this study was to determine the expression of NGAL in different premalignant lesions, stages, grades, organs and degree of differentiation of oral cancer tissues from patients by immunohistochemical analysis. The tissue

microarray contains a total of 160 samples including normal tissues which provided us a deep information about the expression of NGAL and its role in the pathogenesis of oral cancer.

2.2.1. Expression of NGAL is downregulated in oral cancer:

The first aim of this study was to ascertain the expression of NGAL in oral cancer tissues compared to normal tissues. Our results showed a moderate expression of NGAL in normal tissues compared to week to moderate expression in malignant tissues (Figure 2.1). Intriguingly, majority of the well differentiated epithelial cells of both malignant and normal tissues showed moderate expression of NGAL. Moderate expression of NGAL was also observed in tumour infiltrating cells and central cells forming the keratinized horn pearl (Monisha *et al.*, 2018).

Our results were consistent with the previous reports. In a similar study carried out by Wang L *et al*., 2015, it was reported that NGAL is downregulated in malignant head and neck squamous cell carcinoma tissues compared to normal tissues. No significant difference was seen in the expression of NGAL in metastatic and non-metastatic tumours (Wang *et al*., 2015). Contrastingly, in our study we observed a significant decrease in expression of NGAL in metastatic tissues and non-metastatic tissues compared to normal tissues and was strongly associated with lymph node metastases. Similar to our results, Shinriki *et al*., 2014, reported the expression of NGAL in tumour infiltrating cells and also in keratinized horn pearl indicating that NGAL is elevated in well differentiated tumours. However, overall expression of NGAL in well differentiated cells was lower than that of normal tissues (Shinriki *et al*., 2014). Thus from our study we can conclude that NGAL was downregulated in tumour tissues compared to normal tissues.

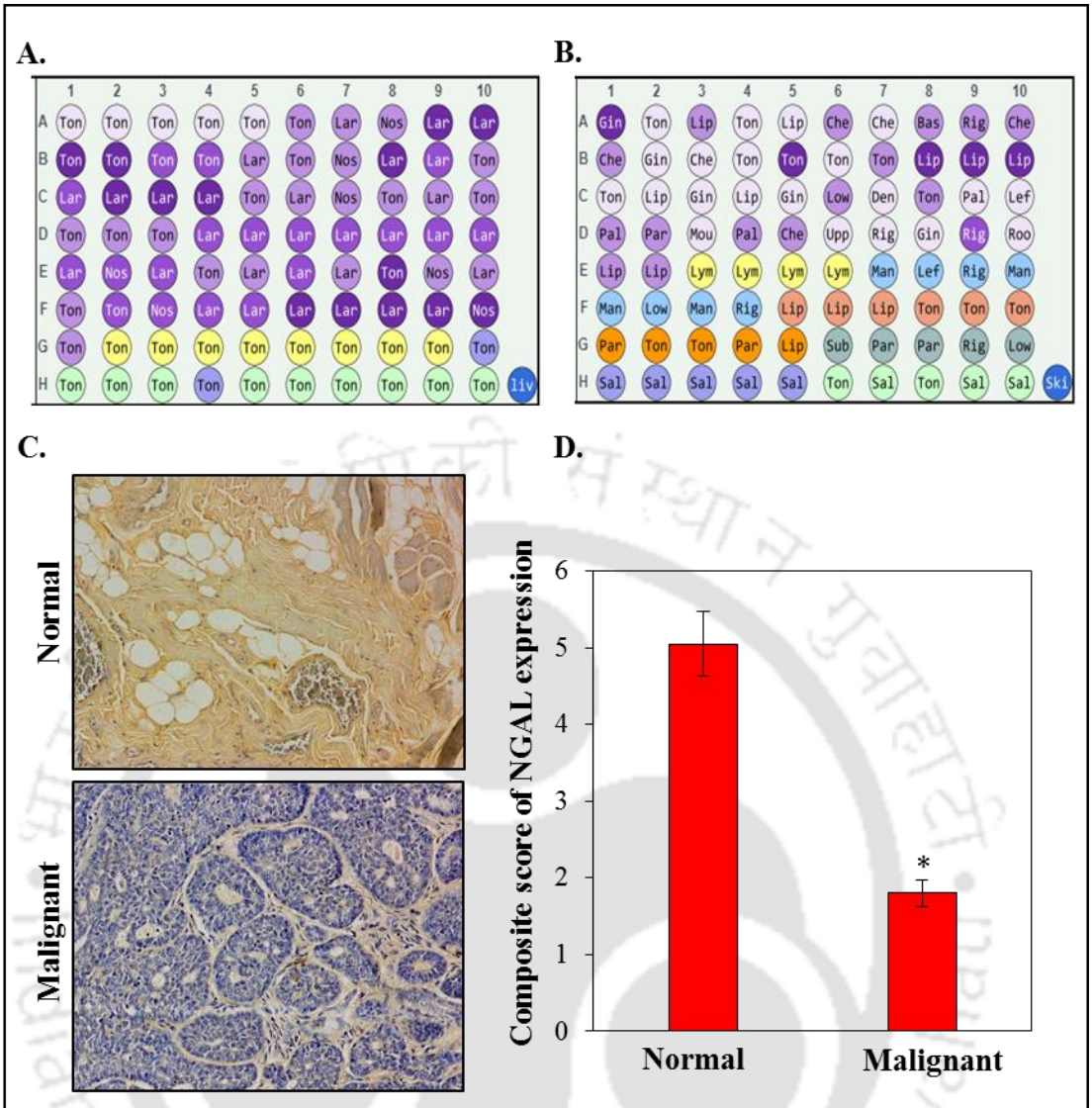

Figure 2. 1: Expression of NGAL in oral cancer.
A) Schematic representation of head and neck cancer microarray slide; Lar: Larynx, Nos: Nose, Ton: Tongue; -malignant tumor; -malignant tumor stage I; -malignant tumor stage II; -malignant tumor stage III; - malignant tumor stage IV; -metastases; -normal adjacent tissue; - normal tissue. B) Schematic representation of oral cancer microarray slide; 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. C) Representative images of expression of NGAL in oral cancer (left panel). D) Expression of NGAL in normal vs malignant oral cancer tissues. Data are mean ± SE. *=$p<0.05$.

2.2.2. Expression of NGAL is downregulated in different tumours of oral cavity:

Oral cancer is a collective term that includes the tumours arising from lips, front two-thirds of the tongue, gums, lining inside the cheeks and lips, floor of the mouth, hard palate, larynx, nasal cavity and salivary glands (NCI, 2017). Consequently, we analyzed the expression of NGAL in tumours arising from different organs of oral cavity. The expression of NGAL was found to be downregulated in majority of the malignant tissues from different organs of the oral cavity compared to normal tissues. Most of the malignant tissues from mandible, cheek, gingiva, lip, palate, parotid gland, tongue and larynx tissues showed week to moderate staining of NGAL except nose where it was negative (Figure 2.2) (Monisha *et al.,* 2018). This result showed that the expression of NGAL varies in different organs of OSCC. This is the first report that shows the expression pattern of NGAL in tumors arising in different regions of oral cavity.

2.2.3. Expression of NGAL is inversely correlated with degree of differentiation of oral cancer:

"Differentiation" is a collective term used to define the appearance of malignant tumors. The tumours are mainly categorized into well-differentiated tumours, moderately differentiated, poorly differentiated and un-differentiated tumours. Well differentiated tumours resemble benign tissues and generally has good prognosis. Keratin pearl are usually seen in well differentiated tumours where these cells produce keratin and are usually present in the center of a group of epithelial cells with a whorled appearance.

Our tissue microarray analysis showed that expression of NGAL was significantly downregulated with increase in degree of differentiation of tumors [normal tissues (n=11), well differentiated (n=5), moderately differentiated (n=12), poorly differentiated (n=3) and undifferentiated (n= 3) tongue tissues] compared to normal tissues. Normal tongue tissues and well differentiated tongue tissues showed moderate

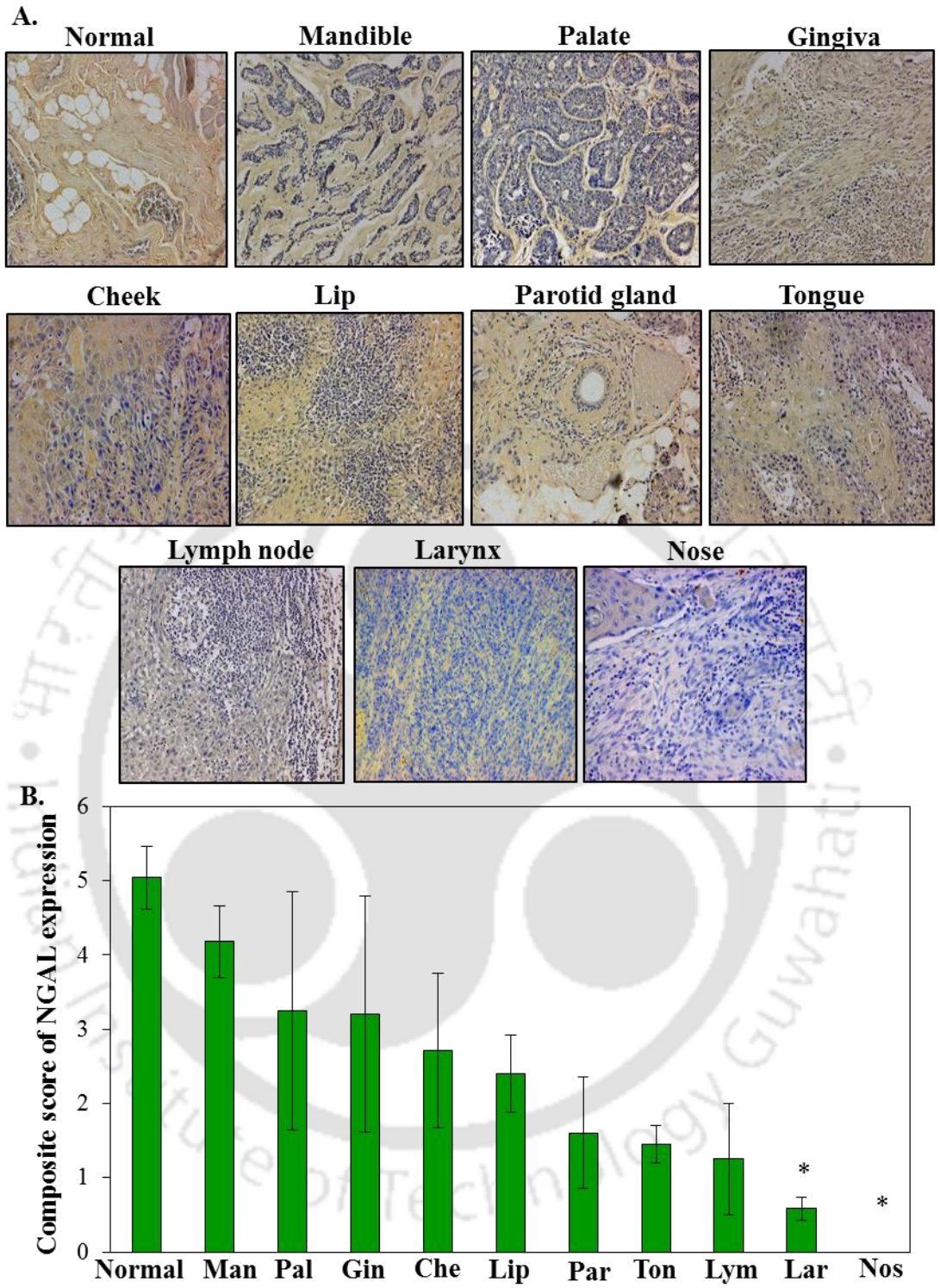

Figure 2.2: Expression of NGAL in different regions of oral cavity. Lar: Larynx, Nos: Nose, Ton: Tongue, Che: Cheek, Gin: Gingiva, Lym: Lymph node, Man: Mandible, Par: Parotid gland, Pal: Palate. A) Representative images of expression of NGAL in oral cancer for each organ. B) Mean composite score of NGAL levels in normal tissues (n=21), malignant mandible (n=11), palate (n=13), gingiva (n= 5), cheek (n=7), lip (n=36), parotid gland (n=8), tongue (n=46), lymph node (n=5) larynx (n=31), and nose (n=7) tissues of oral cancer. Data are mean ± SE. *=$p<0.05$.

Please note that the image of normal control depicted in the figure 2.1 has also been used in figure 2.2

positive staining of NGAL compared to very week positive staining in moderately differentiated and poorly differentiated tongue tissues. However, no positive expression was observed in the undifferentiated tongue tissues. Moreover, the keratin pearl in the well differentiated tissues were positive for the expression of NGAL (Figure 2.3) (Monisha *et al.*, 2018). Our results were consistent with previous studies carried out by Hiromoto *et al*., 2011 and Shinriki *et al*., 2014 where they showed that expression of NGAL was strongly associated with the degree of differentiation of head and neck cancer. Moreover, as it is well known that poorly differentiated and undifferentiated tumours are associated with poor prognosis, loss of expression of NGAL might be an indicator of poor prognosis of oral cancer. This suggests that NGAL can serve as a prognostic biomarker for oral cancer. Yet, further studies are required with large number of patient samples to confirm the prognostic significance of NGAL.

2.2.4. Expression of NGAL is downregulated in different stages of oral cancer:

Staging of tumour is typically based on the results of physical exams, biopsies, endoscopies, and imaging tests, such as MRIs, CT scans, PET scans and/or chest X-rays. Tumours are classified into four stages namely stage I (the primary tumour <2 cm diameter and not invasive), stage II (tumor is 2-4cm and not invasive), stage III (>4 cm and may spread to only one lymph node at the same side of the neck), and stage IV (tumor is highly invasive and metastatic) (The oral cancer foundation, 2017d). As identifying the stage of cancer would help in prescribing the therapeutic options for the patients, we determined correlation of the expression of NGAL with different stages (severity) of the disease. The results showed that the expression of NGAL was correlated with different stages of this disease where stage I showed high expression and stage IV negative expression compared to normal tissues. However, no significant correlation was observed between the stages (Figure 2.4). Similar studies have been

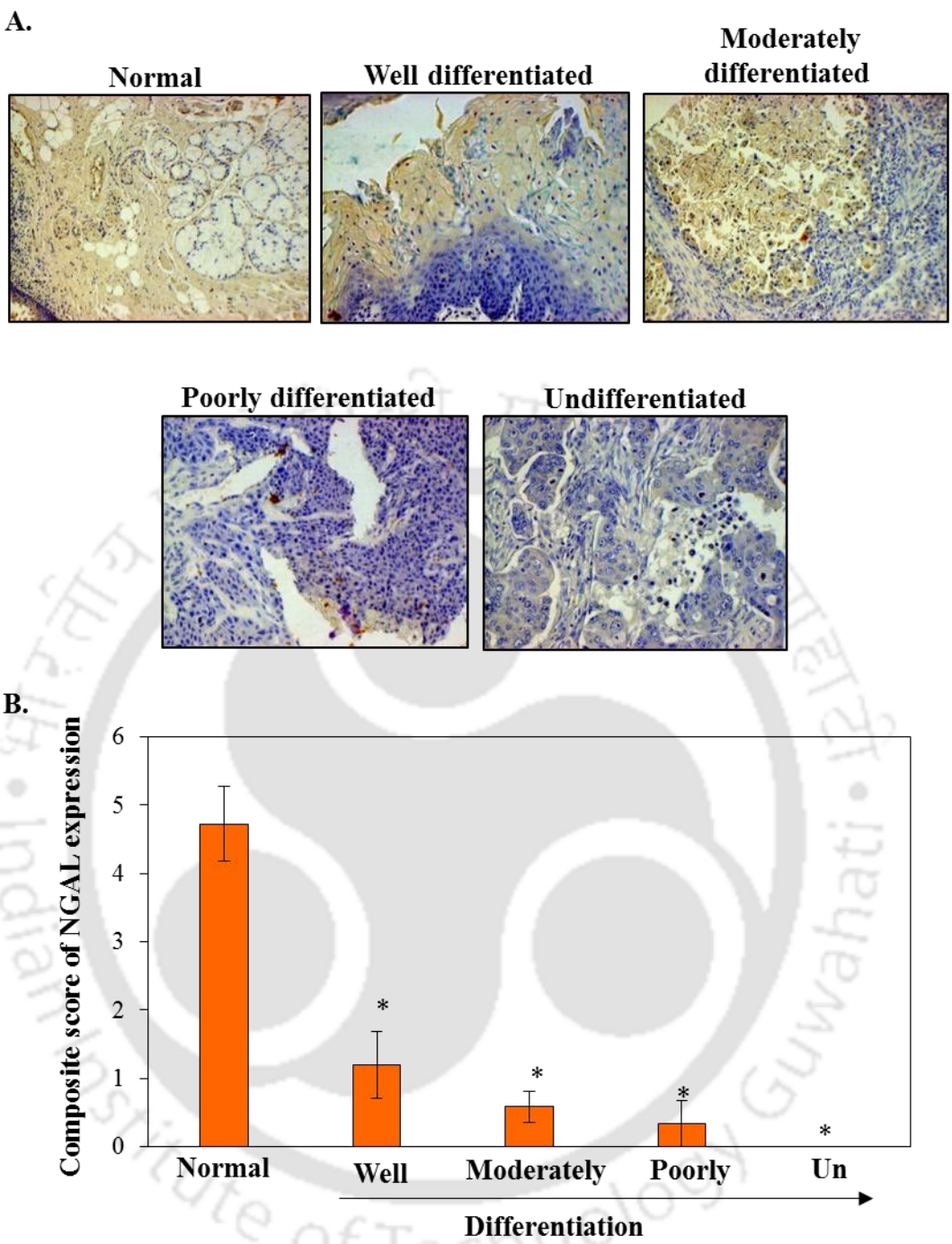

Figure 2.3: Expression of NGAL with degree of differentiation of oral cancer. A) Representative images of expression of NGAL in with the degree of differentiation. B) Mean composite score of NGAL levels in normal (n=11), well- (n=5), moderately (n=23), poorly differentiated (n=19) and undifferentiated (n=14) tongue tissues. Data are mean ± SE. *=p<0.05.

reported in other cancers. For example, expression of NGAL was strongly associated different stages in lung adenocarcinoma which in turn correlated with overall survival,

however, it was contrasting with the expression and stage of the disease in oral cancer (Zhang *et al*., 2012; Ruiz-Morales *et al*., 2015). Likewise, upregulation of NGAL was also strongly associated with the stage of HCC and colon cancer, and can serve as a prognostic marker for stage I patients (Zhang *et al*., 2012; Sun *et al*., 2011; Barresi *et al*., 2010). However, additional studies are required to correlate the survival of oral cancer patients with the expression of NGAL in different stages as this would help in the effective management of this disease.

2.2.5. Expression of NGAL is downregulated in different grades of oral cancer:

Grading of tumours helps to identify the spread of tumour to other organs. Grading is based on the appearance and behavior of tumour cells under microscope. Grade I tumours possess well differentiated cells, grade II, moderately differentiated cells, grade III, and poorly differentiated cells (Pereira *et al*., 2007; Pindborg *et al*., 1997). Therefore, we analyzed the expression of NGAL in different grades of OSCC and our results suggest that NGAL is downregulated in different grades of tumour tissues compared to normal tissues (Figure 2.5). This is the first report that shows the expression of NGAL in different grades of tumors of OSCC. In contrast to our results, other cancers show overexpression of NGAL in different grades such as endometrial cancer, breast cancer, glioma, and esophageal cancer (Li *et al*., 2016; Wenners *et al*., 2012; Liu *et al*., 2010; Du *et al*., 2011a). Thus, expression of NGAL in different grades of tumors also depends on the type of cancer.

2.2.6. Expression of NGAL is downregulated in different process of the development of oral cancer:

It is now well established that inflammation is the early stage of the development of many cancers including oral cancer, pancreatic cancer, prostate cancer, esophageal

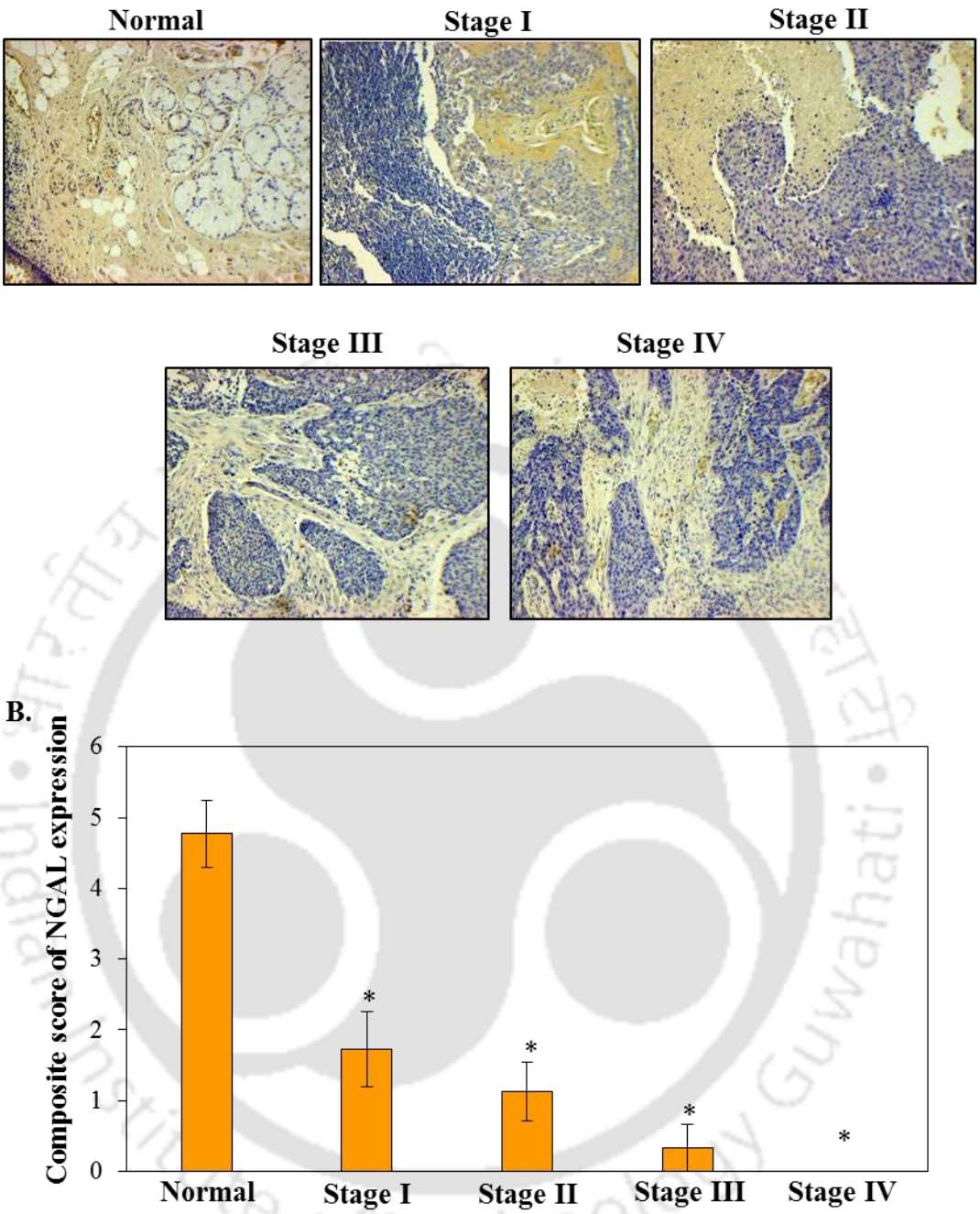

Figure 2.4: Expression of NGAL in different stages of oral cancer.
A) Representative images of expression of NGAL in different stages of oral cancer. B) Mean composite score of NGAL levels in normal tongue tissues (n=11), stage I (n=5), stage II (n=23), stage III (n=18) and stage IV (n=14) malignant tissues of tongue. Data are mean ± SE. *=$p<0.05$.

Please note that the image of normal control depicted in the figure 2.1 has also been used in figure 2.4

cancer etc. as many of the pro-inflammatory molecules are also involved in the different process of cancer such as cell proliferation, survival, invasion, angiogenesis and metastases (Aggarwal *et al*., 2006). Reports also suggest that approximately 18% of oral pre-malignant lesions develop into oral cancer (Reibel, 2003).The preneoplastic lesions of oral cavity include leukoplakia, erythroplakia, dysplasia and carcinoma *in situ*. Leukoplakia and erythroplakia transform to dysplasia with red and white patches (Reibel, 2003). Later, these lesions can develop into a benign tumour which is non-cancerous and does not spread to other parts of the body and are of different types such as hyperplasia (increase in normal cells), ameloblastoma or admantinoma (locally aggressive neoplasms arising from ameloblasts), papilloma, pleomorphic adenoma, etc. (Baker,1972; Hariram *et al*., 2014). Therefore, we analyzed the expression of NGAL in different stages of development of oral cancer. In inflammatory tissues, week to moderate expression of NGAL was observed. In hyperplasia, benign tumours, and in malignant tissues the expression of NGAL was downregulated compared to the normal tissues. However, in malignant and metastatic tissues, significant downregulation of NGAL was observed compared to normal tissues. Lymph node metastatic tissues showed very week focal staining or negative staining for NGAL. Thus, the downregulation of NGAL was found to be associated with different stages of development of oral cancer where the benign tumours showed moderate expression, malignant tissues showed week to moderate staining while the metastatic tissues showed week to negative staining of NGAL (Figure 2.6). Hence, this study indicated that loss of expression of NGAL may drive the progression of oral cancer to lymph node metastases (Monisha *et al.*, 2018).

Nevertheless, overexpression of NGAL has been reported to drive the progression of colorectal, endometrial and esophageal cancers (Odabasi *et al*., 2014; McLean *et al*., 2013; Liao *et al*., 2012; Zhang *et al*., 2007). Thus, modulations in the basal level of

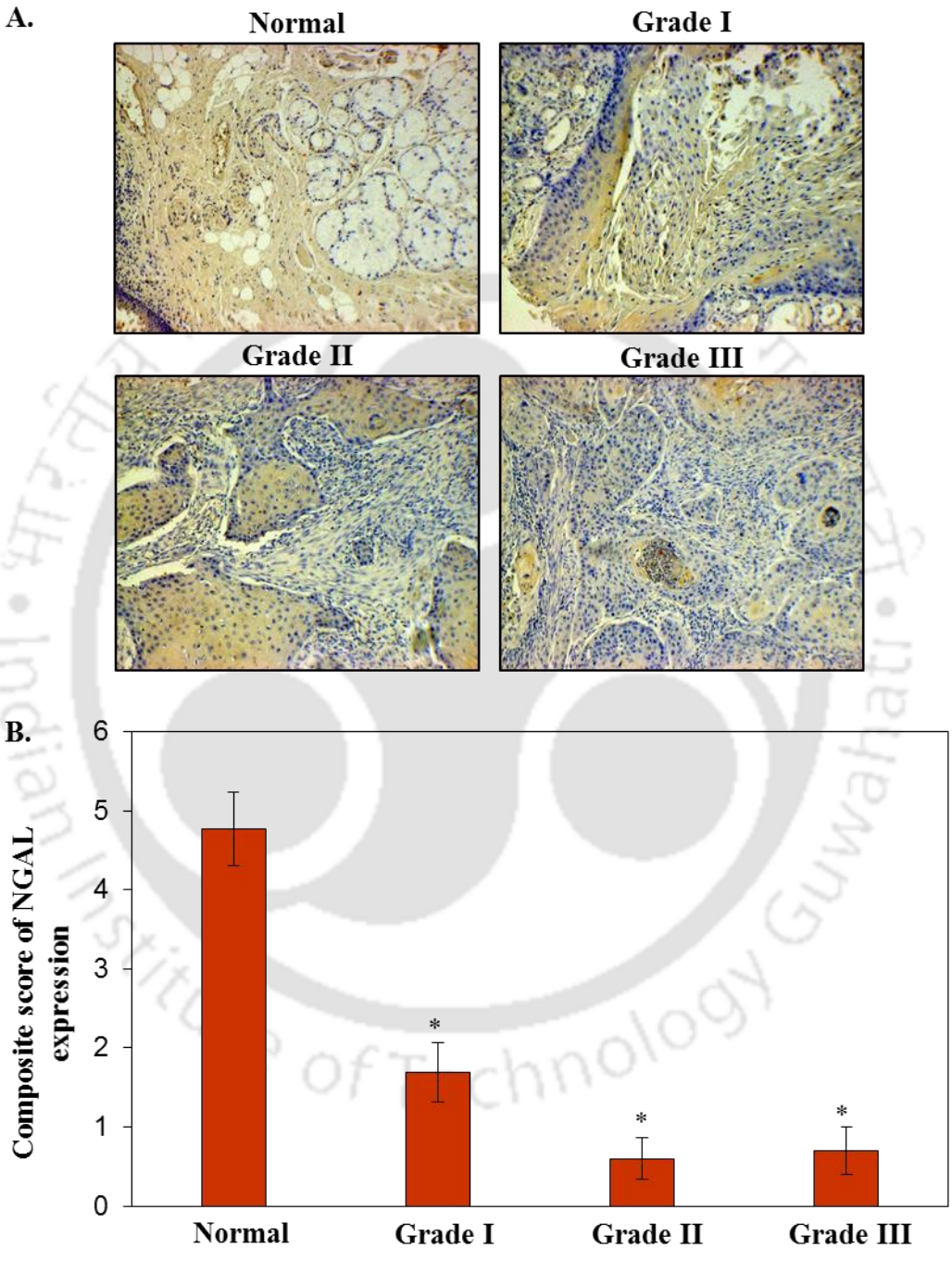

Figure 2.5: Expression of NGAL in different grades of oral cancer.
A) Representative images of expression of NGAL in different grades of oral cancer. B) Mean composite score of NGAL levels in normal tongue tissues (n=11), grade I (n=10), grade II (n=3), and grade III (n=20) malignant tissues of tongue. Data are mean ± SE. *=p<0.05.

Please note that the image of normal control depicted in the figure 2.1 has also been used in figure 2.5

expression of NGAL can drive cancer progression depending on the cancer type. Aforementioned, approximately 18% of premalignant lesions develops into oral cancer. Therefore, analyzing the expression of NGAL in oral cancer would help us to determine whether NGAL can be used as a biomarker for identifying the high risk individuals with preneoplastic lesions.

2.2.7. Expression of NGAL is downregulated in different pathological types of oral cancer:

Oral cancer can be divided into BCC, MEC, ACC and SCC depending on the cell type of origin. To determine the correlation between these different types of tumors and NGAL expression, we studied the expression of NGAL in these different types of tumors. We found that expression of NGAL was downregulated in all the different tumor types and was highly significant in BCC, SCC and MSCC (metastatic squamous cell carcinoma), while, MEC and ACC did not show significant downregulation (Figure 2.7). This is the first report on the expression of NGAL and its comparison with oral cancers of different cellular origin (Monisha *et al.*, 2018).

2.2.8. Expression of NGAL with respect to gender and age of oral cancer patients:

It has been well evinced that oral cancer incidences are twice in men than women. Earlier, excessive consumption of tobacco and alcohol was seen in men compared to women. Although, this scenario has been changed, HPV-linked cancers has been mainly among younger men, and hence mostly men are affected with this cancer (American cancer society, 2017). Therefore, we studied the expression of NGAL in oral cancer tissues from both males and females. The results suggest that there is a 70% reduction in the expression of NGAL in male cancer tissues compared to 53% reduction in female cancer tissues. This shows that downregulation of NGAL is higher in males than females and can be one of the factor of the high occurrence of oral cancer

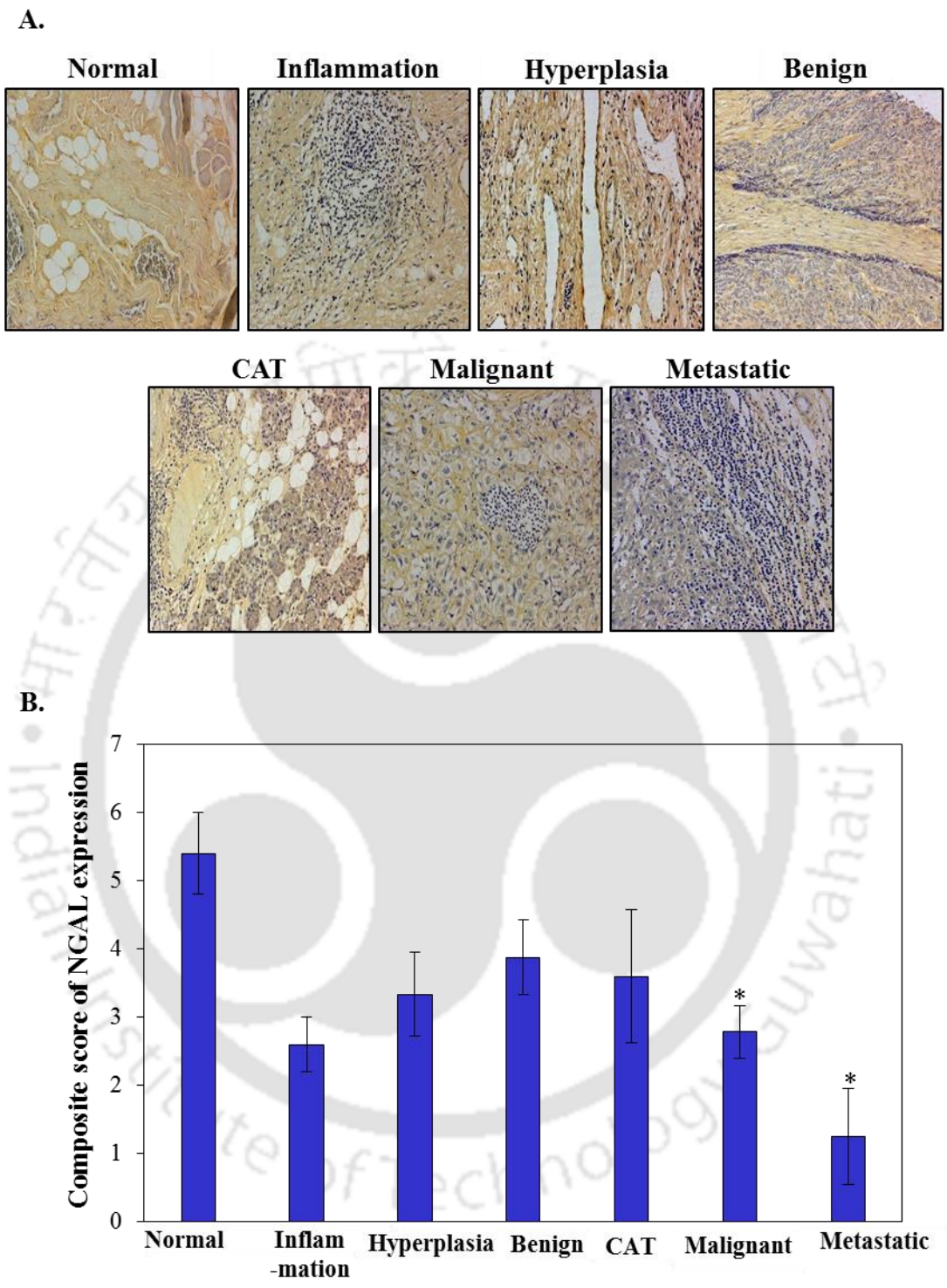

Figure 2.6: Expression of NGAL in different stages of the development of oral cancer. CAT: Cancer adjacent tissue. A)Representative images of expression of NGAL in different stages of development of oral cancer. B) Mean composite score of NGAL levels in Normal tissues (n=10), inflammation (n=5), hyperplasia (n=5), cancer adjacent tissues (n=5), malignant tissues (n=42), metastatic tissues (n=4). Data are mean ± SE. *= $p<0.05$.

Please note that the image of normal control depicted in the figure 2.1 has also been used in figure 2.6

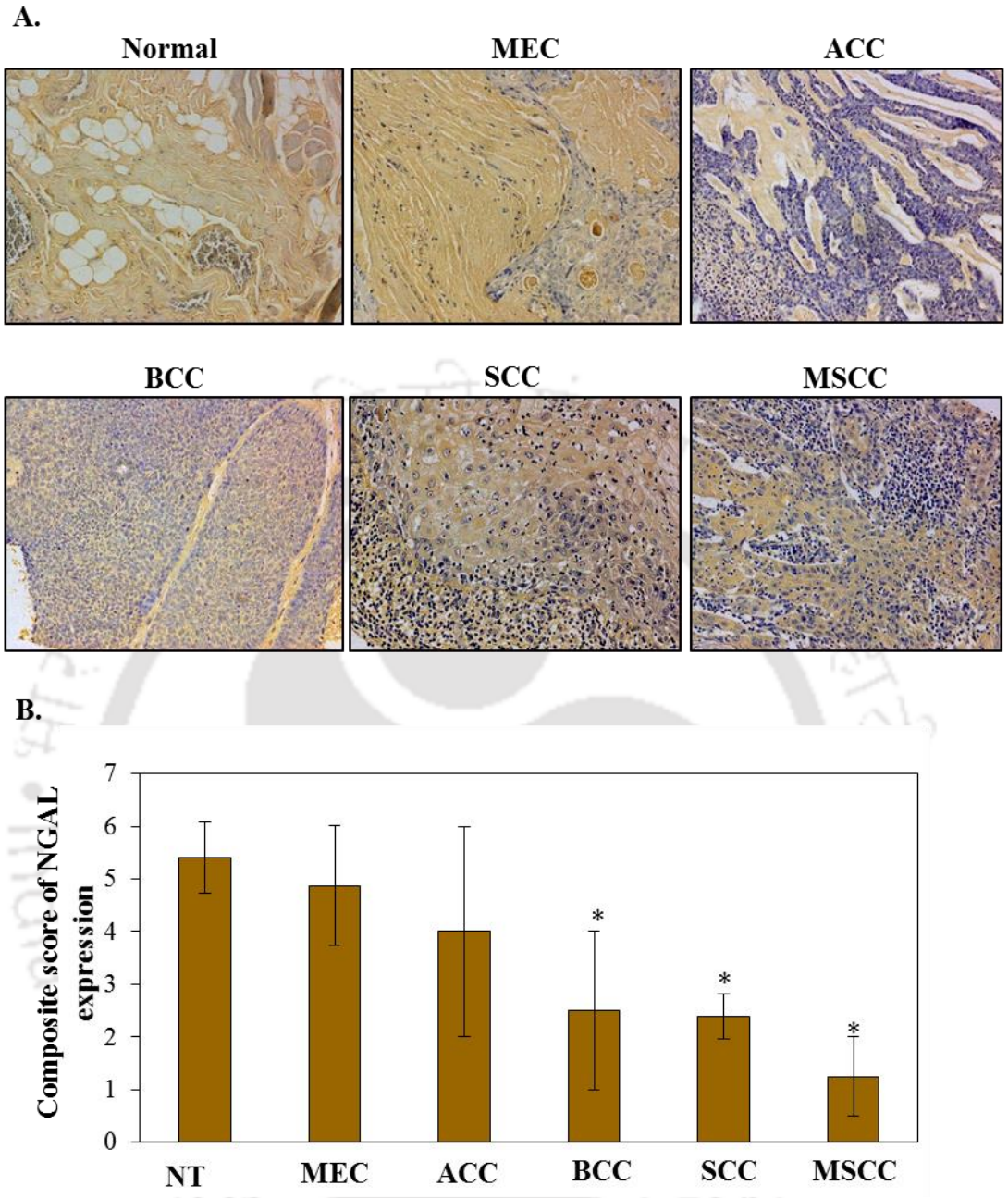

Figure 2.7: Expression of NGAL in different types of oral cancer.
A) Representative images of expression of NGAL in different types of oral cancer. B) Mean composite score of NGAL levels in NT-Normal tissue (n=10), MEC-Mucoepidermoid carcinoma (n=8), ACC- Adenoid cystic carcinoma (n=3), BCC-Basal cell carcinoma (n=2), SCC-Squamous cell carcinoma (n=28), MSCC-Metastatic squamous cell carcinoma (n=4) of oral cancer. Data are mean ± SE. *= $p<0.05$.

Please note that the image of normal control depicted in the figure 2.1 has also been used in figure 2.7

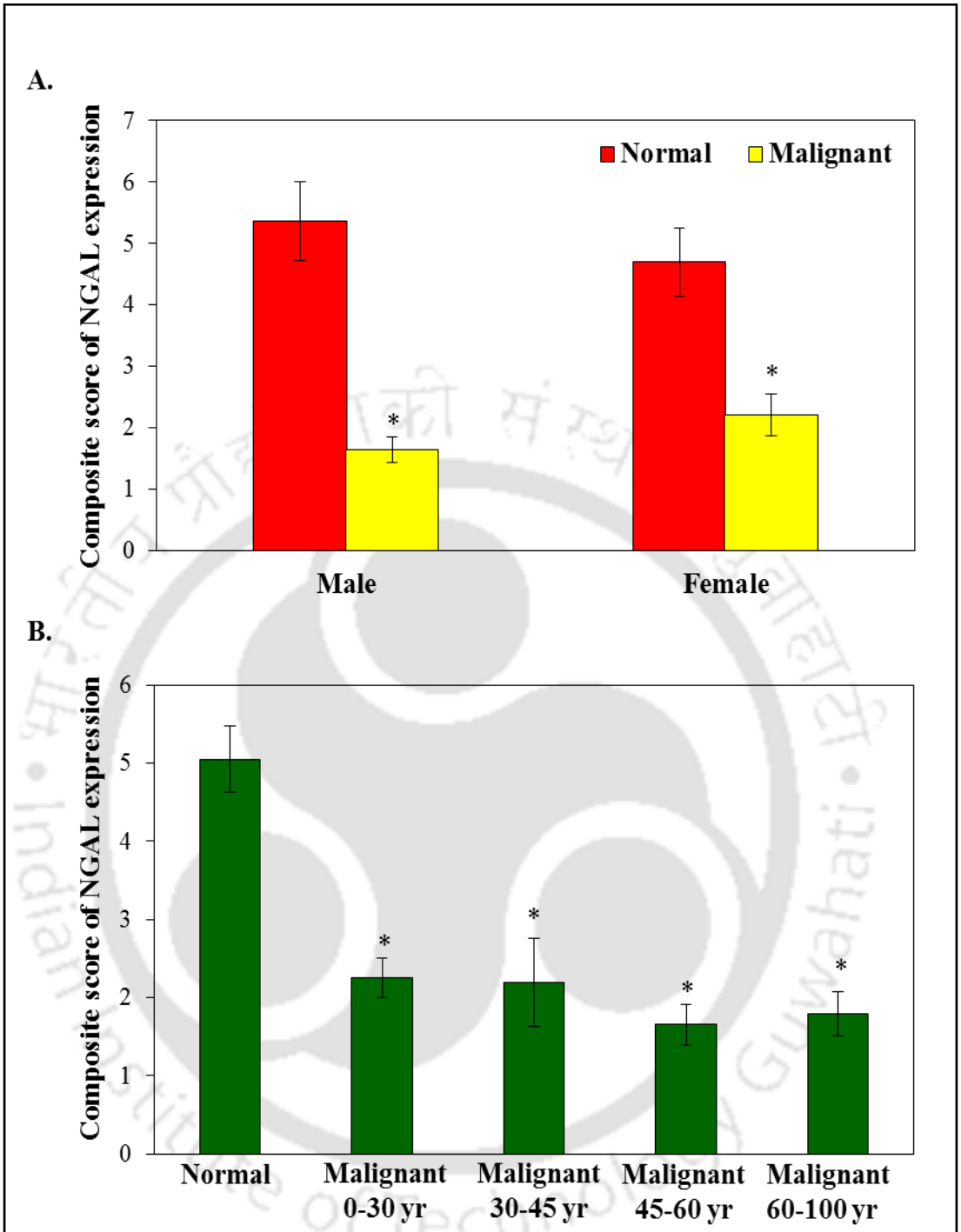

Figure 2.8: Expression of NGAL with age and gender in oral cancer tissues. A) Mean composite score of NGAL levels in tissues from normal male (n=11), normal female (n=10), malignant male (n=100) and malignant female (n=39) patients of oral cancer. Data are mean ± SE. *= $p<0.05$. B) Mean composite score of NGAL levels in normal (n=17), malignant patient tissues of age group 0-30 yr (n=4), 30-45 yr (n=18), 45-60 yr (n=63), 60-100 yr (n=52) in oral cancer. Data are mean ± SE. *= $p<0.05$.

in males (Figure 2.8 A). However, more studies are required to confirm this finding. We next analyzed the expression of NGAL in different age groups; 15-30 years; 30-45 years; 45-60 years and 60-90 years. Our results showed that the expression of NGAL was downregulated in malignant tissues irrespective of age. No significant correlation was observed with the expression of NGAL with increase in age (Figure 2.8 B) (Monisha *et al.,* 2018).

2.3. Conclusion

The aim of this chapter was to study the expression of NGAL in oral cancer tissues of different stages, grades, degree of differentiation, sex, different cellular origin etc. Ascertaining the significance of NGAL expression with these factors would help us to determine whether NGAL can be employed as a predictive marker for oral cancer. Our results suggest that expression of NGAL is different in tumors originated from different tissues and cells. It is noteworthy that expression of NGAL correlated with the degree of differentiation of tumors, stage of the tumor compared to normal tissues. Therefore, from our study we can conclude that loss of expression of NGAL is an early event in malignant transformation and drives the progression of oral cancer. However, further studies are required to undermine this notion.

3
CHAPTER

Effect Of Various Tobacco Components On The Expression Of NGAL In Oral Cancer Cells

3. Introduction

In the previous chapter we have shown that expression of NGAL is downregulated in oral cancer tissues also in oral premalignant lesions compared to normal tissues. This suggest that the risk factors of oral cancer might play a significant role in downregulating the expression of NGAL that leads to inflammation and cancer. The prime risk factors for oral cancer include tobacco use, betel quid chewing, alcohol and low fruit and vegetable consumption. Worldwide, 25% of oral cancers are attributable to tobacco (smoking and/or chewing), 7-19% to alcohol, and 10-15% due to micronutrient deficiency (Petti, 2009; Warnakulasuriya, 2009). It is also reported that smoking tobacco is attributable to 85% of oral cancer deaths (Warnakulasuriya, 2009). This indicates that tobacco presents the major cause for oral cancer. However, the role of tobacco in downregulating the expression of NGAL is not studied thus far. Hence, the main aim of this chapter is to examine the role of tobacco on the expression of NGAL in oral cancer cells. It is now well established that over sixty carcinogens are present in cigarette smoke and sixteen in unburned tobacco. The most important of them are tobacco-specific nitrosamines, such NNK and NNN; PAH such as benzo[a]pyrene, and aromatic amines. In particular, NNK, NNN and PAH are known to induce oral cancer (Petti, 2009). These agents induce carcinogenesis by forming DNA adducts and by activating different oncogenic pathways (Xue *et al*., 2014). CYP's convert nitrosamines to their DNA-reactive metabolites and induce methylation, pyridyloxobutylation and pyridylhydroxybutylation of nucleobases in DNA and form DNA adducts (Xue *et al*., 2014). Nicotine and nitrosamines help in tumor promotion by activating nAChRs and β-AdrRs, resulting in the activation of downstream signaling pathways associated with tumour progression (Warren and Singh, 2013). Therefore, in

this chapter we have analyzed the effect of tobacco components on the expression of NGAL using different cell lines.

3.1. Materials and methods

3.1.1. Materials:

NNK (Cat No. 78013), NNN (Cat No. 75285), Nicotine (Cat No. N3876) and 4-NQO (Cat No. N8141) were purchased from Sigma- Aldrich, Missouri, USA. Antibodies against NGAL (dilution 1:3000; Cat No. ab23477, Abcam, Cambridge, USA), GAPDH (dilution 1:2000; Cat No. 2118S, Cell Signaling Technology, Massachusetts, USA); β-Actin (dilution 1:2000; Cat No. 4967S, Cell Signaling Technology, Massachusetts, USA), anti-mouse secondary antibody (dilution 1:6000; Cat No. ab97040, Abcam, Cambridge, USA), anti-rabbit secondary antibody (dilution 1:6000; Cat No. ab97080, Abcam, Cambridge, USA) were also used.

3.1.2. Cell culture:

SAS cells were procured from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India and KB-CHR-85 were procured from NCCS, Pune, India. These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco™; Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco®, NY, USA) and 1X Penstrep (Invitrogen, CA, USA). The cells were cultured and maintained at 37°C in a 5% CO_2 and 95% humidity.

3.1.3. Cell proliferation:

Non-toxic concentrations of tobacco components were determined by MTT assay. Oral cancer cells (SAS cells and KB-CHR-85) were seeded at a density of $2x10^3$ cells/well in 96-well plates in sextuplicate. After 24h, the cells were treated with different concentrations of tobacco components for 0h and 24h respectively. After each time point, 10µl of MTT (cont. 5mg/ml; Cat No. M2128, Sigma- Aldrich, Missouri, USA)

was added to the cells and was further incubated for 2h at 37°C. The MTT solution along with the media was removed and 100µl of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added to each well and was further incubated for 1h. The absorbance was then measured at 570nm using an Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland). The percentage of proliferation was calculated using the formula:

$$\text{Percent Proliferation} = \frac{\text{Absorbance (Treatment)}}{\text{Absorbance (Control)}} \times 100$$

3.1.4. Western blot analysis:

Western blot was carried out to analyze the expression of NGAL in tobacco treated cells. Briefly, $5x10^5$ cells (SAS and KB-CHR-85) were treated with different concentrations of tobacco components and 4-NQO for 24 or 48 hrs. The cells were harvested and lysed using whole cell lysis buffer (20mM HEPES, 2mM EDTA, 250mM NaCl, 0.1% NP-40) in the presence of protease inhibitors (2µg/ml Leupeptin hemisulfate, 2µg/ml Aprotinin, 1mM PMSF, 1mM DTT). The protein concentration of the lysates were measured using Bradford assay (Cat No. 500-0205; Bio rad, California, USA). 50µg of protein was resolved and mixed with 5X Laemmli Buffer (250mM Tris-HCl, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% Bromophenol blue), electrophoresed in a 12% SDS-acrylamide gel, and transferred to nitrocellulose transfer membrane (Bio rad, California, USA). The membranes were blocked with 5% non-fat milk in tris-buffered saline (TBS: 0.2M Tris base, 1.5M NaCl, H_2O) containing 1% tween 20 (TBST). The blots were probed with appropriate primary antibodies for overnight. The following day the blots were washed with TBST and were incubated in appropriate horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized using Optiblot ECL Detection Kit (Cat No. ab133406, Abcam, Cambridge, USA). β-actin/GAPDH was used as the loading control.

3.1.5. Statistical analysis:

One way ANOVA followed by Tukey test was carried out to determine the statistical significance. *p-value* < 0.05 was accepted as statistically significant.

3.2. Results and Discussion

In this chapter we have examined the effect of different tobacco components such as nicotine, NNK, NNN and the synthetic oral carcinogen 4-NQO on the expression of NGAL in oral cancer cells. However, as most of these agents are toxic to cells, we have determined the non-toxic concentrations of these agents using cell proliferative assay. Then, we have used these concentrations to determine their effect on the expression of NGAL by western blot analysis. This is the first report which shows the effect of tobacco components on the expression of NGAL in any cancers.

3.2.1. Nicotine and the expression of NGAL in OSCC cells:

Nicotine is a major addictive component present in tobacco smoke. Tumour inducing potential of nicotine has been reported in the development of several SCLC, NSCLC, oral, esophageal, pancreatic and colon cancer (Jensen *et al*., 2012; Egleton *et al*., 2008). Nicotine is known to cause structural changes in nAChRs, thus replacing them from their local transmitters. Nicotine also altered the transcriptional/translational control of cell cycle and different genes including Ki-67, PCNA, p21, cyclin D1, and TP53 (Jensen *et al*., 2012; Egleton *et al*., 2008; Arredondo *et al*., 2001). As nicotine altered the expression of many genes associated with tumour development, we analyzed whether nicotine altered the expression of NGAL. First we determined the non-toxic concentrations of nicotine in SAS and KB-CHR-85 cells and found that the concentrations 0.25, 0.5, 0.75, 1 and 2µM did not induce any cytotoxicity in both the cell lines. Therefore, we used these concentrations for our further studies. Then we treated SAS and KB-CHR-85 cells with the above mentioned concentrations of nicotine

for 24hrs and the expression of NGAL was studied using western blot analysis. Our results showed that nicotine downregulated the expression of NGAL in KB-CHR-85 cells in a dose dependent manner. However, in SAS cells the expression of NGAL was found to be upregulated (Figure 3.1). This suggests that NGAL is involved in nicotine induced carcinogenic processes in oral cancer and it is cell line specific. Previous reports suggest that HIF-1α is downregulated in NGAL overexpressing cells, while in NGAL deficient cells HIF-1α is upregulated (Lin *et al*., 2016). Nicotine is known to activate HIF-1α in lung cancer cells and promote tumor angiogenesis and metastasis (Zhang *et al*., 2007). This indicated that downregulation of NGAL may activate HIF-1α and induce tobacco induced oral carcinogenesis. However, more studies are required to establish this finding. Moreover, nicotine is well known to alter the expression of growth factors (VEGF, HGF, VEGF-C, TGF-β, PDGF) growth factor receptors (VEGFR-2, PDGFR, HGFR and EGFR), and other proteins involved in carcinogenesis such as COX-2, VEGF, MMM-2, MMP-9 etc. (Egleton *et al*., 2008). Therefore, it can be concluded that nictotine induced downregulation of NGAL may play a key role in the development of oral cancer. However, additional studies are required to understand the detailed mechanisms involved.

3.2.2. Tobacco-specific nitrosamines and the expression of NGAL:

Two tobacco specific nitrosamines, NNK and NNN are well known carcinogens present in tobacco smoke. Nitrosamines in tobacco products are formed by nitrosation of nicotine and related tobacco alkaloids. Naturally occurring NNK, a procarcinogen, is metabolically activated by many CYP's to reactive metabolites which result in methylation, pyridylhydroxybutylation and pyridyloxobutylation of nucleotides in DNA and form DNA adducts and persuade tumor formation (Sturla *et al*., 2005; Kiyohara *et al*., 2005, 2006; Hecht *et al*., 2004; Xue *et al*., 2014).

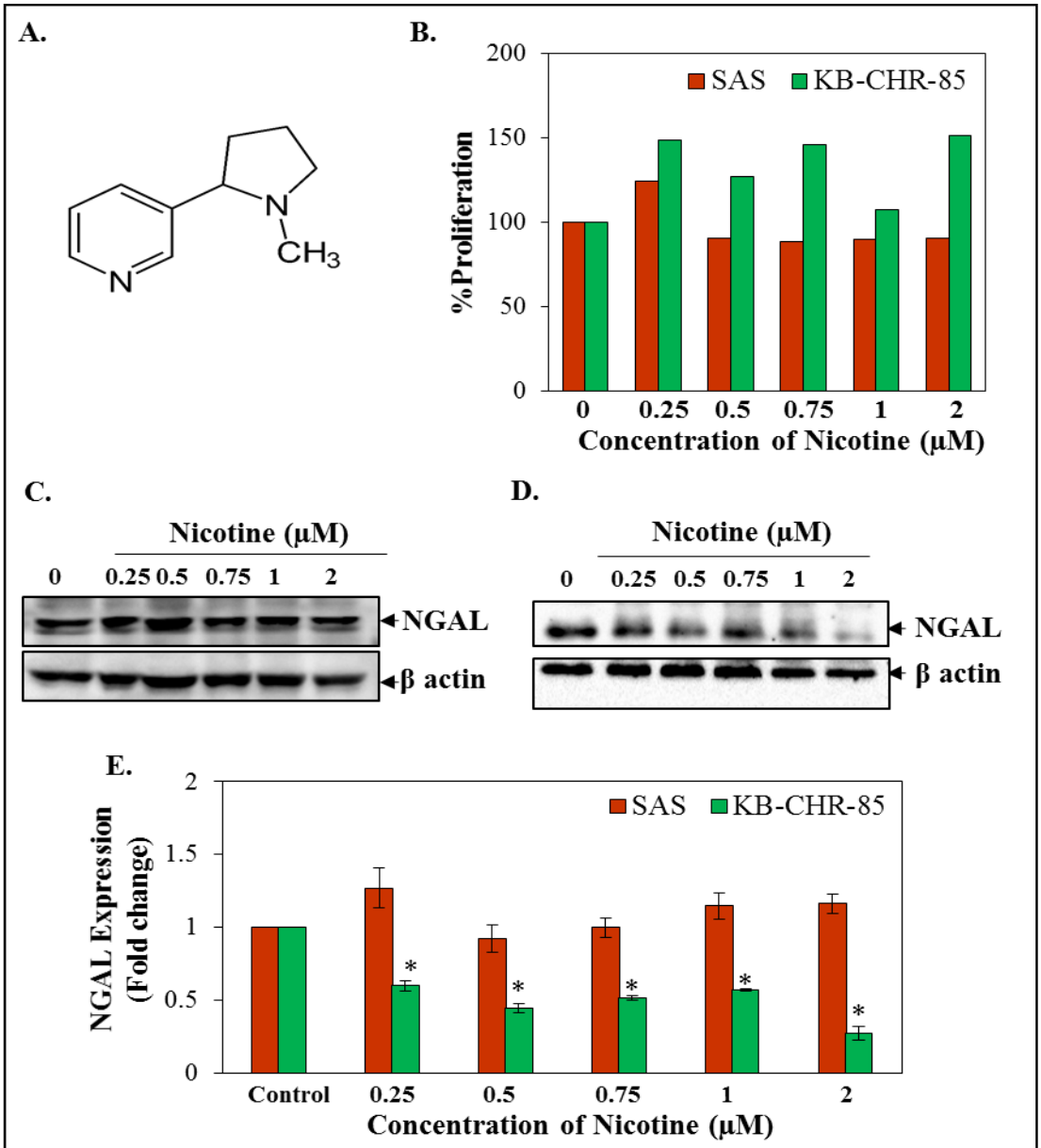

Figure 3.1: Effect of nicotine on the expression of NGAL in oral cancer cells. A) Structure of nicotine. B) MTT assay showing non-cytotoxic concentrations of nicotine in SAS and KB-CHR-85 cells. C) Western blot analysis of expression of NGAL on treatment with nicotine for 24h in SAS cells. D) Western blot analysis of expression of NGAL on treatment with nicotine for 24h in KB-CHR-85 cells. β-actin was used as internal control. E) Graphical representation of NGAL expression in nicotine treated over control as measured by densitometry scanning. Data are means ± SE. *= $p<0.05$.

α-Methylene hydroxylation of NNK produces methane diazohydroxide and/or the methyldiazonium ion, which interacts with DNA and forms 7-*N*-methylguanine and O6-methylguanine and small amounts of O4-methylthymine. α-Hydroxylation of NNK may occur either at the methyl or methylene carbon and the earlier one produces

stable α-hydroxymethyl NNK which undergoes glucuronidation (Boyland *et al*., 1964; Xue *et al*., 2014). During this process it impulsively loses formaldehyde and produces pyridyloxobutyldiazohydroxide, which reacts with DNA yielding bulky pyridyloxobutylation (POB) adducts (Hecht 1999; Xue *et al*., 2014). Metabolic activation of NNN results in pyridine N-oxidation, hydroxylation of the pyrrolidine ring and norcotinine formation (Hecht 1998, 1999; Xue *et al*., 2014). Similar to NNK many CYP's catalyze the hydroxylation of NNN and 2'-hydroxylation of NNN produces pyridyloxobutyldiazohydroxide, which reacts with DNA and forms adducts (Sturla *et al*., 2005; Patten *et al*., 1997; Xue *et al*., 2014). In addition, NNK and NNN can also bind to *α,β*-nAChR and *α*7nAchR. The affinity of NNN to these receptors is 5000 times and 1300 times higher than that of nicotine respectively (Schuller *et al*., 1998; Arredondo *et al*., 2006; Xue *et al*., 2014). Binding of NNK to α7nAChR activates voltage-gated Ca^{2+} channels which in turn activate ERK 1/2, as well as the transcription factors FOS, JUN and MYC and induce proliferation *in vitro* (Jull *et al*., 2001; Xue *et al*., 2014). In NSCLC, PI3K/Akt and NF-κB signaling pathways were activated in response to NNK and inhibited the chemotherapy induced apoptosis (West *et al*., 2004; Tsurutani *et al*., 2005; Xue *et al*., 2014). NNK is known to activate ERK1/2 signaling, STAT1, NF-κB, and GATA3, while NNN activates only GATA3 and STAT1 (Arredondo *et al*., 2006; Xue *et al*., 2014). These studies suggests that NNK and NNN induce carcinogenesis either by forming DNA adducts or by inducing molecular alterations.

Therefore, we determined the effect of these tobacco specific nitrosamines on the expression of NGAL in oral cancer cells. First we determined the non-toxic concentrations of NNK and NNN in SAS and KB-CHR-85 cells and found that the concentrations 100µM, 250µM, and 500µM of NNK and NNN did not induce any

toxicity in both the cell lines. Then, we treated SAS and KB-CHR-85 cells with the above mentioned concentrations of NNK and NNN and analyzed the expression of NGAL. We observed that NNK and NNN downregulated the expression of NGAL in SAS cells while in KB-CHR-85 cells, NNK downregulated the expression of NGAL. On the other hand, we observed that NNN upregulated the expression of NGAL in KB-CHR-85 cells (Figure 3.2 and Figure 3.3). This indicates that regulation of the expression of NGAL by NNK and NNN is both cell line and tobacco component specific. Therefore, it can be concluded that both the cell lines have different molecular characteristics which enables them respond differentially in response to NNK and NNN. However, more studies are required to elucidate the mechanism behind these differences. In the previous chapter we have shown that NGAL is downregulated in preneoplastic tissues and malignant tissues compared to normal oral tissues which support the fact that NNK and NNN may be involved in the downregulation of NGAL in oral cancer. However, further studies are required to understand the signaling which leads to the downregulation of NGAL by NNK and NNN.

3.2.3. 4-nitroquinoline 1-oxide (4-NQO) and the expression of NGAL:

4-NQO is a synthetic carcinogen derivative of a quinoline, soluble in water, sensitive to high temperature and light (Wilkey *et al*., 2009; Kanojia and Vaidya, 2006; Martínez, 2012). Carcinogenic potential of 4-NQO was first reported in the year 1957 by Nakahara and his group (Nakahara *et al*., 1957). This chemical is used as carcinogen in murine models to study the multi-step process of development of oral squamous cell carcinoma and induces tumours both locally and systemically and mimics the sequential stages of development of OSCC including hyperplasia, dysplasia, severe dysplasia, in situ carcinoma and SCC in murine models (Vered *et al*., 2005; Liu *et al*., 1999; Boyd and Reade 1991; Martínez, 2012; Wilkey *et al*, 2009; Kanojia and Vaidya, 2006).

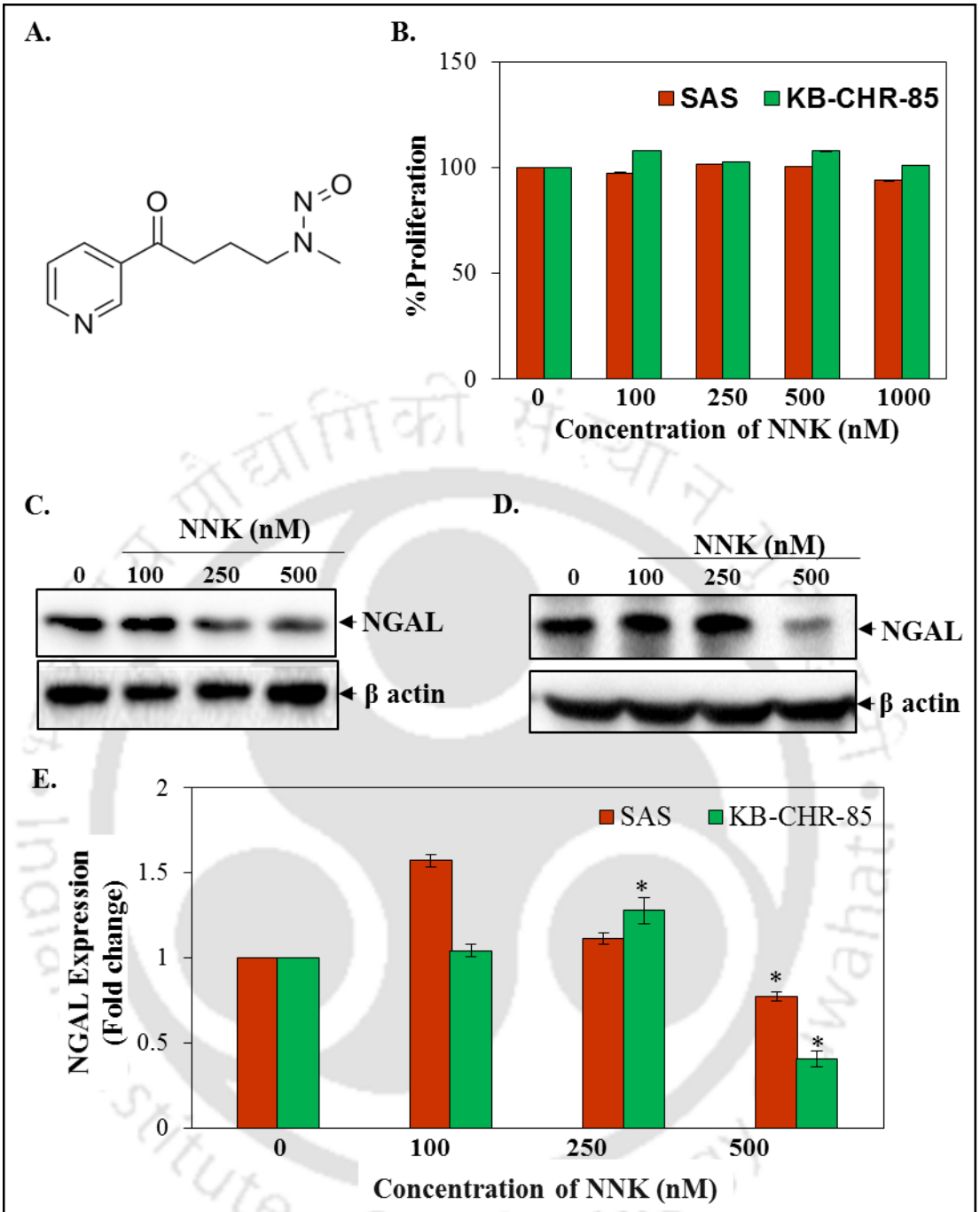

Figure 3.2: Effect of Nicotine-derived nitrosamine ketone (NNK) on the expression of NGAL in oral cancer cells. A) Structure of NNK. B) MTT assay showing non-cytotoxic concentrations of NNK in SAS and KB-CHR-85 cells. C) Western blot analysis of expression of NGAL on treatment with NNK for 48h in SAS cells. D) Western blot analysis of expression of NGAL on treatment with NNK for 48h in KB-CHR-85 cells. β-actin was used as internal control. E) Graphical representation of NGAL expression in NNK treated over control as measured by densitometry scanning. Data are means ± SE. *= $p<0.05$.

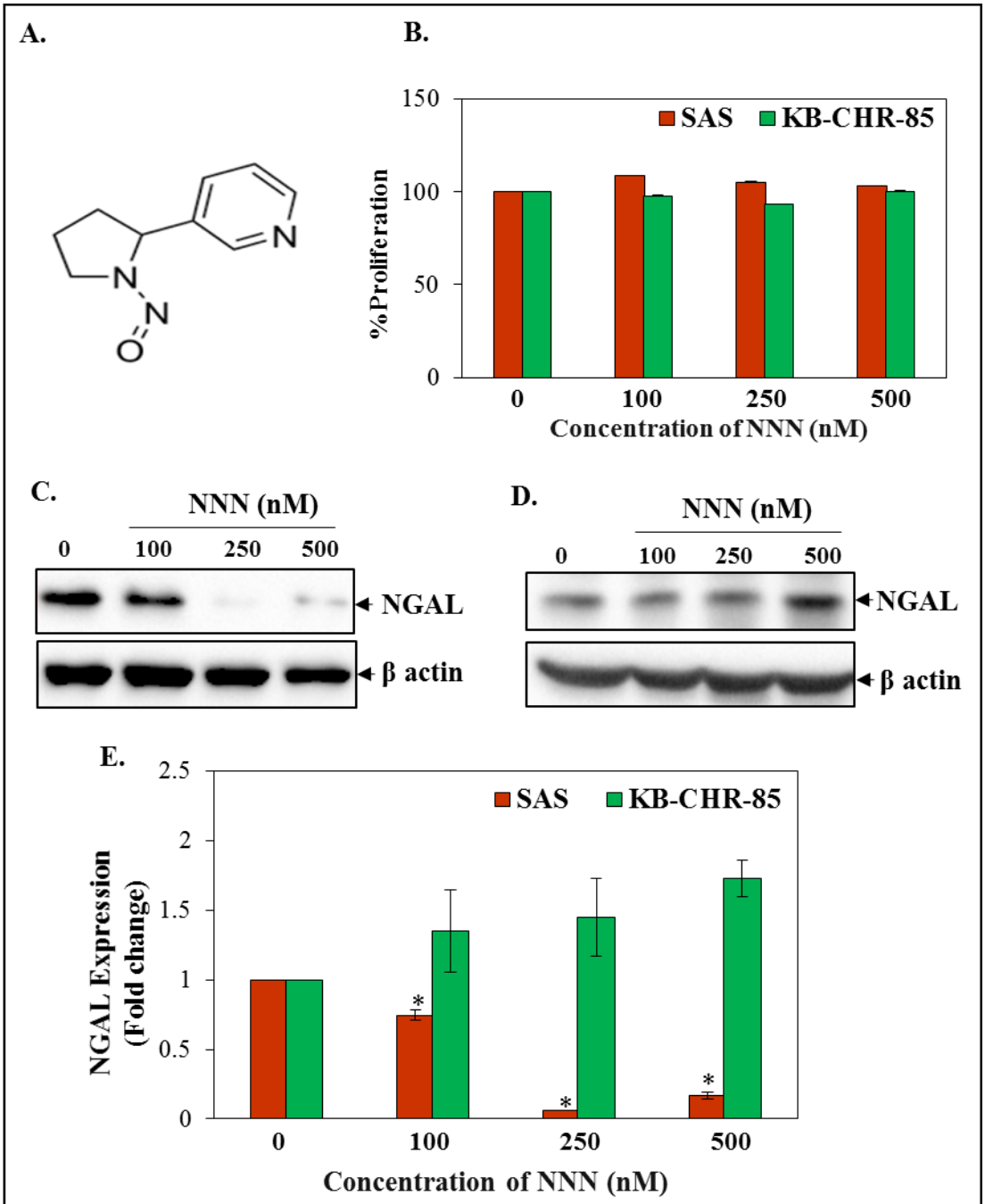

Figure 3.3: Effect of N-Nitrosonornicotine (NNN) on the expression of NGAL in oral cancer cells. A) Structure of NNN. B) MTT assay showing non-cytotoxic concentrations of NNN in SAS and KB-CHR-85 cells. C) Western blot analysis of expression of NGAL on treatment with NNN for 48h in SAS cells. D) Western blot analysis of expression of NGAL on treatment with NNN for 48h in KB-CHR-85 cells. β-actin was used as internal control. E) Graphical representation of NGAL expression in NNN treated over control as measured by densitometry scanning. Data are means ± SE. *= $p<0.05$.

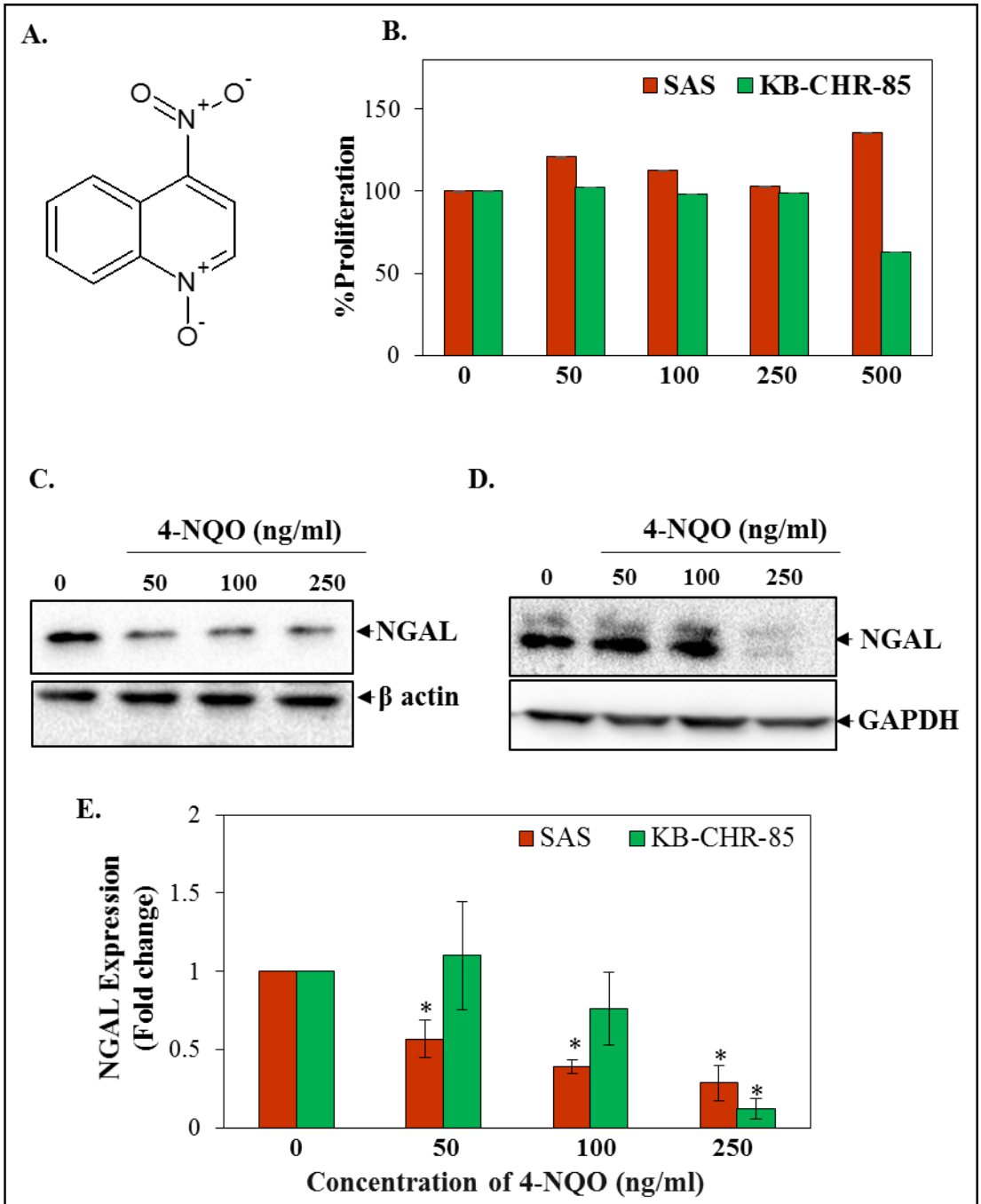

Figure 3.4: Effect of 4-nitroquinoline-1-oxide (4-NQO) on the expression of NGAL in oral cancer cells. A) Structure of 4-NQO. B) MTT assay showing non-cytotoxic concentrations of 4-NQO in SAS and KB-CHR-85 cells. C) Western blot analysis of expression of NGAL on treatment with 4-NQO for 48h in SAS cells. D) Western blot analysis of expression of NGAL on treatment with 4-NQO for 24h in KB-CHR-85 cells. β-actin /GAPDH was used as internal control. E) Graphical representation of NGAL expression in 4-NQO treated over control as measured by densitometry scanning. Data are means ± SE. *= $p<0.05$.

Moreover, the histological and molecular changes are similar as seen in human oral carcinogenesis (Nishimura 1999; Niwa *et al*., 2001; Kaplan *et al*., 2002; Tamura, *et al*., 2003; Kanojia and Vaidya, 2006). NADH and NADPH reduce 4-NQO to 4-hydroxyaminoquinoline-1-oxide (4HAQO), 4-NQO nitroreductase and quinone reductase. 4HAQO is believed to be the potent carcinogenic metabolite of 4-NQO and is involved in the formation of DNA adducts. This active metabolite is further metabolized and acetylated by seryl-tRNA-synthetase to form seryl-AMP enzyme complex (Kanojia and Vaidya, 2006). This complex is found to induce quinoline groups in the DNA. 4-NQO preferentially binds to the N2 and C8 positions of guanine respectively and induces guanine adducts (Galiegue-Zouitina *et al*.,1985; Kanojia and Vaidya, 2006; Martínez, 2012). Similar to the molecular alterations observed in OSCC, 4-NQO also altered p16, cyclin D1, p53, keratin 1 & 14, EGFR, H19, IGF2 and Kip (Tang *et al*., 2004; Yuan *et al*., 1997; Kanojia and Vaidya, 2006). In murine models, 4-NQO upregulated the apoptosis related proteins (Bcl-2 and Bax); increased cell cycle check point proteins (cyclin D1, CDK4, pRb, PCNA) and EMT (P-cadherin, β catenin). Therefore, we determined the effect of 4-NQO on the expression of NGAL in oral cancer cells. We found that concentrations 50ng/ml, 100ng/ml, 250ng/ml of 4-NQO are not toxic to cells and downregulated the expression of NGAL in both the cell lines (Figure 3.4). This indicates the NGAL may be involved in 4-NQO induced OSCC. However, further studies are required to decipher the mechanism of 4-NQO induced downregulation of NGAL.

3.3. Conclusion

It has been well established that tobacco is the main risk factor for oral cancer. There are many carcinogens isolated from tobacco which include benzo[a]pyrene, NNK and NNN etc. which are known to cause cancer. Therefore, we analyzed the effect of

tobacco on the expression of NGAL. This is the first report that shows the effect of tobacco components on the expression of NGAL. Herein, we have shown that exposure of SAS cells with NNN, NNK and 4-NQO downregulated the expression of NGAL indicating that NGAL plays an important role in tumorigenesis of oral cancer. Similarly, in KB-CHR-85 cells nicotine, NNK and 4-NQO downregulated the expression of NGAL suggesting that NGAL is involved in the development of oral cancer. However, in our study we observed that benzo[a]pyrene did not downregulate the expression of NGAL, in both the cell lines, indicating the involvement of alternate pathways in oral cancer tumorigenesis (data not shown). This suggests that the effect of tobacco components on these cell lines was found to be both cell line and tobacco component specific. Thus it can be concluded that downregulation of NGAL might be an early event in malignant transformation of tobacco induced oral cancer.

4

CHAPTER

Role Of NGAL In The Development Of Oral Squamous Cell Carcinoma

4. Introduction

In the previous chapters we have shown that NGAL is downregulated in oral cancer tissues compared to normal tissues and the tobacco components, the main risk factor for oral cancer downregulated the expression of NGAL in oral cancer cell lines. In addition, we have shown that 4-NQO which recapitulate the development of human oral cancer in animals has been shown to repress the expression of NGAL in oral cancer cell lines. Therefore, we hypothesized that downregulation of NGAL may play a major role in the development of oral cancer. In line with our results, the downregulation of NGAL in oral cancer has been reported by other groups (Lin *et al*., 2016; Shrinki *et al*., 2014; Hiromoto *et al*., 2011). However, the role of NGAL in different processes of oral cancer development is poorly understood. In pancreatic cancer, downregulation of NGAL induces aggressiveness of the cancer cells *in vitro* partly by activating EGFR-MEK-ERK signaling pathway (Tong *et al*., 2011). In contrast, pro-tumorigenic role of NGAL has been reported in other cancers. In colon cancer upregulation of NGAL enhanced cell-matrix attachment, and increased cell motility and invasion (Hu *et al*., 2009). Similarly, in esophageal cancer overexpression of NGAL promotes migration and invasion along with the activation of MMP-9 (Li *et al*., 2003). In lung cancer elevated expression of NGAL induces EMT, increases tumour stemness, and tumour metastases by modulating NF-κB (Mongre *et al*., 2016). These substantial reports reveal the oncogenic role on NGAL in different cancers. In addition, studies also suggest that NGAL also is involved in cancer cell chemoresistance. Therefore, in this chapter we have determined the effect of silencing of NGAL in different hall marks of cancer as well as chemoresistance.

4.1. Materials and Methods:

4.1.1. Cell culture:

SAS cells were procured from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India These cells were maintained in DMEM (Gibco™; Life Technologies, NY, USA) supplemented with 10% FBS (Gibco®, NY, USA) and 1X Penstrep (Invitrogen, CA, USA). The cells were cultured and maintained at 37°C in a 5% CO_2 and 95% humidity.

4.1.2. shNGAL stable knockdown :

shRNA mediated knockdown of NGAL was carried out in SAS cell line. Human shNGAL plasmids (**Table 4.1**) and puromycin (Cat No. P8833, Sigma-Aldrich, Missouri, USA) were purchased from sigma. SAS cells were seeded at a concentration of $25x10^4$ cells/well in 1ml of media in a 24-well plate. The next day cells were transfected with shRNA control and shNGAL plasmids (2µg of DNA) using X-treme gene 9 DNA transfection reagent (Cat No. 06365787001, Sigma-Aldrich, Missouri, USA) for 48h. After 48h, the medium containing transfection reagent was replaced with fresh DMEM medium and allowed for recovery for 24h. Then SAS cells were selected with 1µg/ml puromycin and the cells which were resistant to puromycin were allowed to grow into colonies. After the stable colonies were established, the colonies were trypsinized, seeded one colony per well in a 96-well plate and were grown till 90% confluency. Then the cells were cultured in a 24-well plate till 90% confluency, and RNA and cell lysates were prepared and the expression of NGAL was determined by quantitative PCR and western blot analysis. The stable cells which were negative for NGAL were used for further studies.

Table 4.1: Control shRNA (SHC204) and NGAL shRNA sequences used for transfection.

S. No	Clone	Sequence
1	TRCN0000372769	CCGGCAATTCTCAGAGAAGACAAAGCTCGAGCTTTGTCTTCTCTGAGAATTGTTTTTG
2	TRCN0000378896	CCGGGAGTGGTGAGCACCAACTACACTCGAGTGTAGTTGGTGCTCACCACTCTTTTTG
3	TRCN0000372827	CCGGGGAGCTGACTTCGGAACTAAACTCGAGTTTAGTTCCGAAGTCAGCTCCTTTTTG
4	TRCN0000060288	CCGGGCTGGGCAACATTAAGAGTTACTCGAGTAACTCTTAATGTTGCCCAGCTTTTTG
5	TRCN0000060289	CCGGCCAGCATGCTATGGTGTTCTTCTCGAGAAGAACACCATAGCATGCTGGTTTTTG
6	SHC204	CCGGCGTGATCTTCACCGACAAGATCTCGAGATCTTGTCGGTGAAGATCTTTTT

4.1.3. Cell proliferation assay:

Cell proliferation was determined by MTT assay. Control shRNA and shNGAL cells were seeded at a density of $2x10^3$ cells /well in 96-well plates in sextuplicate and was incubated for 24 and 48h respectively. After each time point, 10µl of MTT (cont. 5mg/ml; Cat No. M2128, Sigma- Aldrich, Missouri, USA) was added to the cells and was incubated for 2 h at 37°C. The MTT solution along with the media was removed and 100µl of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added to each well and was incubated for 1h. The absorbance was then measured at 570nm using an Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland).

4.1.4. Cell cycle analysis:

Cell cycle analysis was carried out by flowcytometer. Control shRNA and shNGAL cells were plated at a density of $1x10^5$ cells/well and after 24h cells were trypsinized, washed with phosphate-buffered saline (PBS) and fixed with 75% ethanol at -20°C overnight. The following day, cells were washed with PBS, treated with PI/RNase solution (Cat No. A35126, Invitrogen, CA, USA) for 20min in the dark and analyzed by flow cytometer (FACS Calibur, Becton-Dickinson, New Jersey, USA). 25,000 cells

in each sample were analyzed. The data was analyzed on FCS express 6.

4.1.5. Cell survival assay:

Cell survival was determined by two-dimensional colony formation assay. Control shRNA and shNGAL cells were seeded in a 6-well plate at a density of $1x10^3$ cells/well. The cells were grown for fifteen days, the colonies were fixed with 70% ethanol and were stained with crystal violet. Pictures of individual wells were taken and were analyzed using imageJ software and surviving fraction was calculated using the formulas:

$$\text{Plating efficiency (PE)} = \frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100$$

$$\text{Surviving fraction (SF)} = \frac{\text{PE of treated sample}}{\text{PE of control}} \times 100$$

4.1.6. *In vitro* wound closure assay:

The migratory potential of NGAL knockdown cells were determined by *in vitro* wound healing assay. Control shRNA and shNGAL cells were seeded in 6-well plates and were allowed to grow till confluency. The cells were washed with PBS and were serum starved for 8h. Confluent monolayers were scratched with a pipette tip. Plates were washed with PBS to remove non-adherent cells, and were maintained in serum free media. The wound was photographed at regular time intervals and the percentage of wound area was calculated compared to controls.

4.1.7. Cell invasion and migration assay:

The invasive and migratory potential of NGAL silencing were carried out by Boyden chamber assay. Control shRNA and shNGAL cells were serum starved for 18h before seeding to transwell migration chambers. 24-well, 8mm pore transwell inserts (Cat No. 3422, Corning, New York, USA) pre-coated with matrigel. Post serum starvation, the cells were trypsinized and were seeded at a concentration of $5x10^4$ cells in the upper

chamber of transwell insert in 500µl of serum free media. Then 750µl of media containing 10% FBS was added to the lower chamber as a chemo-attractant. Cells were then incubated for another 24h at 37°C. The non-migrating cells on the upper surface of the membrane were then scraped off with cotton swabs. The migrated cells at the bottom of the transwell insert were fixed in 70% ethanol and were stained with crystal violet solution. Stained cells were visualized under a microscope and photographs were taken using Nikon 500 camera. After the photographs were taken, the membrane was dissolved in 1% SDS solution at 37°C for 1h and absorbance was read at 595nm.

4.1.8. Western blot analysis:

Western blot analysis was carried out to determine the expression of different proteins. Antibodies, for NGAL (dilution 1:3000; Cat No. ab23477, Abcam, Cambridge, USA), S6 Ribosomal protein (dilution 1:2000; Cat No. 2317S), Phospho- S6 Ribosomal protein (Ser235/236) (dilution 1:2000; Cat No. 4858T), p53 (dilution 1:1000; Cat No. 2524T), Redd1 (dilution 1:1000; Cat No. 2516S), LC3B (dilution 1:1000; Cat No. 2775S), Caspase-9 (dilution 1:1000; Cat No. 9508T), Bcl-2 (dilution 1:1000; Cat No. 15071), MMP-9 (dilution 1:1000; Cat No. 13667P), cyclin D1 (dilution 1:1000; Cat No. 2978BC), GAPDH (dilution 1:2000; Cat No. 2118S) were purchased from Cell Signaling Technology, Massachusetts, USA. Anti-mouse secondary antibody (dilution 1:6000; Cat No. ab97040) and anti-rabbit secondary antibody (dilution 1:6000; Cat No. ab97080) were purchased from Abcam, Cambridge, USA. Briefly, Control shRNA and shNGAL cells were lysed using whole cell lysis buffer (20mM HEPES, 2mM EDTA, 250mM NaCl, 0.1% NP40) in the presence of protease inhibitors (2µg/ml Leupeptin hemisulfate, 2µg/ml aprotinin, 1mM PMSF, 1mM DTT). The protein concentration of the lysates were measured by Bradford Assay. 50µg of protein were resolved and mixed with 5X Laemmli Buffer (250mM TrisHCl, 10% SDS, 30% Glycerol, 5% β-

mercaptoethanol, 0.02% Bromophenol blue), electrophoresed in a 12% SDS-acrylamide gel, and transferred to Nitrocellulose Transfer Membrane (Bio rad, California, USA). The membranes were blocked with 5% non-fat milk in tris-buffered saline containing 1% tween 20 (TBST) and for the phospho antibodies the membranes were blocked with 5% BSA in tris-buffered saline containing 1% tween 20. The blots were probed with appropriate primary antibodies overnight. The following day the blots were washed with TBST and were incubated with appropriate horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized using Optiblot ECL Detection Kit (Cat No. ab133406, Abcam, Cambridge, USA). GAPDH was used as the housekeeping control.

4.1.9. RNA isolation and Reverse transcriptase PCR:

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was then performed for 33 cycles with 1µl of cDNA as a template. To avoid genomic DNA contamination, the primers used were intron spanning. PCR conditions, primer sequences and amplicon lengths are mentioned in **table 4.2**.

4.1.10. Propidium Iodide Flow Cytometry (PI/FACS) analysis:

The cell death induced by chemotherapeutic agents was determined by staining with propidium iodide (PI) (conct. 1mg/ml; Cat No. P4170, Sigma-Aldrich, Missouri, USA). Control shRNA and shNGAL cells were seeded in a 6-well plate at a density of $5x10^4$ cells/well. After 24h, the cells were treated with different concentrations of cisplatin and 5-Flurouracil for 48h. After 48h, the cells were harvested and were washed with PBS twice. 10µl of PI was added and was analyzed by flowcytometer (FACSCalibur, Becton-Dickinson, New Jersey, USA). The data was analyzed using FCS Express 6 software.

Table 4.2: List of primers and their sequences used to study mRNA expression.

Gene		Primers	Tm (ºC)	Amplicon Size
NGAL	F	5’ ATGCCCCTAGGTCTCCTGT 3’	55 ºC	597bp
	R	5’ T C A G C C G T C G A T A C A C T G 3’		
LKB1	F	TCAAAATCTCCGACCTGGGC	55 ºC	570bp
	R	TGTGACTGGCCTCCTCTTCT		
αAMPK	F	CGGCAAAGTGAAGGTTGGCAA	59 ºC	227bp
	R	CAAATAGCTCTCCTCCTGAGAC		
P53	F	CTGCCCTCAACAAGATGTTTTG	55 ºC	172bp
	R	CTATCTGAGCAGCGCTCATGG		
Redd-1	F	CTGATGCCTAGCCAGTTGGT	55 ºC	233bp
	R	GAGCTAAACAGCCCCTGGAT		
Caspase-3	F	ATGGAGAACACTGAAAACTCAGTGGATT	65 ºC	782bp
	R	CCACCAACCAACCATTTCTTTAGTG		
GAPDH	F	AGG TCG GAG TCA ACG GAT TTG	60 ºC	532bp
	R	GTG ATG GCA TGG ACT GTG GT		

4.1.11. Statistical analysis:

All the statistical analysis was carried out using Student's *t*-test or one-way ANOVA followed by Tukey test. *p*-value less than 0.05 was considered as statistically significant.

4.2. Results and Discussion

In this chapter we have determined the role of NGAL on different hallmarks of cancer such as cell survival, proliferation, migration, invasion and metastases. First we have established stable knockdown of NGAL in SAS cells. Then we have studied the effect of NGAL knockdown on cell proliferation, cell cycle arrest, colony formation, invasion etc. and also the expression of genes involved in these different processes. We have also studied the effect of knockdown of NGAL in cancer cell chemoresistance.

4.2.1. Confirmation of knockdown of NGAL:

To understand the role of NGAL in oral cancer, we silenced NGAL mRNA expression using shRNA clones. We have used a total of five shRNA clones out of which clone TRCN0000378896 showed a maximum downregulation of NGAL (Figure 4.1). Then, we have confirmed this knockdown using PCR and western blot analysis. We observed complete knockdown of NGAL in transfected cells. These cells were further multiplied and used for different experiments.

4.2.2. Silencing of NGAL increases proliferation and survival of oral cancer cells:

The most cardinal property of cancer cells is their ability to sustain cell survival and proliferation. Normal cells control and carefully scrutinize the growth promoting signals, that direct the entry into and progression through cell growth and cell cycle, thus maintaining the homeostasis and normal architecture (Hanahan and Weinberg, 2011). This homeostasis and normal architecture are deregulated in cancer, and hence the cancer cells proliferate uncontrollably. Therefore, we determined the effect of silencing of NGAL on the proliferation of oral cancer cells. We found that silencing of NGAL increased the rate of proliferation in a time dependent manner (Figure 4.2). To confirm the increase in proliferation, we analyzed the effect of knockdown on different phases of cell cycle (G1, S, G2-M phases, Figure 4.2) by flow cytometer. We found that silencing of NGAL lead to an increase in number of cells in S-Phase and decreased number of cells in G2/M phase compared to control shRNA (Figure 4.2). S-phase also known as synthesis phase where the DNA packaged into chromosomes is replicated. Once the cell crosses the G1 checkpoint, it is irretrievably dedicated to cell division. Increase in number of cells in S-phase, indicates that these NGAL knockdown cancer cells proliferate continuously and passes through G2/M check point smoothly without

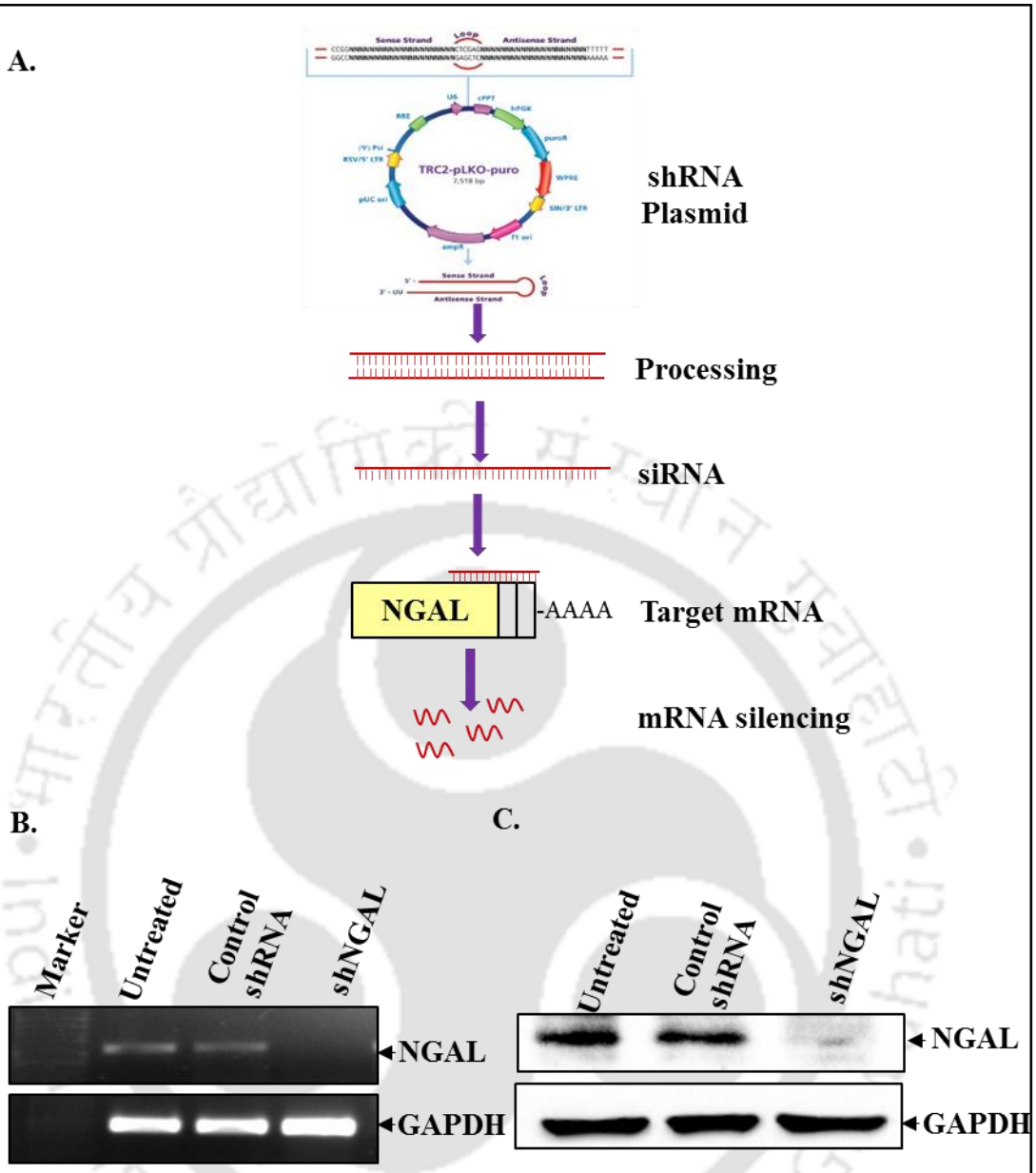

Figure 4.1: Silencing of NGAL in oral cancer cells. A) Mechanism of shRNA knockdown. B) qRT–PCR showing the mRNA expression of NGAL in SAS cells post knockdown. C) Western blot analysis showing the NGAL in SAS cells post knockdown.

any DNA repairs. This indicates that, loss of expression of NGAL deregulated the cell cycle checkpoints, and hence the proliferation of these cells is high. In addition, in NGAL deficient cells we observed that the expression of cyclin D1 is upregulated which is regulated by NF-κB/PI3K-mTOR pathways (Figure 4.4). Therefore, we believe that activation of mTOR is might be the key mechanisms that leads to the

increase in proliferation of oral cancer cells. We also assessed whether knockdown of NGAL increases oral cancer cell survival by clonogenicity assay. We observed a two fold increase in number of colonies in shNGAL group compared to control shRNA group (Figure 4.2). However, no significant difference in size of the colonies was observed. Further, in our study we found that the cell survival protein Bcl-2 were upregulated, which is also regulated by mTOR (Figure 4.4). This is the first report that shows that knockdown of NGAL increases the survival of oral cancer cells. Recently, similar to our findings Kim *et al*., 2017 reported that downregulation of NGAL increased cell proliferation and survival and induced morphological shift from an epithelial to mesenchymal state in colorectal cancer *in vitro* (Kim *et al*., 2017). Thus our study indicates that NGAL negatively regulates cell proliferation and survival *in vitro*.

4.2.3. Silencing of NGAL increases invasion and migration of oral cancer cells

In the second chapter we found that the expression of NGAL is significantly downregulated in lymph node metastases compared to non-metastatic tumors. The fundamental steps in tumour metastasis is the migration and invasions of cancer cells away from the primary tumor (Clark and Vignjevic, 2015). Therefore, we hypothesized that downregulation of NGAL may lead to invasion and metastases of oral cancer. To confirm this, we performed *in vitro* invasion and migration assays using NGAL knockdown cells. The transwell migration assay indicated that the NGAL knockdown cells exhibited higher invasive ability compared to shRNA control cells. We observed that the number of cells that invaded at the lower part of the transwell insert were higher in shNGAL cells compared to the control cells (Figure 4.3). The *in vitro* wound healing assay showed that the migratory potential of NGAL knockdown cells were higher

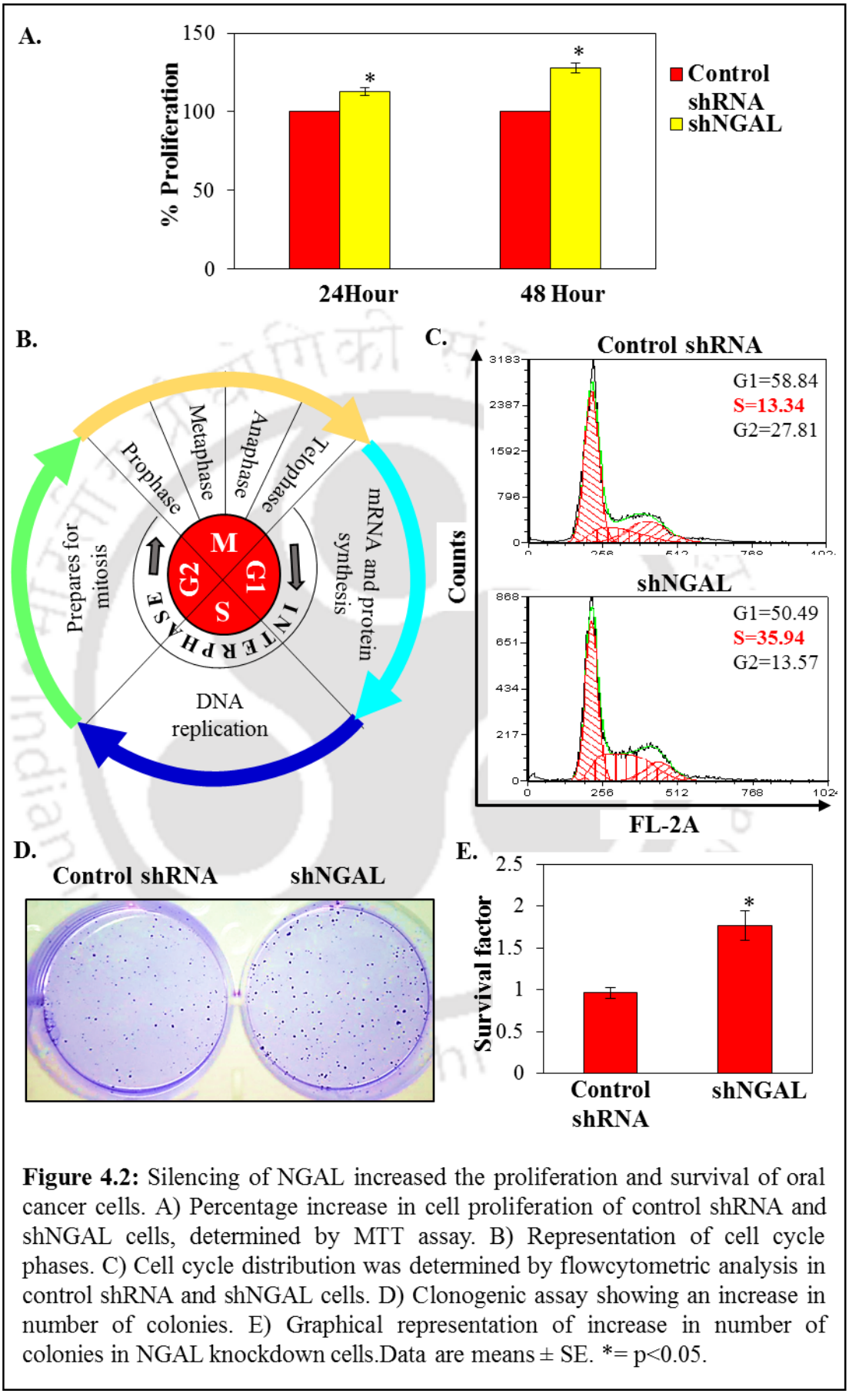

Figure 4.2: Silencing of NGAL increased the proliferation and survival of oral cancer cells. A) Percentage increase in cell proliferation of control shRNA and shNGAL cells, determined by MTT assay. B) Representation of cell cycle phases. C) Cell cycle distribution was determined by flowcytometric analysis in control shRNA and shNGAL cells. D) Clonogenic assay showing an increase in number of colonies. E) Graphical representation of increase in number of colonies in NGAL knockdown cells.Data are means ± SE. *= $p<0.05$.

compared to the shRNA control cells. It was observed that the wound was completely healed in case of shNGAL cells with in 8h compared to the control cells. This suggests that knockdown of NGAL increased the motility of SAS cells. Moreover, in our study we have observed that in NGAL silenced cells, MMP-9 was found to be upregulated (Figure 4.4). The role of NGAL in invasion and metastases has been well studied in different tumours. Overexpression of NGAL is known to induce invasion and metastases in breast, colon, esophageal, cervical and lung cancers (Leng *et al*., 2009, 2011; Hu *et al*., 2009; Du *et al*., 2015; Chung *et al*., 2016; Mongre *et al*., 2016; Tang *et al*., 2015). Contrastingly, in our study we found that downregulation of NGAL increased invasion and migration of SAS cells. Similar to our results, Lin *et al*., 2016 reported that overexpression of NGAL reduced *in vitro* invasion and migration, and *in vivo* metastases, while downregulation of NGAL increases cell motility. Anti-tumorigenic role of NGAL is reported in pancreatic, liver and colorectal cancers. In liver cancer cells *in vitro*, overexpression of NGAL decreased invasion and migration, partly by inhibiting the phosphorylation of JNK and PI3K/Akt signaling pathways (Lee *et al*., 2011). Similarly, in pancreatic cancer, overexpression of NGAL reversed EMT by upregulation of E-cadherin and decreased vimentin (Xu *et al*., 2013).

4.2.4. Silencing of NGAL activates mTOR signaling and suppresses autophagy:

As the above results suggest that loss of NGAL induces oral cancer cell proliferation, survival, invasion and migration, we studied the underlying mechanism involved in these processes. In the previous chapter, we observed that treatment of oral cancer cells with tobacco components downregulated the expression of NGAL (Figure 3.1-3.4). It has been well established that tobacco is one of the major risk factors for oral cancer, and it induces oral cancer by activating Akt/mTOR pathway (Memmott and Dennis, 2009; Du *et al*., 2012). Activation of Akt/mTOR is involved in many cellular

functions such as proliferation, survival, protein synthesis, glucose metabolism, angiogenesis etc. that are linked with tumorigenesis (Gao *et al*., 2012). Therefore, we determined the effect of silencing of NGAL on the activation of S6, a marker of mTOR pathway. Our results suggest that, NGAL knockdown induces the activation of S6 (serine 235/236), the downstream effector of mTOR pathway (Figure 4.4). mTOR directly activates p70S6 kinase, that phosphorylates S6 protein of the 40S ribosomal subunit (phosphorylated S6 ribosomal protein (phospho-S6rp)) at several sites, including serines 235 and 236, leading to the initiation of protein synthesis (Dufner and Thomas, 1999; Ferrari *et al*., 1991). However, the mechanism involved in the activation of mTOR by NAGL is not clearly understood. Recently, it has been reported that metformin, an anti-diabetic drug inhibited activation of S6 in HNSCC cells via activation of AMPK (Lin *et al*., 2014). Therefore, we examined the effect of NGAL knockdown on the expression of AMPK in oral cancer cells and found that NGAL knockdown inhibited the expression of AMPK, indicating AMPK is the intermediate link between NGAL and S6. Furthermore, it is well established that metformin activates AMPK via LKB1 and AMPK is the only substrate for LBK1 (Lin *et al*., 2014; Shackelford and Shaw, 2009). Therefore, we analyzed the expression of LKB1 in NGAL knockdown cells, which showed a clear downregulation of LKB1 suggesting that the silencing of NGAL induces the activation of S6 by inhibiting LKB1-AMPK pathway. Liver Kinase B1 (LKB1), also known as serine-threonine kinase 11 (STK11), controls many cellular functions linked with embryo development, epithelial cell polarity, cell cycle arrest, DNA damage response, apoptosis, and the dynamics and maintenance of hematopoietic stem cells (Bardeesy *et al*., 2002; Shackelford and Shaw, 2009; Marignani *et al*., 2001; Baas *et al*., 2004; Zheng *et al*., 2007; Karuman *et al*., 2001; Gan *et al*., 2010; Gurumurthy *et al*., 2010; Nakada *et al*., 2010). During glucose

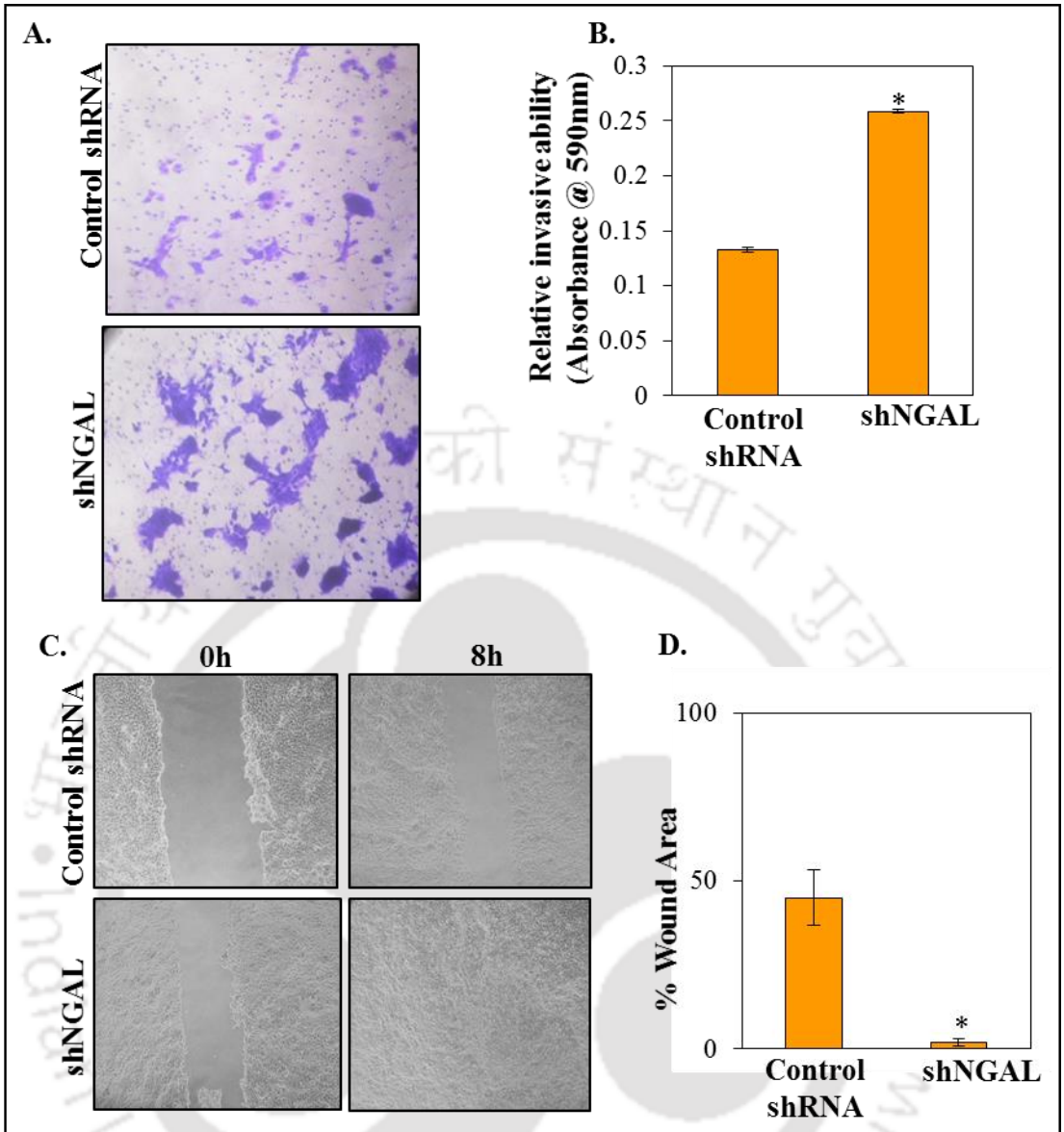

Figure 4.3: Silencing of NGAL increased the invasion and migration of oral cancer cells. A) Cell invasion was determined by transwell invasion assay. Cells invading through the matrigel were fixed, stained and photographed under inverted microscope at a 20x magnification. B) Graphical representation of increase in cells invading the lower surface of transwell insert. C) Cell migration was detected by wound healing scratch assay. Photographs were taken at 10x magnification. D) Graphical representation of decrease in wound area. Data are means ± SE. *= $p<0.05$.

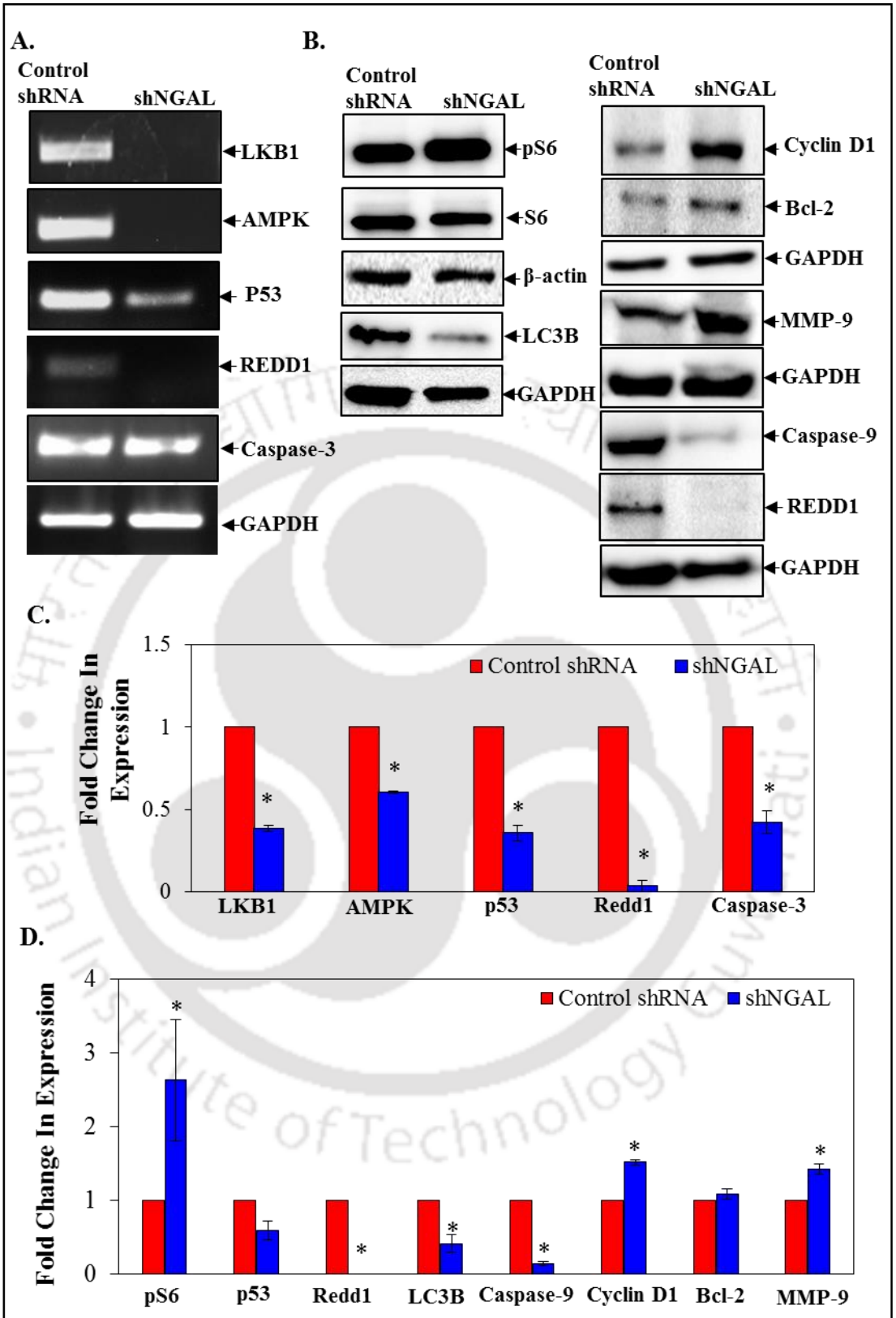

Figure 4.4: Silencing of NGAL activated mTOR signaling and induced autophagy. A) mRNA expression of LKB1-AMPK-P53-Redd1 and Caspase-3 B) Expression of proteins involved in mTOR signaling and autophagy. C) Fold change in mRNA expression as analyzed by image lab software. D) Fold change in expression of proteins as analyzed by image lab software. GAPDH was used as internal control. Data are means ± Stdev. *= $p<0.05$.

starvation, reduction in the ratio of ATP/AMP, activates LKB1 that potentiates the phosphorylation of AMPK (Zhao and Xu, 2014). Consequently, phosphorylation of mTORC1 was inhibited, leading to the activation of tuberous sclerosis complex 2 (TSC2) resulting in inhibition of cell proliferation and induction of cell cycle arrest and autophagy (Inoki *et al*., 2003; Marignani *et al*., 2001; Inoki *et al*., 2012; Li *et al*., 2011; Zang *et al*., 2006; Vingtdeux *et al*., 2011; Zhao and Xu, 2014). Recently, it was reported that '*Regulated in development and DNA damage 1*' also known as Redd1, which is activated during hypoxia or energy stress inhibits mTOR pathway by upregulating AMPK in HNSCC cells (Ellisen, 2005; Schneider *et al*., 2008). Therefore, we analyzed the expression of Redd1 in NGAL knockdown cells and it was downregulated (Figure 4.4). Interestingly, activation of AMPK is known to activate p53 during metabolic stress. During this process, the activated AMPK, phosphorylates and inactivates MDMX (murine double minute X), resulting in stabilization and activation of p53 (He *et al*., 2014). Thus, p53 accumulates and activates target genes involved in programming growth arrest, senescence, and apoptosis, depending on the cell type (Imamura *et al*., 2001; Jones *et al*., 2005; Okoshi *et al*., 2008). Therefore, we analyzed the expression of p53 as it serves as a downstream target of AMPK and found that it was downregulated in NGAL knockdown cells. In a recent study it was reported that Redd1 is a direct transcriptional target of p53 and its promoter region contains a consensus p53 family binding element which is required for the induction of Redd1 by p53 (Vadysirisack *et al*.,2011; Ellisen *et al*., 2002). Thus, our results suggests that p53 can serve as a connecting link between AMPK and Redd1 and AMPK regulates mTOR pathway via p53-Redd1 axis. Therefore, it can be concluded that silencing of NGAL increases survival, proliferation, invasion and migration of oral cancer cells via LKB1-AMPK-p53-Redd1-mTOR axis.

Apart from the significant role of mTOR in cell growth, it is also involved in survival, differentiation and invasion and is known to regulate MMP-2/-9 activities (Tian *et al*., 2010; Brouxhon *et al*., 2014; Cheng *et al*., 2017; Yang *et al*., 2014; Li *et al*., 2017). Overexpression of p70S6K promotes EMT and migration of HNSCC cells by upregulation of MMP-9 (Wu *et al*., 2016). Similarly, in our study we observed that MMP-9 is upregulated in NGAL knockdown cells and that leads to the invasion and migration of SAS cells. It is well established that activation of mTOR results in the increase in cell proliferation by upregulating the expression of cyclin D1 (Alao, 2007; Gao *Et al*., 2004). Hence, we analyzed the expression of cyclin D1 and observed that cyclin D1 was found to be upregulated and leads to the increase in proliferation of NGAL knockdown cells. Moreover, studies also suggest that activation of mTOR inhibits apoptosis (Liu *et al*., 2018; Lian *et al*., 2018; Yang *et al*., 2018). Similarly, in our study, we found that silencing of NGAL upregulated Bcl-2 expression and inhibited caspase-3 and -9 indicating the direct role of NGAL in inducing apoptosis in oral cancer cells. In addition, it is also established that inactivation of p53 suppresses apoptosis via induction of Bcl-2 and suppression of caspases (Schuler and Green, 2001). During the process of p53 dependent activation of caspases, the released cytochrome c, activates apoptosome, which consists of Apaf-1 and caspase -9. Thus, the active caspase -9 cleaves and activates the effector caspases, such as caspases-3 and -7, which execute the death program (Schuler and Green, 2001). Therefore, it can be concluded that inhibition of p53 and activation of mTOR plays an important role in the survival of NGAL silenced oral cancer cells.

Increasing lines of evidences suggest that activation of mTOR reduces autophagy, hence we analyzed the expression of autophagy marker LC3B and observed that knockdown of NGAL reduced expression of LC3B indicating that NGAL knockdown

cells are more resistant to autophagy and that leads to their enhanced survival (Yang *et al*., 2018; Dunlop and Tee, 2014; Gao *et al*., 2012). Aforementioned, NGAL knockdown inhibited the expression of LKB1, AMPK, p53 and Redd1, the well established regulators of autophagy. Therefore, it is clear that the resistance to autophagy in NGAL knockdown cells is via the inhibition of LKB1-AMPK-p53-Redd1 and upregulation of mTOR pathway. Autophagy is a controlled catabolic process that is involved in the maintenance of cellular homeostasis and decrease in metabolic stress by lysosomal degradation of proteins and cellular organelles (Ávalos *et al*., 2014; White and DiPaola, 2009). Dysregulations in the implementation of this process is associated with severe pathological conditions such as neurodegeneration, aging and cancer. As autophagy is directly related with cell proliferation and survival we conclude that inhibition of autophagy plays an important role in the increase in cell proliferation and survival of NGAL knockdown oral cancer cells.

4.2.5. Silencing of NGAL selectively induces resistance against cisplatin:

Chemoresistance is a major hurdle in the treatment of oral cancer. Chemoresistance developed by cancer cells is mainly of two types; 'intrinsic resistance' in which the cells are resistant since the initial stage while 'acquired resistance' is developed in due course of chemotherapy (Monisha *et al*., 2017). Variations in intracellular signal transduction pathways, cross-talk among diverse membrane receptors, alterations in apoptotic signaling, and interruption in cell division are few mechanisms by which tumor cells develop chemoresistance (Monisha *et al*., 2018). Cisplatin and 5-flurouracil (5-FU) are the first line chemotherapeutic agents being used for oral cancer. Cisplatin also known as *cis*-diamminedichloroplatinum (II), interlinks with the purine bases on the DNA; impedes with DNA repair mechanisms and instigates DNA damage (Dasari and Tchounwou, 2014). 5-FU is an analogue of uracil that inhibits the action of

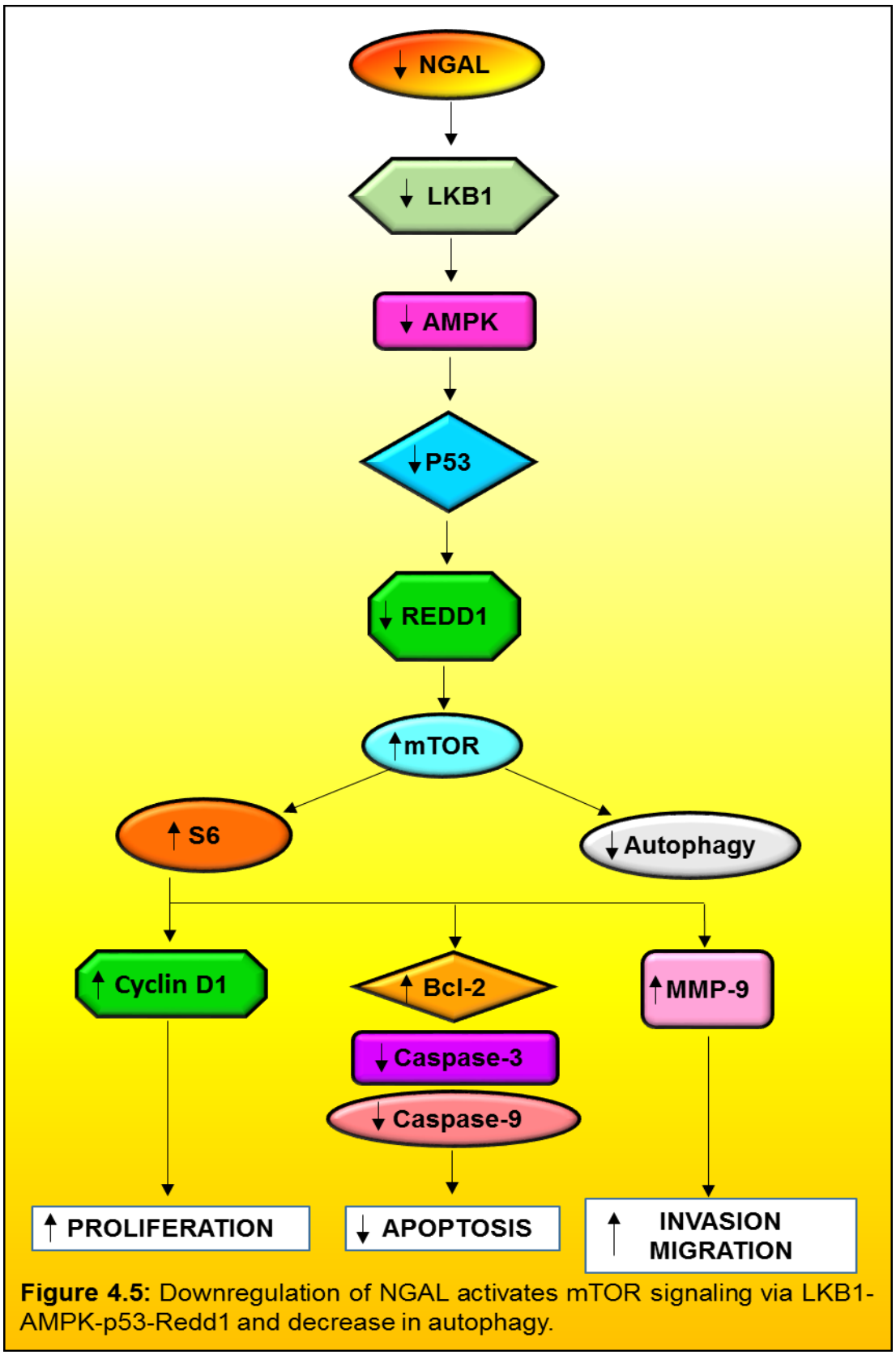

Figure 4.5: Downregulation of NGAL activates mTOR signaling via LKB1-AMPK-p53-Redd1 and decrease in autophagy.

thymidylate synthase (TS) and integrates its metabolites into RNA and DNA (Longley *et al*., 2003). Therefore, we examined whether the knockdown of NGAL induces resistance

against cisplatin and 5-FU. We treated NGAL knockdown cells and control shRNA cells with cisplatin (50µM) and 5-FU (50 µg/ml) for 48h. After 48h, PI-FACS was performed to determine the effect of knockdown on cell death. The data showed that, the NGAL knockdown cells were specifically resistant to cisplatin but not 5FU (Figure 4.6). Our results showed that silencing of NGAL increased the expression of Bcl-2 and inhibited capase-3 and -9 and can be the underlying mechanism responsible for chemoresistance. However, the exact mechanism has to be further studied.

4.2.6. Curcumin restored the expression of NGAL in oral cancer cells

Our results suggest that downregulation of NGAL increased survival, proliferation and induced invasion and metastases in oral cancer. Therefore, the agents that can upregulate the expression of NGAL would be beneficial in developing therapies against this disease. Curcumin is believed to be one such agent that can upregulate the expression of NGAL.

Therefore, we examined the effect of curcumin on the expression of NGAL and oral cancer cell proliferation *in vitro*. Curcumin is the most active component of turmeric and is well known for its biological and pharmacological activities (Anand *et al*., 2007). We treated SAS cells with increase in concentration of curcumin in for 24h. The IC_{50} was found to be 6µM for 24h (Figure 4.7). Next, we found that curcumin induced apoptosis in SAS cells in dose-dependent manner (Figure 4.7). On elucidating the mechanism involved, interestingly we found that curcumin can upregulate the expression of NGAL in SAS cells. We treated SAS cells with 1µM of curcumin for 24h and analyzed the expression of NGAL using western blot. We observed that on treatment with curcumin the expression of NGAL was upregulated (Figure 4.7). This indicates that NGAL is involved in apoptotic effect of curcumin in SAS cells.

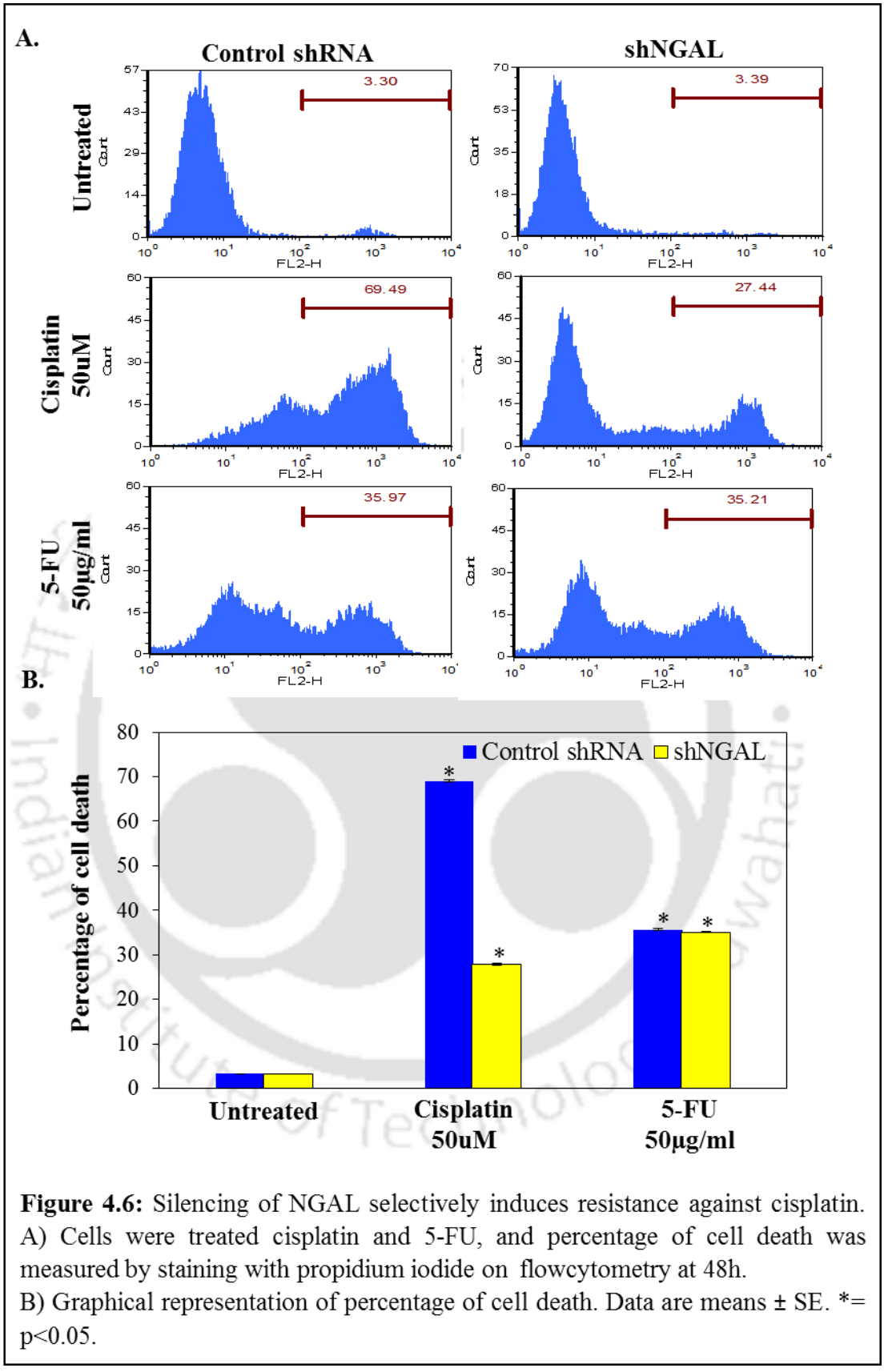

Figure 4.6: Silencing of NGAL selectively induces resistance against cisplatin. A) Cells were treated cisplatin and 5-FU, and percentage of cell death was measured by staining with propidium iodide on flowcytometry at 48h.
B) Graphical representation of percentage of cell death. Data are means ± SE. *= $p<0.05$.

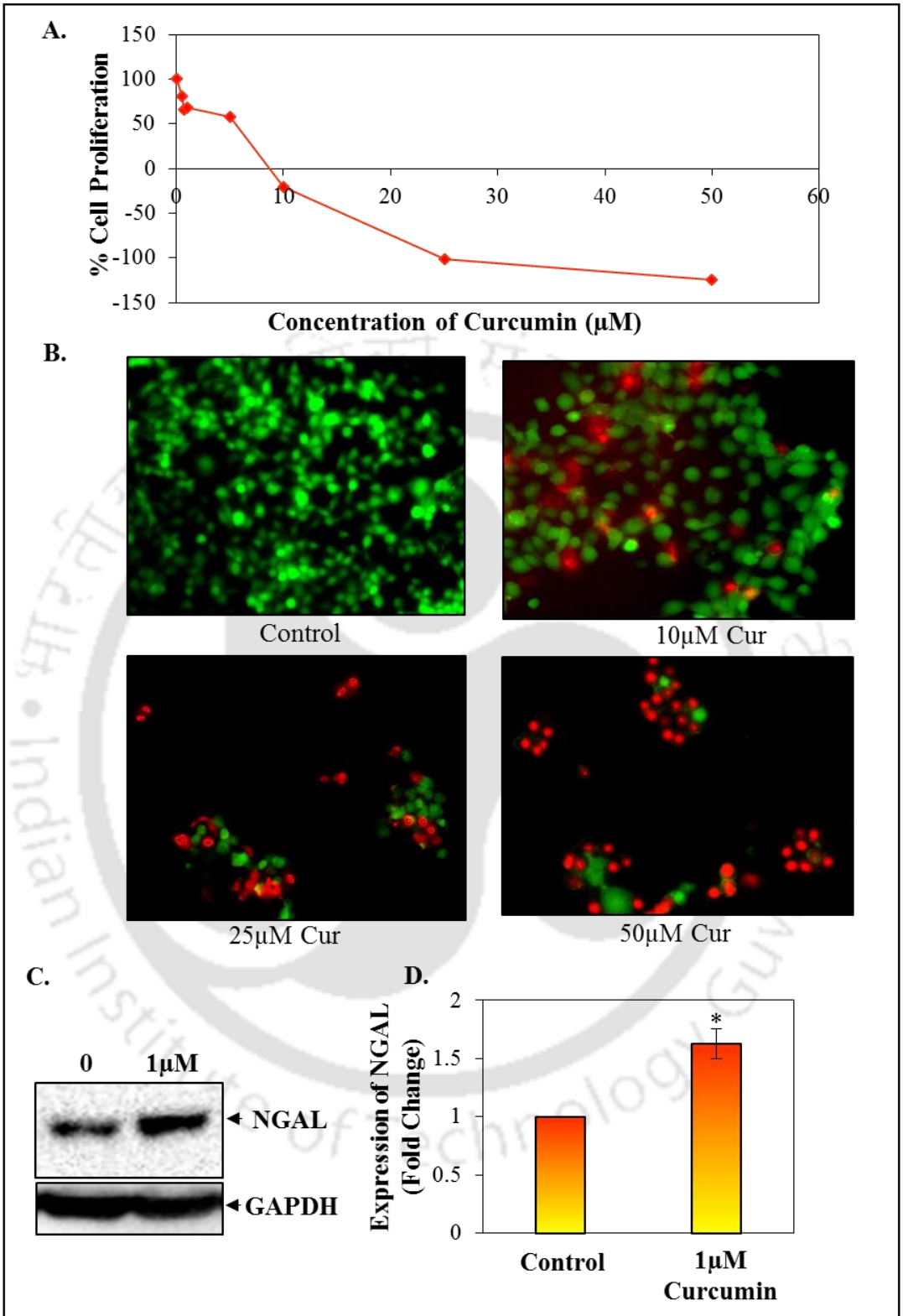

Figure 4.7: Curcumin restored the expression of NGAL in oral cancer cells. A) IC_{50} of curcumin was determined by MTT assay and was found to be 6µM B) Cells were treated with different concentration of curcumin for 24h and were stained with live/dead assay kit. C) Treatment with curcumin increased the expression of NGAL. D) Graphical representation of upregulation of NGAL on treatment with curcumin. Data are means ± SE. *= $p<0.05$.

Therapeutic properties of curcumin have been comprehensively studied over the past two decades. Anti-cancer activity of curcumin has been reported against leukemia, lymphoma, gastrointestinal cancers, genitourinary cancers, breast cancer, ovarian cancer, head and neck squamous cell carcinoma, lung cancer, melanoma, neurological cancers, sarcoma etc. Curcumin is known to target multiple signaling pathways associated with cell survival, invasion, angiogenesis, metastasis and inflammation (Anand *et al*., 2007; Bordoloi *et al*., 2016; Monisha *et al*., 2016).

4.3. Conclusion

In this chapter, we studied the role of NGAL for different hallmarks of cancer. Our results showed that knockdown of NGAL increased the survival, proliferation, invasion and migration of SAS cells. Moreover, silencing of NGAL activated mTOR signaling and reduced autophagy by LKB1-AMPK-p53-Redd1 signaling axis. Thus, activation of mTOR altered the expression of proteins associated with cell proliferation, survival, invasion and metastases. Cyclin D1, Bcl-2 and MMP-9, were upregulated while caspase-3 and caspase-9 were downregulated suggesting that NGAL is a crucial regulator of cell survival, cell motility and apoptosis in oral cancer. Moreover, downregulation of NGAL also induced resistance against chemotherapeutic agent cisplatin. Thus, agents that can rescue the loss of NGAL would be helpful in developing therapies against this dreadful disease. Curcumin, a multi-targeted agent upregulated the expression of NGAL in SAS cells, indicating that curcumin can help in rescuing the loss of NGAL. The future research will focus on validating these *in vitro* results in *in vivo* and clinical settings which would be beneficial to develop potent therapies against oral cancer.

5
CHAPTER

Discussion And Conclusion

5. Discussion and Conclusion

Regardless of the remarkable advances in the field of oncology, oral cancer still remains a major problem with poor prognosis and survival. Lips, cheeks, roof of mouth (hard palate), back of the mouth (soft palate and uvula), floor of the mouth (area under the tongue), gums, teeth, tongue, and tonsils are the regions of oral cancer. The indispensable risk factors of oral cancer are tobacco, alcohol, virus infection, air pollution and immune deficiency syndromes. These factors persuade molecular alterations in oncogenes and tumour suppressor genes, and cause mutations that aid in the development of oral cancer. Development of oral cancer is a multi-stage process, where the early lesions emerge as leukoplakia, erythroplakia, which then advances to dysplasia, carcinoma in situ and metastasize to neighboring and distant organs. This intricate process requires different molecular alterations in each stage for its progression. Complexity of the disease and lack of biomarkers for the early diagnosis and prognosis intensifies the difficulty in the management of this disease. Most of the chemotherapeutic agents such as 5-FU, cisplatin, docetaxel etc. curtail the quality of life of the patients and lead to poor survival. This augments the quest for new molecular signatures for early diagnosis and prognosis and to develop novel therapies for this disease and that would help in increasing the survival rates and the quality of life of the patients.

NGAL, also known as lipocalin-2, was initially identified as a bacteriostatic agent, but later voluminous literature supported its role in the development of cancer. Overexpression of NGAL was strongly implicated in the progression of breast, colorectal, esophageal, gastric and endometrial cancers, chronic myeloid leukemia and hepatocellular carcinoma. Nevertheless, in other cancers such as pancreatic and oral cancers, downregulation of NGAL advocates disease progression (Monisha *et al*.,

2014). Therefore, we hypothesized that downregulation of NGAL may be one of the significant molecular alterations that aid in the development of oral cancer. To ascertain the role of NGAL in oral cancer, we first analyzed the expression of NGAL in primary tumor tissues and also in metastatic tissues of oral cancer. Our results indicated that NGAL was downregulated in the primary tumour and metastatic tissues compared to normal tissues. We found that the downregulation of NGAL was inversely associated with the degree of differentiation where the well differentiated tissues showed moderate positive staining while moderately differentiated and poorly differentiated tissues were weekly positive. Our results were consistent with the previous studies indicating that NGAL is downregulated in the malignant tissues compared to normal tissues (Hiromoto *et al*., 2011). However, there were no reports about the expression of NGAL with respect to age, organs, stages, grades etc. in oral cancer. Decrease in expression of NGAL was pragmatic in malignant tongue, larynx, lip, cheek, gingiva and palate tissues. Furthermore, downregulation of NGAL was observed in all the stages (stage I-IV) and grades (grade I-III) of OSCC. In a recent study, Lin *et al*., 2016 reported that the expression of NGAL was decreased in lymph node metastatic OSCC patients compared non metastatic patients. Congruently, in our study we found that NGAL is significantly downregulated in the tissues of lymph node metastases. Taken together, the present study indicates that NGAL is downregulated in oral cancer and was strongly associated with stage and degree of differentiation of the tumour. From this study, it is evident that loss of expression of NGAL can drive the progression of OSCC from well- to poorly differentiated tumors and also helps in lymph node metastases. Moreover, NGAL can be used as a biomarker for identifying the degree of differentiation, prognosis and severity of the disease.

Secondly, we investigated the effect of tobacco components on the expression of NGAL in oral cancer cell lines as it is now well established that the tobacco usage and alcohol are the main risk factors for oral cancer. These two agents act synergistically, and heavy smokers and heavy drinkers have thirty eight-fold increase in the risk of developing oral cancer than abstainers from both products (Warnakulasuriya *et al*., 2009). The main carcinogens identified from tobacco smoke include benzo[a]pyrene, nicotine, NNK, NNN, dibenzo[a]pyrene, benzene, nitrobenzene, 2-toluidine, 2-6-dimethylaniline, etc. In our study, we analyzed the expression of NGAL in oral cancer cell lines on treatment with tobacco carcinogens, nicotine, NNN, NNK, benzo[a]pyrene and the synthetic carcinogen 4-NQO. The effect of tobacco specific nitrosamines NNK and NNN are unique. Metabolically activated NNK and NNN persuade mutations in oncogenes and tumor suppressor genes by forming DNA adducts, which leads to tumor initiation (Sturla *et al*., 2005; Kiyohara *et al*., 2005, 2006; Hecht *et al*., 2004; Xue *et al*., 2014). These nitrosamines can also bind to nicotinic acetylcholine receptors and promote tumour growth. These two aspects of NNK and NNN synergistically induce cancers in tobacco-exposed individuals (Xue *et al*., 2014). Nicotine, the major addictive component of tobacco is known to promote, proliferation, invasion, and angiogenesis in cancer (Jensen *et al*., 2012; Egleton *et al*., 2008; Arredondo *et al*., 2001). 4-NQO is a quinoline derivative and a tumorigenic compound that induces oral cancer in mouse and is most frequently used *in vivo* model to study the development of squamous cell carcinoma of the oral cavity (Kanojia and Vaidya, 2006; Martínez, 2012). Exposure of SAS cells with NNN, NNK and 4-NQO downregulated the expression of NGAL in a dose-dependent manner but not with NNK. In KB-CHR-85 cells, downregulation of NGAL was seen on treatment with nicotine, NNN and 4-NQO. These results suggest that the downregulation of NGAL was found to be cell line specific and tobacco

component specific and the molecular characteristic of the cell determines the effect of tobacco components on these cells. However, further studies are essential to decipher the role of molecular signatures that intricate the alteration of NGAL in oral cancer cell lines. These studies indicate that NGAL plays a prime role in tobacco induced carcinogenesis. However, the exact mechanism involved warrants further investigation. Sizeable literature suggest that NGAL is involved in cancer cell proliferation, survival, invasion and metastases. Therefore, we next determined the role of NGAL in these different processes by shRNA silencing of NGAL. Many studies have reported that downregluation of NGAL decreased *in vitro* cell motility, invasion, metastases and *in vivo* tumour growth and metastases (Li *et al*., 2003; Hu *et al*., 2009; Leng *et al*., 2011 Du *et al*., 2015; Chung *et al*., 2016). In breast cancer, downregulation of NGAL reduced the activity of MMP-9 and that lead to the inhibition of EMT, invasion and metastases. Similarly, in colon cancer, silencing of NGAL decreased cell–matrix attachment, cell motility and invasion (Hu *et al*., 2009). Contrastingly, in our study, we observed that knockdown of NGAL in oral cancer cells increased cell proliferation, survival, invasion and migration. The results were consistent with previous reports where it was observed that the presence of NGAL reduced *in vitro* invasion and migration while knockdown increased cell motility (Lin *et al*., 2016). Similar results were observed in colon cancer, where upregulation of NGAL suppresses the invasion and liver metastasis (Lee *et al*., 2006). However, the present study was the first report which showed that knockdown of NGAL induces cell proliferation and survival in OSCC.

Next we studied the mechanism involved in these different processes. We have observed that loss of NGAL induced activation of mTOR pathway and its downstream targets. mTOR, also known as FRAP (FKBP12-rapamcyin-associated protein), RAFT1

(rapamycin and FKBP12 target), RAPT 1 (rapamycin target 1), or SEP (sirolimus effector protein), is a 289 kDa serine/threonine kinase belonging to the PI3K-related protein kinase (PIKKs) family (Brown *et al*., 1994; Pópulo *et al*., 2012). mTOR comprises of two protein complexes: mTOR complex 1 and mTOR complex 2. Many factors regulate the activity of mTORC1 including growth factors, nutrients, energy status, and cellular stress (Liao *et al*., 2011; Zaytseva *et al*., 2012). Activation of mTORC1, promotes protein synthesis, proliferation, cell survival, ribosome biogenesis, angiogenesis, migration, invasion, and metastasis by phosphorylating ribosomal protein S6 kinase 1 (S6K1) (Kim *et al*., 2002; Zhang *et al*., 2005; Sarbassov *et al*., 2005; Gao *et al*., 2012). mTORC2 is involved in actin remodeling, cell-cycle progression, and cell survival via protein kinase Cα (PKCα) and glucocorticoid-induced protein kinase 1 (SGK1) (Jacinto *et al*., 2004, García-Martínez and Alessi, 2008; Gao *et al*., 2012). Aberrant activation of mTOR is seen in OSCC and was associated with poor prognosis (Hay and Sonenberg., 2004; Monteiro *et al*., 2013; Hirashima *et al*., 2010; Bakarakos *et al*., 2010; Xu *et al*., 2010). The downstream target of mTOR, S6 also serves as a potential diagnostic biomarkers for oral cancer (Chaisuparat *et al*., 2013). High levels of phosphorylated S6 is predominant in oral epithelial dysplasia and OSCC (Chaisuparat *et al*., 2013). Likewise, increased phosphorylation of S6 is a frequent event in head and neck cancer cell lines and tumour tissues (Chakraborty *et al*., 2008; Amornphimoltham *et al*., 2005; Molinolo *et al*., 2012; Gao *et al*., 2012). In laryngeal carcinoma patients treated with postoperative radiotherapy, overexpression of mTOR serves as a prognostic marker for high risk of recurrence (Lionello *et al*., 2012). A meta-analysis carried out by Marques *et al*., 2016 suggested that the mTOR pathway proteins can serve as predictive markers for head and neck cancers, as their expression was strongly associated with poor disease free and overall survival (Marques *et al*., 2015).

As mTOR signaling is known to play a key role in tumerogenesis, many inhibitors of this pathway have been proven beneficial in preclinical and clinical studies (Gao *et al*., 2012).

Liver kinase B1 (LKB1, also known as STK11), was initially identified as the casual mutation in Peutz–Jeghers Syndrome (PJS), a rare inherited autosomal dominant disorder characterized by the development of benign gastrointestinal hamartomas and the early onset of cancer (Hemminki *et al.*,1998; Momcilovic and Shackelford, 2015). Later, many studies suggested that LKB1 is a critical tumour suppressor gene, and was found to be mutated or deleted in many cancers (Zhao and Xu, 2014). LKB1 is a serine threonine kinase that directly phosphorylates and regulates the AMPK and twelve other AMPK-like kinases, and regulates key cellular processes such as growth, metabolism and autophagy (Shackelford and Shaw, 2009; Momcilovic and Shackelford, 2015). AMPK is the main substrate of LKB1 and is the central metabolic regulator in all the eukaryotes that regulates glucose and lipid metabolism and autophagy in response to nutritional deprivation or alterations in energy levels. Activation of LKB1 stimulates AMPK which phosphorylates and inactivates many metabolic enzymes associated with ATP- regulated processes such as fatty acid, cholesterol and protein synthesis, and stimulates ATP-generating processes and helps in the maintenance of cellular energy balance (Inoki *et al*., 2012; Kim *et al*., 2011; Mihaylova *et al*., 2011; Egan *et al*., 2011; Dagon *et al*., 2012; Li *et al*., 2011; Zhao and Xu., 2014). Moreover, activated AMPK may also regulate cell cycle, inhibit cell proliferation, induce autophagy, either by phosphorylating its substrates or by regulating gene expression (Marignani *et al*., 2001; Inoki *et al*., 2012; Li *et al*., 2011; Zang *et al*., 2006; Zang *et al*., 2004; Vingtdeux *et al*., 2011; Zhao and Xu, 2014). During hypoxia or energy starvation, LKB1 is activated, which in turn phosphorylates AMPK. Thus, the activated AMPK, phosphorylates

TSC2, which results in switching off the mTOR signaling (Laplante and Sabatini, 2012; Zhao and Xu, 2014).

Redd1, also known as Dig2, RTP801, and DDIT4, is a highly conserved stress response gene and is found to be upregulated during cellular stress including energy stress, ER stress, gamma radiation, and nutritional and serum deprivation (Sofer *et al*., 2005; Jin *et al*., 2009; Whitney *et al*., 2009; McGhee *et al*., 2009; Li *et al*., 2012; Dennis *et al*., 2013; Yanagawa *et al*., 2013; Reuschel *et al*., 2015). It is well established that activation of mTOR signaling inhibits autophagy, and studies also suggest that Redd1 regulates autophagy (Alvarez-Garcia *et al*., 2016). Similar mechanism was observed in our study, indicating that downregulation of NGAL regulates autophagy via Redd1. Interestingly, in our study silencing of NGAL downregulated the expression of a well-known tumour suppressor gene TP53. Studies reported a complicated regulatory loop between NGAL and p53. Recent reports suggest that, NGAL serves upstream of p53 and regulates its expression (Wang *et al*., 2011; Miyamoto *et al*., 2016). P53 is a key tumour suppressor gene that is found to be deregulated in oral cancer (Schantz, 1995). Loss of heterozygosity of the *TP53* gene has been reported in 20% of OSCCs, and 22% of premalignant oral lesions (Mehrotra and Yadav, 2006). Mutations in p53 inhibits its ability to prevent cancer and is strongly associated with the development of OSCC and its progression (Muller and Vousden, 2014; Miyamoto *et al*., 2016). Moreover, the p53 gene locus is a critical target site for carcinogens present in tobacco (Brennan *et al*., 1995; Rowley *et al*., 1998). P53 is regulated by a number stimuli such as DNA damage, stress, hypoxia, nitric oxide, deregulated growth signals etc. AMPK that is activated during energy stress also regulates p53. During metabolic stress, activated AMPK phosphorylates human MDMX on Ser342 resulting in greater association between MDMX and 14-3-3, which leads to the inhibition of p53 ubiquitylation and

subsequently activates p53 (He *et al*., 2014). Moreover, activated AMPK phosphorylates p53 at serine-15 and mediates cell cycle arrest (Okoshi *et al*., 2008). Furthermore, p53 is also known to regulate Redd1 and was reported by Ellisen *et al*., in the year 2002. Ectopic expression of p53 in cells either lacking p53 or destabilized p53, the endogenous Redd1 activity was found to be induced. Moreover, using a luciferase reporter assay, it was found that the promoter region of Redd1 also consists of p53 binding sites, confirming that Redd1 is a direct transcriptional target of p53 (Ellisen *et al*., 2002). Thus our study demonstrates that knockdown of NGAL activates mTOR pathway via LKB1-AMPK-p53-Redd1 signaling axis.

Aforementioned, activation of mTOR, induces key cellular processes involved in tumorigenesis including, protein synthesis, proliferation, cell survival, ribosome biogenesis, angiogenesis, invasion, migration and metastasis. mTOR induces cell proliferation by regulating cyclin D1 and ornithine decarboxylase. Cyclin D1, a cell cycle regulatory protein that regulates the transition of cell cycle from G1 to S phase was also found to be overexpressed in oral cancer tissues compared to normal tissues and is strongly associated with degree of differentiation of oral tumours, lymph node metastases, advancement of clinical stages and poor survival of the patients (Saawarn *et al*., 2012; Zhao *et al*., 2014). Amplification, polymorphism and mutations on the gene encoding cyclin D1 are the main reasons for its upregulation in oral cancers (Ramos-García *et al*., 2017). Aberrant activation of different pathways such as RTKs, Ras-Raf-MEK-ERK, or PI3K, Wnt or NF-κB also result in the activation of cyclin D1 in oral cancers (Tetsu and McCormick, 1999; Guttridge et al, 1999; Caicedo-Granados *et al*, 2012; González-moles *et al*, 2014; Ramos-García *et al*., 2017). Similarly, accumulating evidences suggest that upregulation of mTOR promotes cell survival by activation of anti-apoptotic genes (Advani, 2010). Bcl-2 is a key regulator of apoptosis

and upregulation of Bcl-2 is known to inhibit apoptosis. Studies reported that Bcl-2 was overexpressed in oral precancerous lesions and oral cancer tissues and strongly associated with differentiation of tumours and leads to poor survival (Teni *et al*., 2002; Jordan *et al*., 1996; Sudha and Hemavathy, 2011; Juneja *et al*., 2015; Arya *et al*., 2016; Popović *et al*., 2007). Altered expression of Bcl-2 is one of the early molecular event which provides survival advantage, increases the accumulation of genetic alterations, and results in the malignant transformation of oral cancer (Juneja *et al*., 2015). Similarly, caspase-3 and caspase-9 were downregulated in NGAL silenced cells. Moreover, these caspases were also found to be directly regulated by p53. Two groups of caspases, initiators (e.g. Casp-2, -8, or -9) and effectors (e.g. Casp-3, 6, or 7), are involved in intact caspase cascades, and it appears that each initiator caspase is activated in response to a subset of signals (Cohen, 1997). The expression of caspase-3 was found to be downregulated or completely absent in dysplastic epithelium and majority of OSCC (Veeravarmal *et al*., 2016). Likewise, the expression of caspse-9 is downregulated in oral cancer and is activated in response to apoptotic stimuli. mTOR signaling axis, also regulates metastases and many of the inhibitors of this pathway such as rapamycin, metformin etc. are known to inhibit MMP-2/-9. MMP's, the gelatinases play an important role in tumour invasion and angiogenesis and are associated with the aggressiveness of many cancers by degrading the type IV collagen (Chen *et al*., 2007; Giannelli *et al*., 1997). Serum and protein levels of MMP-9 was found to be upregulated in oral cancer patients compared to healthy controls and a positive correlation was found with lymph node metastases (Lotfi *et al*., 2015; Ravi *et al*., 2012 ; Patel *et al*., 2005; Vilen *et al*., 2013). Overexpression of MMP-9 was detected in 85% of oral dysplasia and all oral cancer tissues (Tamamura *et al*., 2013; Jordan *et al*., 2004; Ogbureke *et al*., 2012).

Apart from the increase in protein synthesis and regulation of cellular processes, activation of mTOR also inhibits autophagy. Autophagy is a catabolic degradation process during starvation or in response to stress, where the proteins, organelles and cytoplasm are engulfed, digested and recycled to maintain homeostasis (Mathew *et al*., 2007). The protective role of autophagy in tumorigenesis was seen in prostate, breast, and ovarian cancers, where the autophagy gene ATG6/BECN1 was monoallelically lost. Reports suggest that *in vitro* suppression of autophagy in certain cells induce cancer growth and beclin-1 heterozygous mutant mice likely to develop liver, lung tumors and lymphomas (Qu *et al*., 2003; Yue *et al*., 2003). LC3 also known as microtubule-associated protein 1A/1B-light chain 3 is a well-accepted marker for autophagy. In hypopharyngeal squamous cell carcinoma decreased expression of LC3 and beclin-1 was associated with poor prognosis (Wang *et al*., 2013). Activation of autophagy by many chemotherapeutic agents in HNSCC induced apoptosis and downregulated mTOR pathway. Many small molecule tyrosine kinase inhibitors such as gefitinib, erlotinib, dasatinib etc. induced autophagy and suppressed mTOR signaling, indicating that increase in autophagy suppresses tumour growth *in vitro* and *in vivo* (Rikiishi, 2012). These studies indicate that, autophagy serves as a tumour suppressor. Thus conclusively our study suggests that knockdown of NGAL induces oral cancer cell proliferation, survival, invasion and migration by activating mTOR signaling via LKB1-AMPK-p53-Redd1 and reduced autophagy. Moreover, knockdown of NGAL also induced resistance against chemotherapeutic drug cisplatin. Upregulation of cyclin D1 also induces resistance in oral cancer, against the chemotherapeutic drug cisplatin, while knockdown of cyclin D1 reversed the resistance (Zhou *et al*, 2009; Ramos-García *et al*., 2017). From the *in vitro* studies we can conclude that loss of NGAL is one of the factor that increases the aggressiveness of oral

cancer. Therefore, agents that can upregulate the expression of NGAL would be helpful in developing efficacious therapies. We observed that curcumin, a well-known anticancer agent increased the expression of NGAL and was also found to decrease the proliferation of SAS cells in a dose and time dependent manner.

Overall, from the present study, we can conclude that NGAL is downregulated in oral cancer tissues and is strongly associated with degree of differentiation, stage of the tumours and lymph node metastases. The tobacco components mainly nicotine, NNK, NNN, and the synthetic carcinogen 4-NQO were involved in the downregulation of NGAL. Mechanistic studies revealed that, knockdown of NGAL increased cell survival, proliferation, invasion and migration by activating mTOR pathway and reduced autophagy via LKB1-AMPK-p53-Redd1 signalling axis. Therefore, agents that can restore the expression of NGAL would be beneficial in developing effective therapies against this dreadful disease. We found that the curcumin, a natural multi-targeted agent was found to upregulate NGAL and induce apoptosis in oral cancer cells. This suggests the NGAL is one of the key molecule involved in oral cancer tumorigenesis.

5.1. Limitations of the study:

Although we have established the expression and role of NGAL in oral cancer, few limitations exists that needs to be studied further. The expression of NGAL in oral cancer tissues was studied using TMA that does not contain tissues from Indian population. Establishing the expression of NGAL in oral cancer tissues from Indian population from different stages, grades, types etc. warrants further investigation. Moreover, our study lacks the expression of NGAL in different stages and grades of tumours from different regions of oral cavity such as lip, cheeks, palate, gingiva etc. and also in metastases to different regions like bone and lung. This would help in

understanding the expression pattern of NGAL meticulously. Next, we reported that tobacco carcinogens, nicotine, NNK, NNN, and 4-NQO, which are well known causative agents of oral cancer, altered the expression of NGAL, however underlying mechanism should be further explored. Besides tobacco, the involvement of NGAL in HPV linked oral cancers has to be established. Subsequently, we silenced the expression of NGAL and detailed the role NGAL in oral cancer tumorigenesis *in vitro* but, whether silencing of NGAL induces oral cancer *in vivo* was not evinced. Mechanistic studies revealed the downstream targets of NGAL, nevertheless the upstream mediators responsible for the downregulation of NGAL in oral cancer remained unascertained. The study was narrowed to understand the potential of inducing resistance against the first line therapeutic agents for oral cancer, which further needs to be validated with other agents such as docetaxel, cetuximab etc. Furthermore, global gene and protein expression profiling of NGAL knockdown cells by microarray would provide us valuable insights in the expression pattern and intricate network of interactions among the genes/proteins involved in the pathogenesis of oral cancer. As most of the above mentioned studies focused on silencing of expression of NGAL, it is noteworthy to study whether restoring or overexpression of NGAL would result in beneficial outcomes. These critical unresolved concerns are the priorities for future research.

5.2. Future prospective:

Future research focusing on the above mentioned issues would be beneficial in understanding the comprehensive role of NGAL in oral cancer. Deciphering the expression pattern of NGAL in large cohorts would establish the prognostic significance of NGAL in oral cancer. Furthermore, studying the expression profile in preneoplastic lesions like leukoplakia, erythroplakia, OMSF etc. would enable the

clinicians to differentiate the high risk and low risk of patients developing oral cancer. Currently, it is well-known that NGAL is downregulated in oral cancer, the next critical step is to determine whether overexpression of NGAL might play a protective role in oral cancer. If the protective role of NGAL is established, then ascertaining agents that can upregulate the expression of NGAL would be fruitful in developing potent therapeutics against this devastating disease.

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List of Abbreviations

µg	: Microgram
µM	: Micromolar
4-NQO	: 4-nitroquinoline-1-oxide
ABCB1	: ATP binding cassette subfamily B member 1
ABCC1	: ATP binding cassette subfamily C member 1
ABCG2	: ATP-binding cassette sub-family G member 2
ACC	: Adenoid cystic carcinoma
AML	: Acute myeloid leukemia
AMP	: Adenosine monophosphate
AMPK	: AMP-activated protein kinase
ANOVA	: Analysis of variance
Apaf-1	: Apoptotic protease activating factor 1
APC	: Adenomatous polyposis coli
ATG6	: Autophagy-specific gene 6
ATP	: Adenosine triphosphate
ATP7B	: Copper-transporting P-type adenosine triphosphate
BARD1	: BRCA1 associated RING domain 1
BCC	: Basal cell carcinoma
Bcl-1	: B-cell CLL/Lymphoma 1
Bcl-2	: B-cell lymphoma 2
Bcl-xL	: B-cell lymphoma-extra large
BCR-ABL	: Breakpoint cluster region abelson murine leukemia viral oncogene
BCRP	: Breast cancer resistance protein
BECN1	: Beclin 1
BIM	: Bcl-2-like protein 11
CAIX	: Carbonic anhydrase IX
CASP-10	: Caspase-10
CASP-2	: Caspase-2
CASP-8	: Caspase-8
CEBPβ	: CCAAT-enhancer binding protein β
CML	: Chronic myeloid leukemia
COX-2	: Cyclooxygenase-2
CT	: Computed tomography
CXCR-4	: C-X-C chemokine receptor type 4
CYP	: Cytochrome
DAB	: 3,3'-diaminobenzidine
DDIT4	: DNA-damage-inducible transcript 4
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DOC-1	: Deleted in oral cancer 1
DTT	: Dithiothreitol
EBRT	: External beam radiotherapy
EBV	: Epstein – Barr virus
ECL	: Enhanced chemiluminescence
EDTA	: Ethylenediaminetetraacetic acid
EGF	: Epidermal growth factor
EGFR	: Epidermal growth factor receptor
EMT	: Epithelial–mesenchymal transition

EpCAM : Epithelial adhesion molecule
ER stress : Endoplasmic reticulum stress
ER : Estrogen-receptor
ERK : Extracellular signal-regulated kinase
ER-α : Estrogen receptor alpha
ESCC : Esophageal squamous cell carcinoma
EtOH : Ethanol
FAK : Focal adhesion kinase
FGF-2 : Fibroblast growth factor
FOS : FBJ murine osteosarcoma
FOXP3 : Forkhead box P3
FRAP : FKBP12-rapamcyin-associated protein
GAPDH : Glyceraldehyde-3-phosphate dehydrogenase
GATA3 : GATA-binding protein 3
GVHD : Graft-versus-host disease
H_2O : Water
HCC : Hepatocellular carcinoma
hCG2 : Human cyclin G2
HCl : Hydrochloric acid
HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HER-2 : Human epidermal growth factor receptor 2
HGF : Hepatocyte growth factor
HGFR : Hepatocyte growth factor receptor
HIF-1α : Hypoxia-inducible factor alpha
HNSCC : Head and neck squamous cell carcinoma
HO-1 : Heme oxygenase-1
HPV : Human papillomavirus
HSP70 : Heat shock protein 70
HST-1 : Hybrid sterility 1
HSV : Herpes simplex viruses
IHC : Immunohistochemistry
ILKAP : ILK associated serine/threonine phosphatase
ING5 : Inhibitor of growth family member 5
JNK : c-Jun NH2-terminal kinase
JUN : Jun proto-oncogene
K-RAS : Kirsten rat sarcoma virus
LC3 : Microtubule-associated protein 1A/1B-light chain 3
LCN2 : Lipocalin-2
LKB1 : Liver kinase B1
LRP1B : LDL receptor related protein 1b
MDMX : Murine double minute X
MDR1 : Multidrug resistance protein 1
MEC : Mucoepidermoid carcinoma
MEK : Mitogen-activated protein kinase
miR : microRNA
ml : Milliliter
mM : Millimolar
Mm : Millimeter
MMP : Matrix metalloproteinase
MMP-2 : Matrix metalloproteinase-2

MMP-9 : Matrix metalloproteinase-9
MPO : Myeloperoxidase
MR : Magnetic resonance
MRI : Magnetic resonance imaging
mRNA : Messenger RNA
MSCC : Metastatic squamous cell carcinoma
mTOR : Mammalian target of rapamycin
MTT : 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide
MUC4 : Membrane mucin 4
MVD : Microvessel density
MXR : Multixenobiotic resistance protein
nAChR : Nicotinic acetylcholine receptors
NaCl : Sodium chloride
NADH : Nicotinamide adenine dinucleotide reduced
NADPH : Nicotinamide adenine dinucleotide phosphate reduced
NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL : Neutrophil gelatinase associated lipocalin
NGALR : Neutrophil gelatinase-associated lipocalin receptor
nM : Nanomolar
NMR : Nuclear magnetic resonance spectroscopy
NNK : 4 [methylnitrosoamino]-1-[3- pyridyl]-1-butanone
NNN : N-nitrosonornicotine
NNO : Nicotine-*N*-oxide
NOS2 : Nitric oxide synthase 2
NP-40 : 4-Nonylphenyl poly (ethylene glycol)
Nrf2 : Nuclear factor (erythroid-derived-2)-like 2
NSCLC : Non-small-cell lung carcinoma
OLP : Oral lichen planus
OSCC : Oral squamous cell carcinoma
OSMF : Oral submucous fibrosis
PAH : Polycyclic aromatic hydrocarbons
PCNA : Proliferating cell nuclear antigen
PDAC : Pancreatic ductal adenocarcinoma
PD-ECGF : Platelet-derived endothelial cell growth factor
PDGF : Platelet-derived growth factor
PDGFR : Platelet-derived growth factor receptor
PET : Positron emission tomography
PGF : Placental growth factor
P-gp : P-glycopretein
pH : Potential of hydrogen
PI3K : Phosphoinositide 3-kinase
PJS : Peutz–Jeghers syndrome
PKCα : Protein kinase Cα
PMSF : Phenylmethylsulfonyl fluoride
POB : Pyridyloxobutylation
PPP1R7 : Protein phosphatase 1 regulatory subunit 7
pR6 : Pathogenesis-related protein 6
PRAD-1 : Parathyroid neoplasia gene 1
Raf : Rapidly accelerated fibrosarcoma
RAFT1 : Rapamycin and FKBP12 target

RAPT 1	: Rapamycin target 1
Rb	: Retinoblastoma
Redd1	: Regulated in development and DNA damage responses -1
RTK	: Receptor tyrosine kinase
S6	: Ribosomal protein S6
S6K1	: Ribosomal protein S6 kinase beta-1
SCC	: Squamous cell carcinomas
SCLC	: Small cell lung cancer
SDS	: Sodium dodecyl sulfate
SEP	: Sirolimus effector protein
SGK1	: Serum glucocorticoid-induced protein kinase 1
shRNA	: Short hairpin RNA

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PRESENTATION AND PUBLICATION LIST

1. **Monisha J,** Roy NK, Padmavathi G, Banik K, Bordoloi D, Khwairakpam AD, Arfuso F, Chinnathambi A, Alahmadi TA, Alharbi SA, Sethi G, Kumar AP, Kunnumakkara AB. NGAL is Downregulated in Oral Squamous Cell Carcinoma and Leads to Increased Survival, Proliferation, Migration and Chemoresistance. Cancers (Basel). 2018; 10;10(7).
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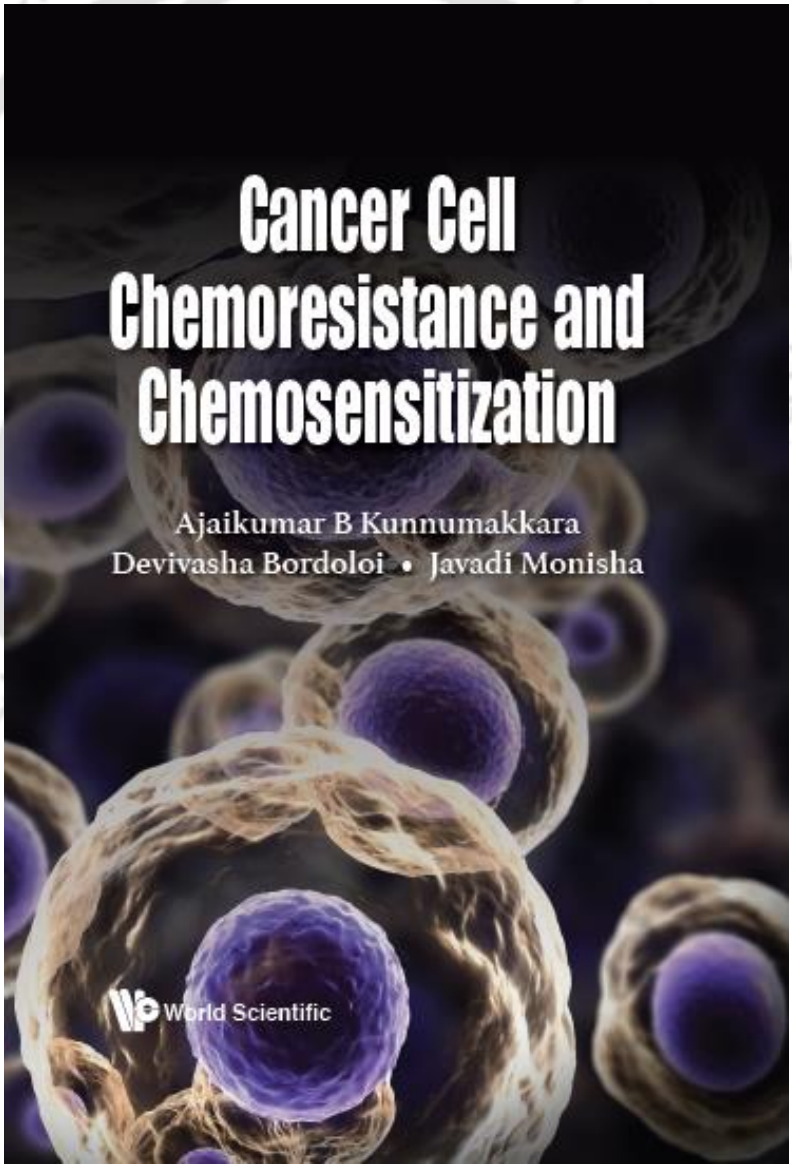

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1. **Javadi Monisha**, Sajin Fransis K, Nand Kishor Roy, Ganesan Padmavathi, Mangalam S. Nair, Ajaikumar B. Kunnumakkara, Chemosensitization potential of Azadiradione, isolated from *Azadiractha indica* against oral cancer, in 2nd International Conference on Nutraceuticals and Chronic Diseases (2nd INCD-2017), 2017 at Goa, India.
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1. **Javadi Monisha**, Nand Kishor Roy, Khwairakpam Amrita Devi, Ganesan Padmavathi and Ajaikumar B. Kunnumakkara, Role of NGAL in the development of human oral squamous cell carcinoma, in Translational Cancer Research-2018 at Chennai, India
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33. Nand Kishor Roy, Anusmita Das, Sudeshna Gupta, **Javadi Monisha**, Ganesan Padmavathi, Elancheran Ramakrishnan, Jibon Kotoky and Ajaikumar B. Kunnumakkara, Biosynthesis of Gold Nanoparticle and their Characterization, Research Conclave-2015 organized by the PhD Council of the Students' Academic Board (SAB), Indian Institute of Technology, Guwahati ,India,2015.
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Conferences, workshops and trainings attended:

1. Participated in the "**2nd International Conference on Nutraceuticals and Chronic Diseases (2nd INCD-2017)**", organized by Society for Nutraceuticals and Chronic Diseases held on 1st, 2nd and 3rd September at Goa, India, 2017.
2. Participated in Indo-japan symposium on "**Hope from Herbs: research based Care and cure Potentials**" jointly organized by IIT Guwahati and AIST, Japan, held on 8th May, 2017.
3. Participated in the "**1 International Conference on Nutraceuticals and Chronic Diseases (2nd INCD-2017)**", organized by Society for Nutraceuticals and Chronic Diseases at held on 9th, 10th and 11th September at Cochin, India, 2016.
4. Participated in the National conference on **'Recent Developments in Medical Biotechnology and Structure Based Drug Designing'** organized by Department of Biosciences and Bioengineering, IIT Guwahati, India, held on 6 th & 7 th December, 2015.

5. Participated in the National Conference on **'Recent Advances in Cancer Biology and Therapeutics'** organized by Department of Biotechnology, IIT Guwahati, India, held on 5 th December, 2014.
6. Participated in the National Conference on **'Advances in Cancer Genomics'** organized jointly by Mizoram State Cancer Institute, Aizwal and Department of Biotechnology, Mizoram University held from 30-31 May, 2014.
7. Participated in a 5 day national course on '**Theoretical and Practical aspects of Cancer Research'** conducted under the Technical Education Quality Improvement Programme sponsored by the Ministry of Human Resource Development, Govt. of India, from February 4 th – 8 th , 2015.
8. Participated in a two day national workshop on **'Flow Cytometry Data Analysis'** organized by Department of Biotechnology, Indian Institute of Technology Guwahati from 23 rd – 24 th January, 2015.
9. Participated in a four day national workshop on **'Next Generation Sequencing and Data Analysis'** organized by Biotech Hub, Centre for the Environment, Indian Institute of Technology Guwahati held during May 14-17, 2014.
10. Hands on training on flowcytometry **"Indo-US Clinical Cytometry Symposium and Wet labs on Haematological Malignancies: Challenges & Management Strategies"** conducted by Cachar Cancer Hospital, February 8th-9th, 2014.

Awards:

1. Received '**Best Oral Presentation Award'** for the paper entitled "Chemosensitization potential of Azadiradione, isolated from *Azadiractha indica* against oral cancer" in Second International Conference on Nutraceuticals and Chronic Diseases, at Goa, India 2017.
2. Received '**Institute Best Poster Presentation Award**' for the paper entitled "Downregulation of NGAL and Its Role in Head and Neck Squamous Cell Carcinoma" in Research Conclave 2017, IITG, Guwahati, India 2017.
3. Received '**Departmental Best Poster Presentation Award'** for the paper entitled "Downregulation of NGAL and Its Role in Head and Neck Squamous Cell Carcinoma" at Research Conclave 2017, IITG, Guwahati, India 2017.
4. Received '**Best Oral Presentation Award'** for the paper entitled "Insights into anticancer activity and mechanism of action of azadiradione against triple negative breast cancer" in First International Conference on Nutraceuticals and Chronic Diseases, at Kerala, India 2016.

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Neutrophil Gelatinase-Associated Lipocalin (NGAL): A Promising Biomarker for Cancer Diagnosis and A Potential Target for Cancer Therapeutics

Review Article

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Abstract

NGAL, also known as lipocalin-2, is a secretory glycoprotein encoded by *LCN2* gene and found to be expressed majorly in kidney diseases. In recent years, NGAL is procuring enormous attention as a cancer biomarker with its differential expression pattern. In breast cancer, NGAL binds with MMP9 and promotes invasion. High expression of NGAL is strongly involved in the progression of colorectal cancer, esophageal cancer, chronic myeloid leukemia, hepatocellular carcinoma, gastric and endometrial cancers. However, in other cancers such as pancreatic cancer and oral cancer, downregulation of NGAL leads to disease progression. Interestingly, a cell surface receptor for NGAL, NGALR was identified and the co-expression of both NGAL and NGALR has been implicated in different cancers. Therefore, understanding the function of NGAL in the development of cancer would provide us new insights to cancer development. This would also help us to develop biomarkers for cancer diagnosis and prognosis. This review article discusses the role of NGAL and NGALR in malignant diseases.

Keywords: NGAL; NGALR; LCN2; Cancer; Biomarker; Invasion; Metastasis

Abbreviations

CML: Chronic Myeloid Leukemia; C/EBP: CCAAT-Enhancer-Binding Proteins; EGFR: Epidermal Growth Factor Receptor; ESCC: Esophageal Squamous Cell Carcinoma; iAS: Inorganic Arsenic; MMP9: Matrix Metalloproteinase 9; NF-κB: Nuclear Factor Kappa B; NGAL: Neutrophil Gelatinase- associated Lipocalin; NGALR: Receptor NGAL; PaCa: Pancreatic Cancer; pNGAL: Plasma NGAL; STAT3- Signal Transducer and activator of Transcription 3; SV40: Simian Virus 40; TPA: 12-O tetradecanoylphorbol-13-acetate; TRE: TPA Responsive Element.

Introduction

The lipocalin protein family is a huge group of small secreted glycoproteins with functional diversity. Various functions of these proteins include retinol transport, cryptic coloration, olfaction, pheromone transport, enzymic synthesis of prostaglandins, regulation of the immune response and mediation of cell homoeostasis [1]. Members of this family share a common secondary and tertiary structure known as "the lipocalin fold". The lipocalin fold is a barrel like structure, consisting of eight antiparallel beta sheets linked with hydrogen bonds and helps enormously in ligand binding. The connection of one beta sheet to another is facilitated by seven short loops [2]. Literature reveals that few members of this protein family exhibit association with cancer. In this review, we mainly focus on NGAL (Neutrophil gelatinase-associated lipocalin), an important member of lipocalin protein family and its role in cancer.

NGAL, also known as lipocalin-2 (*LCN2*), is a 24 kDa secreted glycoprotein in humans encoded by *LCN2* gene located on

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chromosome locus 9q34.11. It was first purified from a culture of mouse kidney cells infected with simian virus 40 (SV40) and subsequently from supernatant of human neutrophils [3]. NGAL is a small molecule which binds and aids in the transport of hydrophobic ligands. It plays a significant role in generating innate immune response and safeguards against bacterial infections by sequestering iron [4]. In addition to ligand binding, it is also involved in protein-protein interactions and hence, sometimes appears to be complexed with matrix metalloproteinase-9 (MMP9). Depending on the free state or bound state of this glycoprotein, it is termed as "apo" or "holo" NGAL respectively. It is expressed in neutrophils, kidney, prostate, epithelia of the respiratory and alimentary tracts and is majorly used as a biomarker in kidney injury [5,6]. The putative mouse and rat homologues of NGAL are 24p3 and neu-related lipocalin (NRL) respectively. Mouse 24p3 is synthesized in the liver and exerts elevated expression in response to inflammation [7]. A comparison between amino acid sequence of NGAL homologues in different species revealed that human NGAL is highly similar to chimpanzees (98% identity) than mouse and rat (62% and 63% identity respectively) [2].

NGAL is also expressed in several adult normal tissues like breast ducts, liver, lungs, trachea, small intestine, bone marrow, thymus, adipose tissue and macrophages. Negligible expression of NGAL is observed in the normal pancreas, endometrial glands and peripheral blood leukocytes. However, NGAL is completely absent in brain, heart, skeletal muscle, spleen, testes, colon and ovary [2].

NGALR- Receptor NGAL

NGALR is highly conserved in human and is predicted to have twelve transmembrane helices [8,9]. A murine NGAL receptor known as 24p3R was first isolated from FL5.12 cells. This receptor binds to both apo and holo NGAL and enables their internalization. Although, 24p3R internalizes both apo and holo NGAL by endocytosis, interestingly each results in totally different biological processes. The internalization of holo-NGAL by 24p3R receptor donates iron to the cells and thus prevents apoptosis successfully, whereas in the case of apo-NGAL it creates an iron efflux inducing apoptosis [8,9].

Role of NGAL in malignancy

Overexpression of NGAL was first identified in SV40 tumour virus infected quiescent mouse primary kidney cells [10]. It is also referred as protumorigenic protein and, induces tumor progression and chemoresistance in cancer cells. Recently, it has emerged as a biomarker for several benign and malignant diseases. However, it showed differential expression pattern in different cancers (Figure 1). COSMIC database has reported few mutations in *LCN2* gene which encodes this protein [13] (Figure 2).

NGAL in breast cancer

Breast cancer represents the most prevalent type of malignancy in women and led to 14% of the total cancer deaths in 2008 [14]. Despite the significant advances made by constant investigation in the development of biomarkers for the diagnosis of breast cancer, the severity of the disease remains the same. Recently, NGAL was detected in the urine sample of breast cancer patients and has been identified as a biomarker for this cancer [15,16]. In the urine of breast cancer patients, NGAL was found to be complexed with MMP-9, protecting it from degradation and thereby preserving its enzymatic activity [17,18]. This urinary detection of MMP-9/NGAL might open new avenue for non-invasive prediction of disease status in

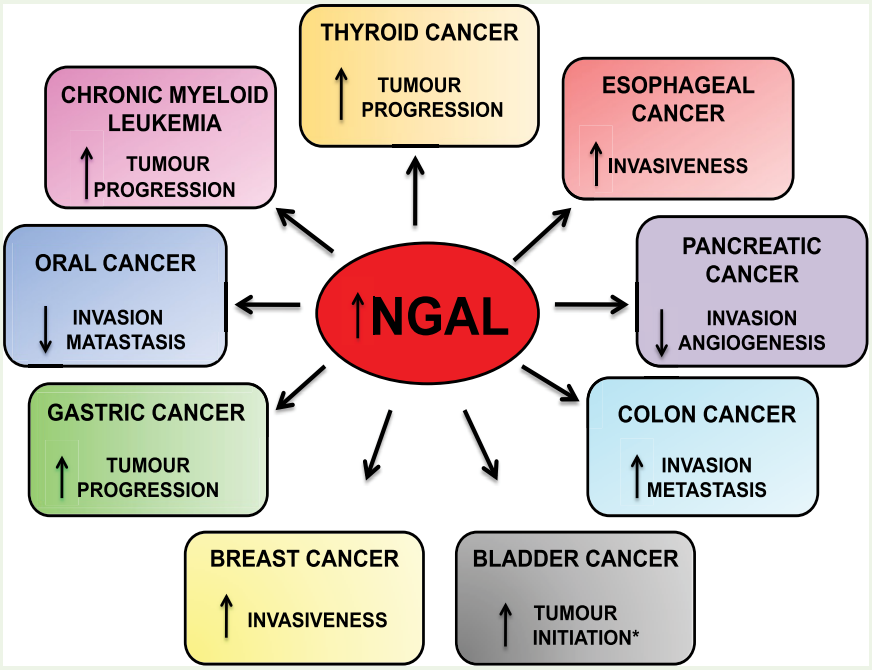

Figure 1: Differential expression and role of NGAL in various cancers; ↑ Upregulation, ↓ Downregulation
* in tumor relapse patients reduced levels of NGAL leads to tumor progression

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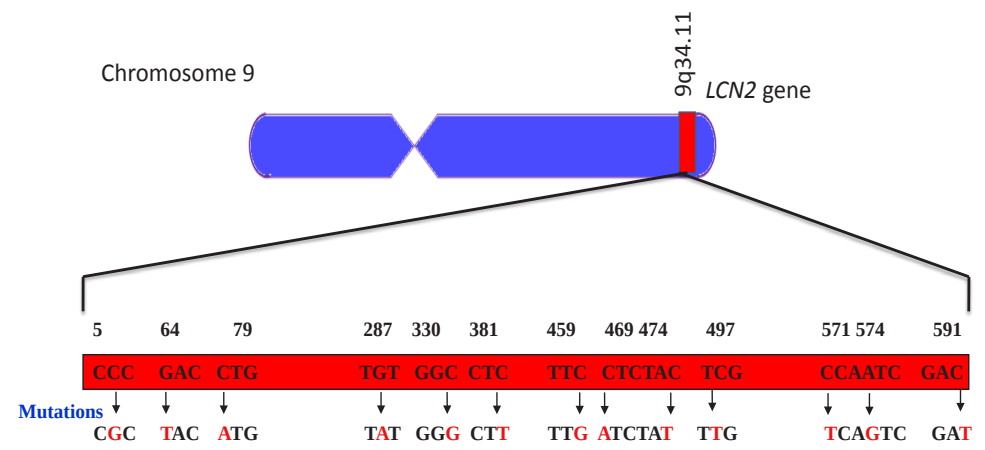

Figure 2: Mutations detected in the gene *LCN2*. Data based on cosmic database [13].

breast cancer patients [18]. MMP9/NGAL complex in the urine of breast cancer patients showed no relationship with the tumor size, TNM stage, patient's age, menopause status and ER status, but it showed a strong correlation with lymph node metastasis and can be used as a prognostic factor in early screening of disease status [19]. Provatopoulou et al., 2009 [16] reported the presence of NGAL and MMP9 in the serum of breast cancer patients and concluded that it can be used in non-invasive monitoring of breast cancer progression, supporting its role as a biomarker in breast cancer. In a study, MMTV-ErbB2 (V664E) mice lacking *LCN2* gene showed notable delay in mammary tumor formation, metastasis and reduced MMP9 activity in blood [20]. Moreover, NGAL expression was enhanced by HER2/phosphoinositide 3-kinase/AKT/NF-κB pathways [20,21]. Reporter assays of full-length or deletion constructs of the NGAL promoter suggested that the production of NGAL is STAT3 and C/EBPβ dependent [22]. The dependency of NGAL on C/EBPβ was confirmed as the overexpression of C/EBPβ downregulated NGAL and inhibited the migration and invasion of breast cancer [23]. These studies indicate that NGAL plays a crucial role as a biomarker in breast cancer and also imply its significance in increasing the invasiveness of this disease.

NGAL in colorectal cancer

Colorectal cancer is the third most common cancer in the world. It is equally prevalent in both men and women and accounts for 9% of total cancer incidences [24]. There are many proteins that are responsible for driving the progression of this disease to the metastatic stage. NGAL is one such protein that is overexpressed and led to the poor prognosis of colorectal cancer patients [25,26]. High expression of NGAL was observed in both colorectal carcinoma cell lines and xenograft mouse models of this disease [26,27]. Plasma NGAL (pNGAL) levels were remarkably higher in colorectal cancer patients when compared to healthy individuals. This elevated pNGAL was associated with higher tumor volume, characteristics of invasion and recurrence, evidencing its prognostic utility in metastatic colorectal cancer patients [28]. In colon carcinoma cell lines, overexpression of NGAL led to subcellular localization of E-cadherin and catenins, decreased E-cadherin mediated cell-cell adhesion, enhanced cell-matrix attachment, increased cell motility and *in vitro* invasion while downregulation of NGAL entailed reduced *in vitro* invasion [26]. Contrastingly, KM12SM- a highly metastatic human colon cancer cell line showed decreased expression of NGAL while ectopically expressing, it suppressed *in vitro* invasion and repressed liver metastasis in BALB/c nude mice [29]. In infectious colitis, downregulation of PPARγ led to the accumulation of lipocalin-2, hence stabilizing MMP9 causing stern colitis. Moreover, mice lacking *LCN2* gene stayed protected even at the later stages of infection [30].

However, NGAL cannot be used as a biomarker for determining the disease progression in colorectal cancer as the marked difference in its expression was found only in its early stages [31].

NGAL in esophageal squamous cell carcinoma

Esophageal squamous cell carcinoma is the seventh most common cancer in the world. Upregulation of NGAL led to the activation of differentiation pathway and invasive progression of esophageal squamous cell carcinoma [32]. Zhang et al., 2007 [32] immunohistochemically stained the tissues of different stages of ESCC and reported a weak positive signal in normal esophageal epithelium and altered expression in dysplasia. However, in esophageal squamous cell carcinoma, NGAL was complexed with MMP9 and the enzymic activity of this complex was much elevated in ESCC than in normal mucosa and significantly correlated with tumor invasion [33]. High expression of NGAL has been observed to be responsible for malignant transformation of human immortalized esophageal epithelial cell line SHEE to esophageal carcinoma cell line SHEEC [33-35]. Blocking the expression of NGAL in SHEEC cell lines suppressed invasion in nude mice [34].

NGALR is also overexpressed in esophageal squamous cell carcinoma [35,36]. Cui et al., 2008 [36] reported a high level of NGALR expression on tumor cell membrane and in the cytoplasm. NGALR hypomethylation contributed to its overexpression in esophageal squamous cell carcinoma [36]. This overexpression of both NGAL and NGALR led to the poor survival of esophageal squamous cell carcinoma patients [37].

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NGAL in pancreatic cancer

Pancreatic cancer is one of the main leading causes of death and has the lowest survival rate [38]. The low survival rates are due to difficulty in diagnosis at its early stage as the patients show non-specific symptoms. This necessitates immediate identification of biomarkers for proper diagnosis of this disease at initial stage. Various reports suggested that NGAL can successfully be used as a biomarker in pancreatic cancer [39,40]. Moniaux et al., 2008 [39] reported that NGAL was upregulated 27 fold in pancreatic cancer cells as compared to normal ductal cells. Differential expression of NGAL in different stages of pancreatic cancer was examined by immunostaining. The results showed that NGAL was strongly upregulated in pancreatic cancer and moderately in pancreatitis, but relatively weak expression was detected in healthy pancreas. However, its expression was observed only in well and moderately differentiated pancreatic adenocarcinoma but not in poorly differentiated carcinomas. Serum NGAL level was found to be high in both pancreatic cancer and acute and chronic pancreatitis patients [39]. In an *in vitro* study, remarkably high level of expression of NGAL was observed in well to moderately differentiated PaCa cells (AsPC-1, BxPC-3, and Capan-2), whereas in the case of moderate to poorly differentiated PaCa cells (PANC-1 and MIAPaCa-2) expression level of NGAL was nearly undetectable. Overexpression of NGAL reduced focal adhesion kinase (FAK) tyrosine-397 phosphorylation without effecting PaCa cells' survival, viability and their response to chemotherapeutic drugs *in vitro*. It also suppressed metastasis, reduced tumor volume and angiogenesis *in vivo*. These results indicated NGAL to be a potential suppressor of invasion and angiogenesis in advanced pancreatic cancer [41]. Later the same group [42] showed that EGF efficiently blocked NGAL expression through activation of the EGFR signaling pathway, which in turn downregulated E-cadherin along with subsequent reduction in NF-κB activation. It has also been reported that NGAL is responsible for gemcitabine resistance in pancreatic ductal adenocarcinoma [43].

NGAL in thyroid carcinoma

Thyroid cancer is a rare type of cancer originating from follicular or parafollicular thyroid cells. It is much more common in women than in men. Barresi et al., 2012 [44] described NGAL to be a potent marker in the identification of malignant follicular cell derived thyroid tumors. NGAL is highly expressed in the human thyroid carcinoma FRO cell line and other poorly differentiated thyroid cancer cell lines [45]. Iannetti et al., 2008 [45] reported that NF-κB helps in thyroid tumor cell survival by controlling iron uptake via NGAL.

NGAL in chronic myeloid leukemia (CML)

Chronic myeloid leukemia (CML), also known as chronic granulocytic leukemia is a cancer of the white blood cells. Fusion protein BCR/ABL formed due to reciprocal translocation of chromosomes 9 and 22 plays a key role in the development of CML [46]. Levels of NGAL expression is correlated with BCR/ABL induced tumorigenesis. Leng et al., 2008 [47] studied the role of 24p3 (Mouse NGAL) and BCR/ABL in leukemia and reported that the mice receiving both BCR/ABL and 24p3 developed leukemia. In addition, primary stable cell line from wild-type mouse marrow cells expressing BCR/ABL caused solid tumors in nude mice whereas a similar BCR/ABL$^+$ cell line from 24p3 null mice did not develop any tumor. Plasma levels of NGAL in both leukemic mice and CML patients were high [47]. Similarly, blood and serum of different stages of CML patients showed elevated NGAL mRNA and protein expression [48].

It was also evidenced that BCR/ABL oncoprotein upregulated apo-NGAL and at the same time suppressed the receptor NGAL (NGALR), which resulted in cells unresponsiveness to secreted apo-NGAL. Therefore, treating the cells with BCR/ABL inhibitor- imatinib upregulated NGALR resulting in apo-NGAL mediated apoptosis [8].

NGAL in oral cancer

Oral cancer is the sixth most common cancer in the world. The 5 year survival rate of this cancer is only 50%. The death rate due to this cancer has been increasing tremendously over past 30 years. So there is an urgent need for the development of novel biomarkers for early diagnosis of this fatal disease. NGAL holds enormous prospects to serve as a biomarker with an expression profile associated with the degree of differentiation of tumors. Interestingly, it was found that the expression of NGAL decreases from well differentiated to moderately differentiated and completely lost in poorly differentiated tumors [49]. Similar results have also been observed in oral cancer cell lines of different grades of invasiveness. Expression of NGAL was found to be high in weakly invasive (SCCKN, HSC-2 and OSC-20), weak in mildly invasive (HSC-3, OSC-19, and SCC-25) and negative in highly invasive (HOC-313 and TSU) oral squamous cell carcinoma cell lines. Moreover, the expression of NGAL was found to be inversely correlated with E-cadherin and MMP-9 [49].

NGAL in other common cancers

In addition to the above mentioned cancers, expression of NGAL was studied in other commonly occurring cancers like kidney tumors, hepatocellular carcinoma, lung cancer, endometrial cancer and gastric cancer. In hepatocellular carcinoma, overexpression of NGAL and/ or NGALR resulted in poor prognosis and poor survival. The overexpression of NGALR can serve as an independent prognostic factor for this disease [50]. In the case of non-small cell lung cancer, NGAL was responsible for the development of erlotinib resistance [51]. In A549 lung cancer cell line, NGAL served as a survival factor by reducing PDK1 inhibitor induced cell death [52]. The expression of NGAL was also increased with the progression of endometrial cancer [53]. In bladder cancer, high expression of NGAL was detected in the urine of patients. Elevated mRNA transcript and protein levels were found in bladder cancer tissues compared to normal tissues [54,55]. In inorganic arsenic (iAs) treated urothelial cells and bladder cancer tissues expression of LCN2 was enhanced by promoter hypomethylation and mutations at the binding sites for NF-κB and c/eBP-α remarkably decreased LCN2 promoter activity [56]. Moreover, overexpression of LCN2 in inorganic arsenic treated urothelial cells enhanced their oncogenic potential. Thus, NGAL offers beneficial prospect to be used as an early diagnostic marker in bladder cancer. High expression of NGAL was observed in advanced gastric cancer patients resulting in poor prognosis [57]. Du et al., 2011 [58] studied the mechanism of expression of NGAL in gastric cancers before and after 12-O-tetradecanoylphorbol-13-acetate (TPA) induction. Overexpression of NGAL upon induction with TPA evidenced the

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binding of C/EBPβ to the TPA responsive element (TRE) which is present upstream to the transcription initiation site of NGAL thereby boosting its mRNA level [58].

Conclusion

Despite the phenomenal development made in the field of cancer diagnostics and therapy through continual investigation and appraisal, there is no significant improvement in the survival rate of cancer patients. Lack of biomarkers for the early detection of disease, tumor relapse and chemoresistance are the major factors that contribute to the poor prognosis. It is well established that alterations in the expression of many proteins are responsible for the development of cancer. NGAL is one such vital protein which plays a crucial role in various cellular processes and can be successfully used as a biomarker. Elevated expression of NGAL increases the invasiveness of certain cancers, on the contrary, decreases the invasiveness of some other cancers. It also serves as a biomarker and usually detected in the urine of patients with breast and kidney tumors. However, the role of this protein in some cancers is poorly understood. Therefore, deciphering the role of NGAL can provide new insights to the cancer diagnosis and treatment.

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cancers

MDPI

Article

NGAL is Downregulated in Oral Squamous Cell Carcinoma and Leads to Increased Survival, Proliferation, Migration and Chemoresistance

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Abstract: Oral cancer is a major public health burden worldwide. The lack of biomarkers for early diagnosis has increased the difficulty in managing this disease. Recent studies have reported that neutrophil gelatinase-associated lipocalin (NGAL), a secreted glycoprotein, is upregulated in various tumors. In our study, we found that NGAL was significantly downregulated in primary malignant and metastatic tissues of oral cancer in comparison to normal tissues. The downregulation of NGAL was strongly correlated with both degree of differentiation and stage (I–IV); it can also serve as a prognostic biomarker for oral cancer. Additionally, tobacco carcinogens were found to be involved in the downregulation of NGAL. Mechanistic studies revealed that knockdown of NGAL increased oral cancer cell proliferation, survival, and migration; it also induced resistance against cisplatin. Silencing of NGAL activated mammalian target of rapamycin (mTOR)signaling and reduced autophagy by the liver kinase B1 (LKB1)-activated protein kinase (AMPK)-p53-Redd1 signaling axis. Moreover, cyclin-D1, Bcl-2, and matrix metalloproteinase-9 (MMP-9) were upregulated, and caspase-9 was downregulated, suggesting that silencing of NGAL increases oral cancer cell proliferation, survival, and migration. Thus, from our study, it is evident that downregulation of NGAL activates the mTOR pathway and helps in the progression of oral cancer.

Keywords: oral cancer; mTOR pathway; secreted glycoprotein; drug resistance

1. Introduction

Despite significant advancements in the management of oral cancer, it is one of the prime concern worldwide, accounting for approximately 128,000 deaths annually [1,2]. The five-year survival rate of oral cancer is 62.1% (2003–2009); nevertheless, survival rates worsen with advancement in clinical stages (SEER 2003–2009) [3]. Regardless of the unquestionable benefits from the available therapeutic modalities, chemoresistance and recurrence are major complications that reduce the quality of life in patients. This demands the development of novel biomarkers for its early diagnosis and novel targets for the discovery of more potent chemotherapeutic agents for this disease.

Over the past two decades, neutrophil gelatinase-associated lipocalin (NGAL) has received enormous attention in the clinic as a biomarker of kidney injury, cardiovascular injuries, and cancer [4–8]. NGAL, also known as Lipocalin-2 (LCN2), is a 24 kDa glycoprotein in humans encoded by the LCN2 gene located on chromosome 9 at the locus 3p11. Recently, it has emerged as a biomarker for several benign and malignant diseases. Upregulation of NGAL increases the invasiveness of breast, bladder, gastric, gynecological, thyroid, lung, esophageal, colon cancer, and chronic myelogenous leukaemia; however, in pancreatic and oral cancer, it decreases the invasiveness [9–11]. Upregulation of NGAL increases cell proliferation of cervical and lung cancer cells, while downregulation reduces cell proliferation [12,13]. NGAL is a well-known modulator of epithelial–mesenchymal transition (EMT), invasion, and migration. Overexpression of NGAL persuades EMT via activation of snail, twist, N-cadherin, fibronectin, MMP-9, and NF-κB; it then upregulates the genes associated with stemness, adhesion, motility, and drug efflux [14–16]. Likewise, silencing of NGAL reduces migration and invasion via the downregulation of vimentin, MMP-2, and MMP-9 and increases the expression of E-cadherin [11]. These findings suggest that NGAL plays a key role in the development and progression of cancer. However, the role of NGAL in oral cancer has not been well established thus far. Although several studies have shown that NGAL is downregulated in oral cancer, its expression and role in different types and process of oral cancer development have not been studied thoroughly [17,18]. Therefore, a study on the expression of NGAL in different processes of development of oral cancer can help us to comprehend whether NGAL can serve as a diagnostic and prognostic biomarker for oral cancer.

In the present study, we have examined the expression of NGAL in different stages, grades, tumours from different tissues, degree of differentiation, and different processes of development of oral cancer. We found that NGAL plays a pivotal role in different processes of oral cancer development such as survival, proliferation, invasion, migration, and resistance to chemotherapeutic agents.

2. Materials ad Methods

2.1. Tissue Microarray

Tissue microarray (TMA) slides for head and neck squamous cell carcinoma (Cat no: HN803b) and oral squamous cell carcinoma (Cat no: OR802) were purchased from US Biomax, Derwood, MA USA.

2.2. Immunohistochemistry (IHC)

Expression of NGAL was determined by immunohistochemical analysis. Histostain plus kit (Cat no: 859043, Life Technologies, Carlsbad, CA, USA) was used according to the manufacturer's protocol. Anti-hNGAL monoclonal antibody was purchased from (Cat no: ab23477, Abcam, Cambridge, MA, USA). The TMAs were deparaffinised and rehydrated using xylene and ethanol and were blocked with 3% hydrogen peroxide in methanol for 30 min. After the antigen retrieval, the sections were incubated in blocking solution for 30 min and then were incubated with primary antibody (1:10 dilution) at 4 °C overnight. The following day, the sections were incubated with secondary antibody for 1 h at room temperature, stained with DAB (3,3′-Diaminobenzidine)counter stained with haematoxylin, and were mounted using DPX.

2.3. Scoring

All slides were observed under a Nikon Eclipse Ti-E microscope, and the intensity of immunoreactivity for NGAL was examined. The staining intensity was graded on a scale of 0 to 3+ (0 for no staining; 1+ for weak immunoreactivity; 2+ for moderate immunoreactivity; and 3+ for strong immunoreactivity). The percentage of cells positive for NGAL were graded by the following protocol: grade 0 intensity (<10% positive cells); grade 1+ intensity (10–25% positive cells), grade 2+ intensity (25–50%), grade 3 intensity (50–75% positive cells), and grade 4 intensity (75–100% positive cells). The staining intensity score and the percent immunoreactivity score were then multiplied to obtain a composite score.

2.4. Materials

4-(Methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK, Cat No. 78013), *N*′-Nitrosonornicotine (NNN, Cat No. 75285), 4-Nitroquinoline *N*-oxide (4-NQO, Cat No. N8141), Cisplatin (PHR1624), and 5-Flurouracil (5-FU) (F6627) were purchased from Sigma-Aldrich, Saint Louis, MO, USA.

2.5. Cell Culture

Human squamous cell carcinoma of the tongue, SAS cells were procured from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India. These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco™; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco®, Grand Island, NY, USA) and 1× Penstrep (Invitrogen, Carlsbad, CA, USA). The cells were cultured and maintained at 37 °C in 5% CO_2 and 95% humidity.

2.6. Antibodies

S6 Ribosomal protein (dilution 1:2000; Cat No. 2317S), Phospho-S6 Ribosomal protein (Ser235/236) (dilution 1:2000; Cat No. 4858T), LC3B (dilution 1:1000; Cat No. 2775S), Caspase-9 (dilution 1:1000; Cat No. 9508T), Bcl-2 (dilution 1:1000; Cat No. 15071), MMP-9 (dilution 1:1000; Cat No. 13667P), and cyclin D1 (dilution 1:1000; Cat No. 2978BC), GAPDH (dilution 1:2000; Cat No. 2118S) were purchased from Cell Signaling Technology, Danvers, MA, USA. Antibodies against NGAL (dilution 1:3000; Cat No. ab23477), anti-mouse secondary antibody (dilution 1:6000; Cat No. ab97040), and anti-rabbit secondary antibody (dilution 1:6000; Cat No. ab97080) were purchased from Abcam.

2.7. shNGAL Knockdown

shRNA-mediated knockdown of NGAL was carried out in the SAS cell line. Human shNGAL plasmids (Table 1) and puromycin (Cat No. P8833, Sigma-Aldrich) were purchased from Sigma. SAS cells were seeded at a concentration of 25×10^4 cells/well in 1 mL of medium in a 24-well plate. The next day, cells were transfected with shRNA control and shNGAL plasmids (2 µg of DNA) using X-treme gene 9 DNA transfection reagent (Cat No. 06365787001, Sigma-Aldrich) for 48 h. The medium containing transfection reagent was replaced with fresh DMEM medium and the cells were allowed to recover for 24 h. Then SAS cells were selected with 1µg/mL puromycin and knockdown clones were established.

Table 1. Control shRNA (SHC204) and NGAL shRNA sequences used for transfection.

S. No	Clone	Sequence
1	TRCN0000372769	CCGGCAATTCTCAGAGAAGACAAAGCTCGAGCTTTGT CTTCTCTGAGAATTGTTTTTG
2	TRCN0000378896	CCGGGAGTGGTGAGCACCAACTACACTCGAGT GTAGTTGGTGCTCACCACTCTTTTTG
3	TRCN0000372827	CCGGGGAGCTGACTTCGGAACTAAACTCGAG TTTAGTTCCGAAGTCAGCTCCTTTTTG
4	TRCN0000060288	CCGGGCTGGGCAACATTAAGAGTTACTCGAGT AACTCTTAATGTTGCCCAGCTTTTTG
5	TRCN0000060289	CCGGCCAGCATGCTATGGTGTTCTTCTCGAG AAGAACACCATAGCATGCTGGTTTTTG
6	SHC204	CCGGCGTGATCTTCACCGACAAGATCTCG AGATCTTGTCGGTGAAGATCTTTTT

2.8. Cell Viability

Briefly, 2 × 10^3 cells/well were seeded in 96-well plates in sextuplicate and incubated for 24 h and 48 h time points. After each time point, 10 µL of 3-(4,5-dimethylthiazol-) 2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL; Cat No. M2128, Sigma-Aldrich) was added to the cells and was further incubated for 2 h at 37 °C. The MTT solution was removed and 100 µL of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added to each well and the absorbance was then measured at 570 nm using an Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland) after 1 h.

2.9. Cell Cycle Analysis

Control shRNA and shNGAL cells were plated at a density of 1 × 10^5 cells/well, and, after 24 h, cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed with 75% ethanol at −20 °C overnight. The following day, cells were washed with PBS, treated with PI/RNase solution (Cat No. A35126, Invitrogen) for 20 min in the dark and analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ, USA). 25,000 cells in each sample were analyzed to obtain a measurable signal. The data were analyzed on FCS express6 (De Novo Software, Glendale, CA, USA).

2.10. Cell Survival Assay

Control shRNA and shNGAL cells were seeded in a 6-well plate at a density of 1 × 10^3 cells/well. The cells were grown for fifteen days, then colonies were fixed with 70% ethanol and were stained with crystal violet. Pictures of individual wells were taken and were analyzed using imageJ 1.x software [19], and the surviving fraction was calculated.

2.11. In Vitro Wound Closure Assay

Control shRNA and shNGAL cells were seeded in 6-well plates and were allowed to grow till confluency, and then serum starved for 8 h. Confluent monolayers were scratched with a pipette tip. Plates were washed with PBS to remove non-adherent cells, and the wound was photographed at 0 h and 8 h, respectively. The percentage of wound area was calculated.

2.12. Cell Invasion and Migration Assay

Control shRNA and shNGAL cells were serum starved for 18 h before seeding onto transwell inserts (Cat No. 3422, Corning, New York, NY, USA) pre-coated with matrigel. Following serum starvation, the cells were trypsinized and were seeded at a concentration of 5 × 10^4 cells in the upper chamber of the transwell insert; in the lower chamber, medium containing 10% FBS was added as a chemo-attractant. Cells were then incubated for another 24 h at 37 °C. The migrated cells at the

bottom of the transwell insert were fixed in 70% ethanol and were stained with crystal violet solution. Stained cells were visualized under an inverted microscope and photographs were taken using a Nikon 500 camera. After the photographs were taken, the membrane was dissolved in 1% sodium dodecyl sulphate (SDS) (Cat. No. 436143, Sigma-Aldrich) solution at 37 °C for 1 h and absorbance was read at 595 nm in a Tecan plate reader.

2.13. RNA Isolation and Reverse Transcriptase PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was then performed with 1 µL of cDNA as a template. Primer sequences and amplicon lengths are listed in Table 2.

Table 2. List of primers and their sequences used to study mRNA expression.

Gene		Primers	Tm (°C)	Amplicon Size
NGAL	F	5′ATGCCCCTAGGTCTCCTGT3′	55 °C	597 bp
	R	5′TCAGCCGTCGATACACTG3′		
LKB1	F	TCAAAATCTCCGACCTGGGC	55 °C	570 bp
	R	TGTGACTGGCCTCCTCTTCT		
AMPK	F	CGGCAAAGTGAAGGTTGGCAA	59 °C	227 bp
	R	CAAATAGCTCTCCTCCTGAGAC		
P53	F	CTGCCCTCAACAAGATGTTTTG	55 °C	172 bp
	R	CTATCTGAGCAGCGCTCATGG		
Redd1	F	CTGATGCCTAGCCAGTTGGT	55 °C	233 bp
	R	GAGCTAAACAGCCCCTGGAT		
GAPDH	F	AGGTCGGAGTCAACGGATTTG	60 °C	532 bp
	R	GTGATGGCATGGACTGTGGT		

2.14. Western Blot Analysis

Whole cell lysates were prepared by lysing the cells in whole cell lysis buffer (20 mM HEPES, 2 mM EDTA, 250 mM NaCl, 0.1% NP-40) in the presence of protease inhibitors (2 µg/mL Leupeptin hemisulfate, 2 µg/mL Aprotinin, 1 mM PMSF, 1 mM DTT). The protein concentration of the lysates was measured using the Bradford assay (Cat No. 500-0205; Bio-rad, Hercules, CA, USA) and 50 µg of protein was mixed with 5× Laemmli Buffer (250 mM TrisHCl, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% Bromophenol blue), electrophoresed in a 12% SDS-acrylamide gel, and transferred to nitrocellulose transfer membrane (Bio-rad). The membranes were blocked with 5% non-fat milk in tris-buffered saline (TBS: 0.2 M Tris base, 1.5 M NaCl, H_2O) containing 1% tween 20 (TBST). The blots were probed with appropriate primary antibodies overnight. The following day, the blots were washed with TBST, were incubated in appropriate horseradish peroxidase-conjugated secondary antibody, and were visualized using an Optiblot ECL Detection Kit (Cat No. ab133406, Abcam). β-actin/GAPDH was used as the loading control.

2.15. Propidium Iodide Flow Cytometry (PI/FACS)

The cell death induced by chemotherapeutic agents was determined by staining with propidium iodide (PI) (conct. 1 mg/mL; Cat No. P4170, Sigma-Aldrich). Control shRNA and shNGAL cells were seeded in a 6-well plate at a density of 5×10^4 cells/well. After 24 h, the cells were treated with different concentrations of cisplatin and 5-Flurouracil for 48 h. After 48 h, the cells were harvested and were washed with PBS twice. 10 µL of PI was added and was analyzed by flow cytometry (FACSCalibur, Becton-Dickinson). The data were analyzed using FCS Express 6 software.

2.16. Statistical Analysis

All the statistical analysis was carried out using Student's *t*-test or one-way ANOVA followed by Tukey test [20]. *p*-value less than 0.05 was considered as statistically significant.

3. Results

To understand the role of NGAL in oral cancer, we carried out immunohistochemical analysis in oral cancer patient tissues. The tissue microarray contained tissues of different premalignant lesions, stages, grades, tissues, and degree of differentiation of oral cancer. Next, we examined the effect of potent tobacco carcinogens such as NNK, NNN, and the synthetic oral carcinogen 4-NQO on the expression of NGAL in oral cancer cells. Then, we established the role of NGAL on different hallmarks of cancer and elucidated the mechanisms involved.

3.1. NGAL Expression Was Found to Be Downregulated in Oral Cancer

To understand the role of NGAL in oral cancer, we first determined the expression of NGAL in oral cancer tissues. Our results showed moderate expression of NGAL in normal tissues compared to weak to moderate expression in malignant tissues (Figure 1A). Intriguingly, the majority of the well-differentiated epithelial cells of both malignant and normal tissues showed moderate expression of NGAL. Expression of NGAL was observed in all the tumours arising from the oral cavity, including mandible, cheek, gingiva, lip, palate, parotid gland, tongue, lymph node, and larynx, and was found to be downregulated (Figure 1B). Weak to moderate staining of NGAL was observed in the above-mentioned tissues, except the nose, where it was negative. Moreover, the expression of NGAL was inversely associated with the degree of differentiation of tumours. Normal- and well-differentiated tongue tissues showed positive staining of NGAL in comparison to very weak positive staining observed in moderately differentiated and poorly differentiated tongue tissues (Figure 1C). However, no positive expression was observed in the undifferentiated tongue cancer tissues. This suggests that NGAL can serve as a prognostic biomarker for oral cancer. The expression of NGAL also correlated with different stages of tongue cancer tissues where stage I showed high expression and stage IV negative expression in comparison to normal tissues. Similarly, the expression of NGAL was downregulated significantly with an increase in grade of oral cancer in comparison to normal tissues (Figure 1D,E). Furthermore, NGAL was also downregulated in different processes and pathological types of oral cancer and was strongly associated with lymph node metastases (Supplementary Figures S1–S3).

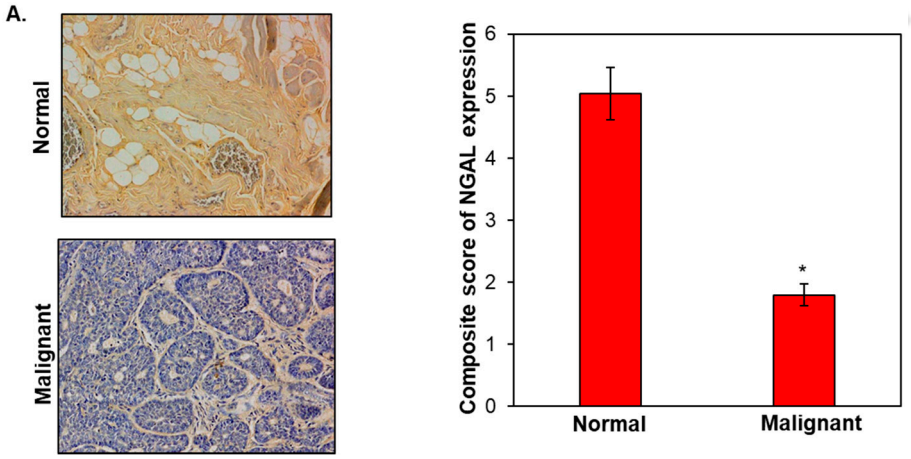

Figure 1. *Cont.*

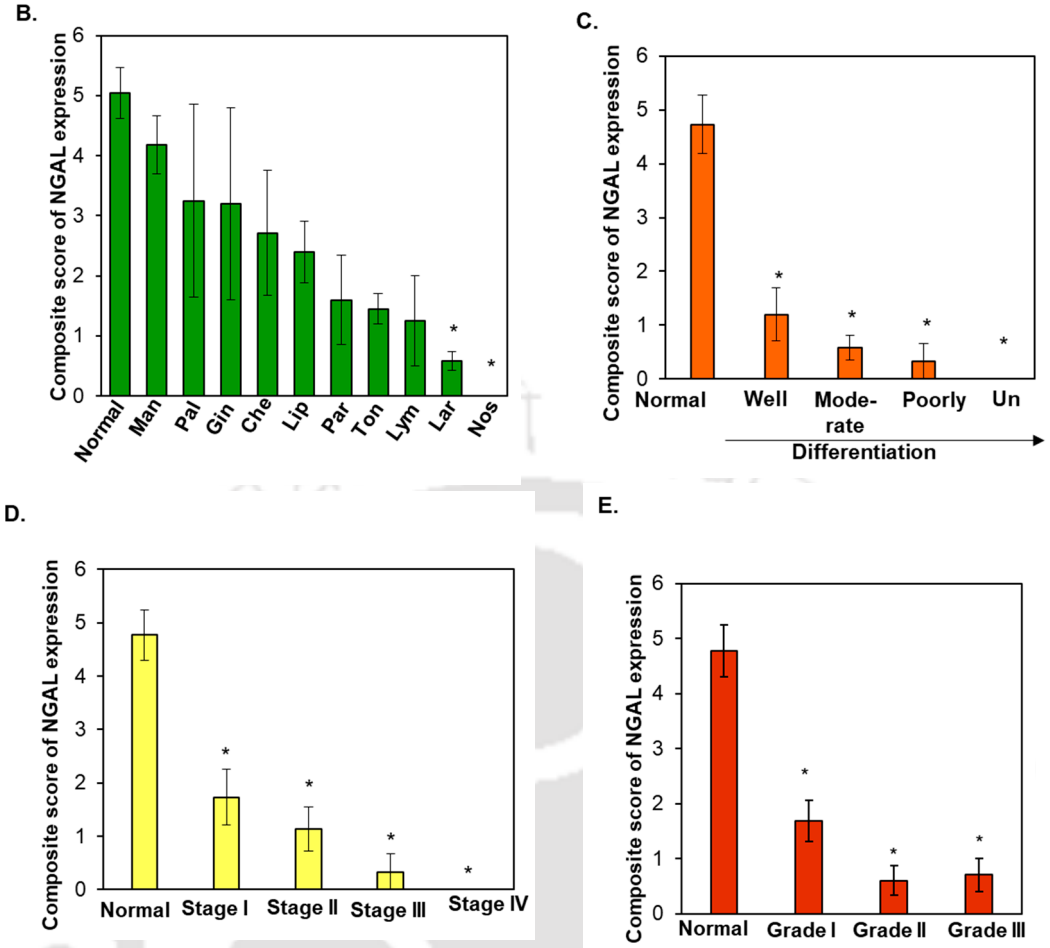

Figure 1. Expression of NGAL (neutrophil gelatinase-associated lipocalin) in oral cancer. (**A**) Representative images of expression of NGAL in oral cancer (left panel). Expression of NGAL in normal (no. of samples (n) = 21) vs. malignant (n = 139) oral cancer tissues (right panel). (**B**) Expression of NGAL in different tissues of oral cancer. Lar: Larynx, Nos: Nose, Ton: Tongue, Che: Cheek, Gin: Gingiva, Lym: Lymph node, Man: Mandible, Par: Parotid gland, Pal: Palate. (**C**) Expression of NGAL with degree of differentiation of oral cancer. (**D**) Expression of NGAL in different stages of oral tongue cancer tissues. (**E**) Expression of NGAL in different grades of oral tongue cancer tissues. Data are mean ± SE. * = $p < 0.05$.

3.2. Tobacco Components Downregulated the Expression of NGAL

NGAL is downregulated in oral cancer tissues and it is well established that tobacco is the prime risk factor for oral cancer [17,21]. Therefore, we determined whether tobacco carcinogens are involved in the downregulation of NGAL. We treated SAS cells with different concentrations of NNK (Figure 2A), NNN (Figure 2B), and the synthetic carcinogen 4-NQO (Figure 2C) and observed that these tobacco components downregulated the expression of NGAL in a dose-dependent manner. This suggests that tobacco carcinogens play a key role in regulating the expression of NGAL.

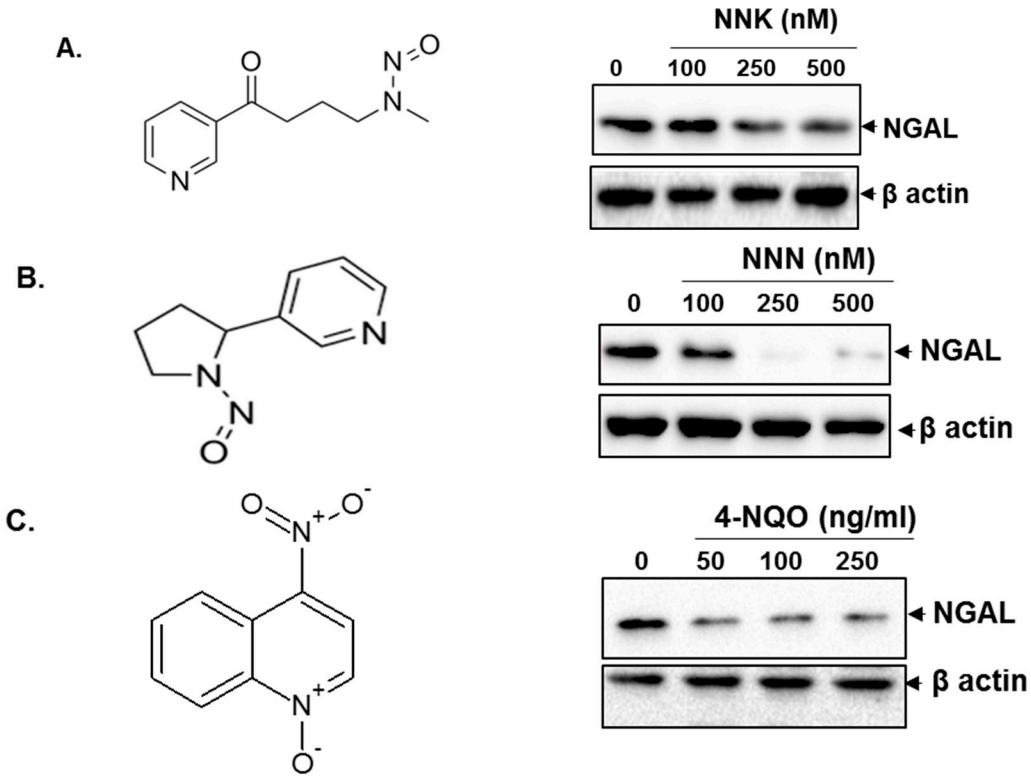

Figure 2. Tobacco components downregulated the expression of NGAL in oral cancer cell line SAS. (**A**) Structure of NNK (left panel). Western blot analysis of expression of NGAL after treatment with NNK for 48 h in SAS cells (n = 2) (right panel). (**B**) Structure of NNN (left panel). Western blot analysis of expression of NGAL after treatment with NNN for 48 h in SAS cells (n = 2) (right panel). (**C**) Structure of 4-NQO (left panel). Western blot analysis of expression of NGAL after treatment with 4-NQO for 48 h in SAS cells (n = 2) (right panel).

3.3. Silencing of NGAL Increased Proliferation and Survival of Oral Cancer Cells

The fundamental property of cancer cells is to sustain cell survival and proliferation. Therefore, we sought to study the effect of silencing of NGAL on the proliferation and survival of oral cancer cells. To study the role of NGAL in oral cancer cell proliferation and survival, we silenced the expression of NGAL (Figure 3A). We carried out an MTT assay and observed that knockdown of NGAL increased cell viability in a time-dependent manner (Figure 3B). To confirm that knockdown of NGAL increases cell viability, we studied its effect on different phases of the cell cycle. We found that silencing of NGAL led to an increase in the number of cells in S-phase and reduced the number of cells in G2/M phase in comparison to control shRNA (Figure 3C). The increase in number of cells in S-phase suggests that NGAL knockdown allows cancer cells to proliferate uninterruptedly and pass through the G2/M check point. In addition, in NGAL deficient cells, we observed that the expression of cyclin D1 is upregulated, which is regulated by the NF-κB/PI3K/mTOR pathways [22,23]. We also assessed if knockdown of NGAL increases oral cancer cell survival by using a clonogenicity assay (Figure 3D). We observed a two-fold increase in the number of colonies in the shNGAL group in comparison to control shRNA group.

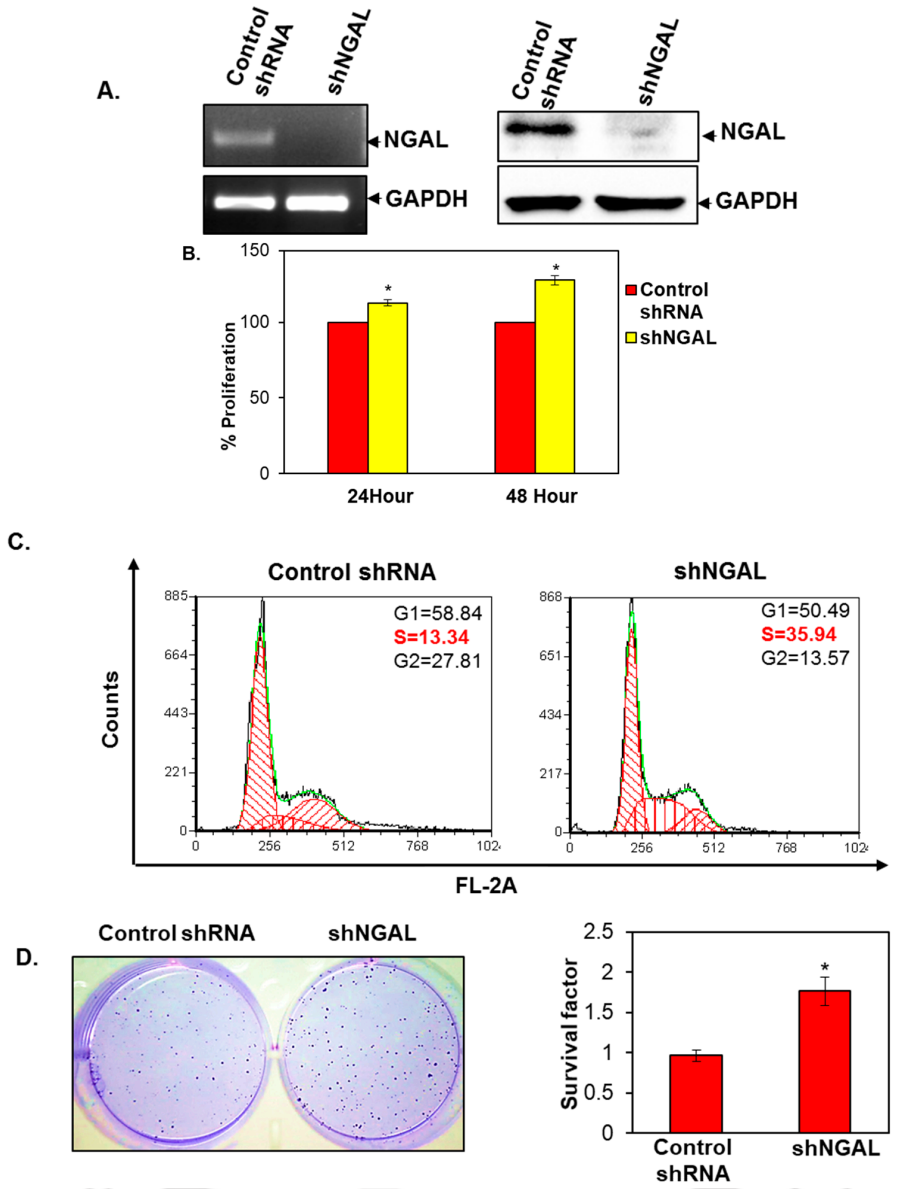

Figure 3. Silencing of NGAL in oral cancer cells. (**A**) qRT–PCR showing the mRNA expression of NGAL in SAS cells post knockdown (left panel). Western blot analysis showing the expression of NGAL in SAS cells post knockdown (right panel). (**B**) Percentage increase in cell viability of control shRNA and shNGAL cells, determined by MTT assay ($n = 2$). (**C**) Cell cycle distribution was determined by flow cytometric analysis in control shRNA and shNGAL cells ($n = 3$). (**D**) Clonogenic assay showing an increase in number of colonies (left panel). Graphical representation of increase in number of colonies in NGAL knockdown cells ($n = 2$) (right panel). Data are means ± SE. * = $p < 0.05$.

3.4. Silencing of NGAL Increases Invasion and Migration of Oral Cancer Cells

Our IHC results advocate that downregulation of NGAL is strongly associated with metastases; accordingly, we hypothesized that knockdown of NGAL may induce invasion and migration of oral cancer cells. To confirm this, we performed in vitro invasion and migration assays using NGAL knockdown cells. Results from the transwell migration assay suggested that the NGAL knockdown cells possessed higher invasive ability than shRNA control cells. The number of cells that invaded the lower part of the transwell insert was higher in the case of shNGAL cells in comparison to control cells (Figure 4A). Similarly, in the in vitro wound healing assay, the wound was healed within 8 h in the case of shNGAL cells in comparison to control cells. This indicates that shNGAL cells have

higher migratory potential (Figure 4B). Similarly, in NGAL silenced cells, MMP-9 was found to be upregulated, which might be responsible for the increase in cell motility.

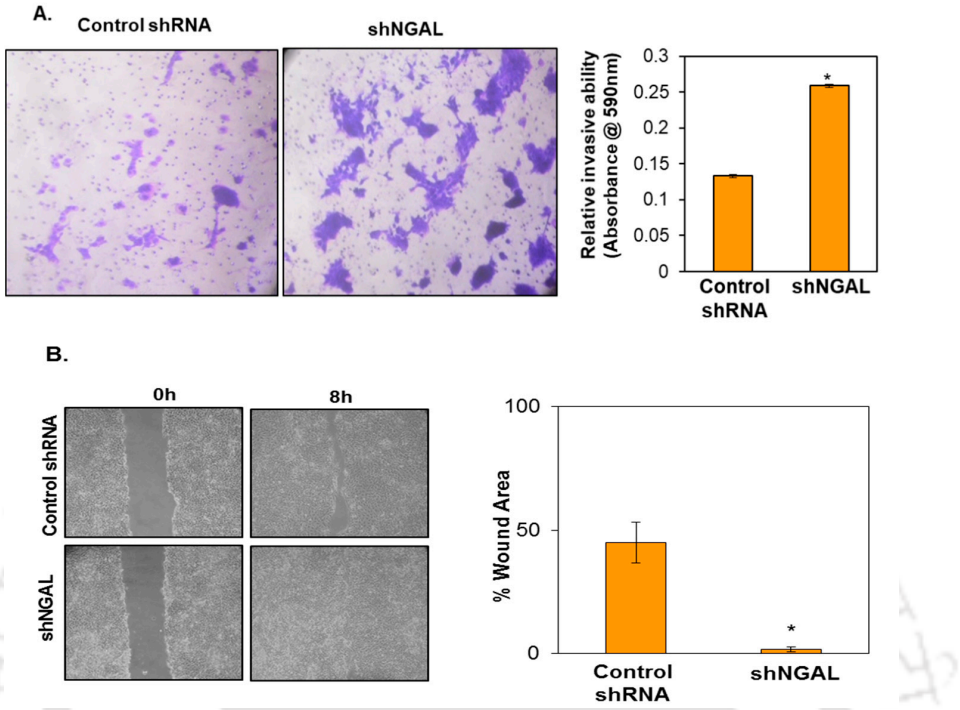

Figure 4. Silencing of NGAL increased the invasion and migration of oral cancer cells. (**A**) Cell invasion was determined by a transwell invasion assay. Cells invading through the matrigel were fixed, stained, and photographed under an inverted microscope at a 20× magnification. Graphical representation of increase in cells invading the lower surface of transwell insert (right panel). (**B**) Cell migration was detected by scratch wound healing assay. Photographs were taken at 10× magnification. Graphical representation of decrease in wound area (right panel). Data are means ± SE. * = $p < 0.05$ ($n = 4$).

3.5. Silencing of NGAL Activates mTOR Signalling and Suppresses Autophagy

Our previous results suggest that tobacco components downregulated the expression of NGAL, and loss of NGAL increased oral cancer cell proliferation, survival, invasion, and migration. However, the underlying mechanism is not clear. Increasing evidences suggest that tobacco components play a key role in the development of oral cancer and are known to regulate the Akt/mTOR pathway. Therefore, we studied the effect of silencing of NGAL on the activation of S6, a well-established marker of the mTOR pathway. We observed that knockdown of NGAL activated S6 (serine 235/236) (Figure 5A,B). Recently, Dowling et al., 2007 reported that metformin inhibited the activation of S6 via the AMP-activated protein kinase (AMPK) pathway [24]. Hence, we studied the expression of AMPK in NGAL-silenced cells and observed that the expression of AMPK was downregulated, indicating that AMPK is the intermediate link between NGAL and S6. LKB1 is upstream of AMPK, and, as AMPK is the only substrate, we analyzed the expression of LKB1 and found that it was downregulated [25]. Thus, knockdown of NGAL activates mTOR signalling via the AMPK-LKB pathway. Reports suggest that, during hypoxia or energy stress in the head and/or neck, squamous cell carcinoma (HNSCC) cells, regulated in developmentand DNA damage responses -1 (Redd1) inhibits mTOR signalling by upregulating AMPK [26,27]. Hence, we studied the expression of Redd1 in NGAL knockdown cells and found that the expression of Redd1 is completely inhibited (Figure 5C,D). Besides Redd1, AMPK is also known to regulate and activate p53 during metabolic stress [28]. Thus, p53 serves as a downstream target of AMPK, and we found that in NGAL knockdown cells the expression of p53 was found to be downregulated (Figure 5C,D).

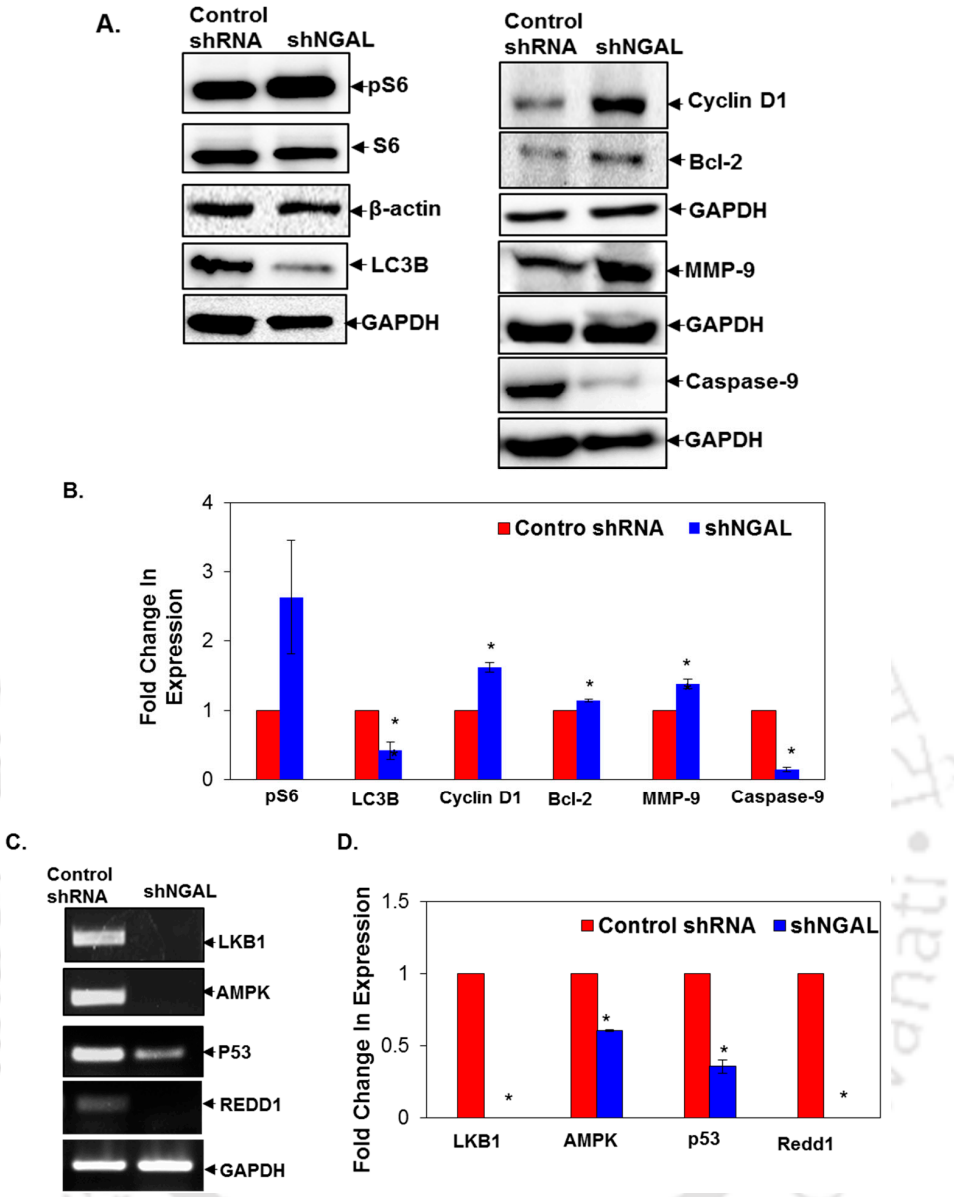

Figure 5. Silencing of NGAL activated mTOR signalling and induced autophagy. (**A**) Expression of proteins involved in mTOR signalling and autophagy. (**B**) Fold change in expression of proteins as analyzed by image lab software (n = 2). (**C**) mRNA expression of LKB1-AMPK-P53-Redd1 in NGAL knockdown cells. (**D**) Fold change in mRNA expression as analyzed by image lab software (n = 3). Data are means ± SE. * = $p < 0.05$.

Moreover, the promoter region of Redd1 is known to possess the consensus p53 family binding element that is required for regulation of Redd1 by p53 [26,29]. This suggests that Redd1 is a direct transcriptional target of p53 and can be a connecting link between AMPK and Redd1. Therefore, silencing of NGAL increases survival, proliferation, invasion, and migration of oral cancer cells via the LKB1-AMPK-p53-Redd1-mTOR axis (Figure 5C,D). We observed that knockdown of NGAL upregulated cyclin D1, Bcl-2, and MMP-9 as well as downregulated caspase-9, confirming the same (Figure 5A,B). In addition to the significant role of mTOR in cancer progression, activation of mTOR downregulates autophagy [30–32]. Thus, we studied the expression of LC3B, an autophagy marker, and observed that the expression of LC3B was found to be downregulated. This suggests that NGAL-silenced cells are more resistant to autophagy-induced cell death, and decreased autophagy

provides a survival advantage. Overall, our results suggest that NGAL knockdown cells were more resistant to autophagy, which was mediated via the LKB1-AMPK-p53-Redd1 axis and activation of mTOR signalling.

3.6. Silencing of NGAL Selectively Induces Resistance Against Cisplatin

Development of resistance is the major reason for the failure of chemotherapeutic agents in the clinic. Therefore, we studied the role of NGAL in the development of resistance against the first-line therapeutic agents, cisplatin and 5-FU. We observed that knockdown of NGAL selectively induced resistance against cisplatin, while both control shRNA and shNGAL cells were sensitive to 5-FU (Figure 6A,B). Upregulation of cyclin D1 and Bcl-2 as well as downregulation of caspase-9 might be the reason for the development of chemoresistance. However, the mechanism requires further study.

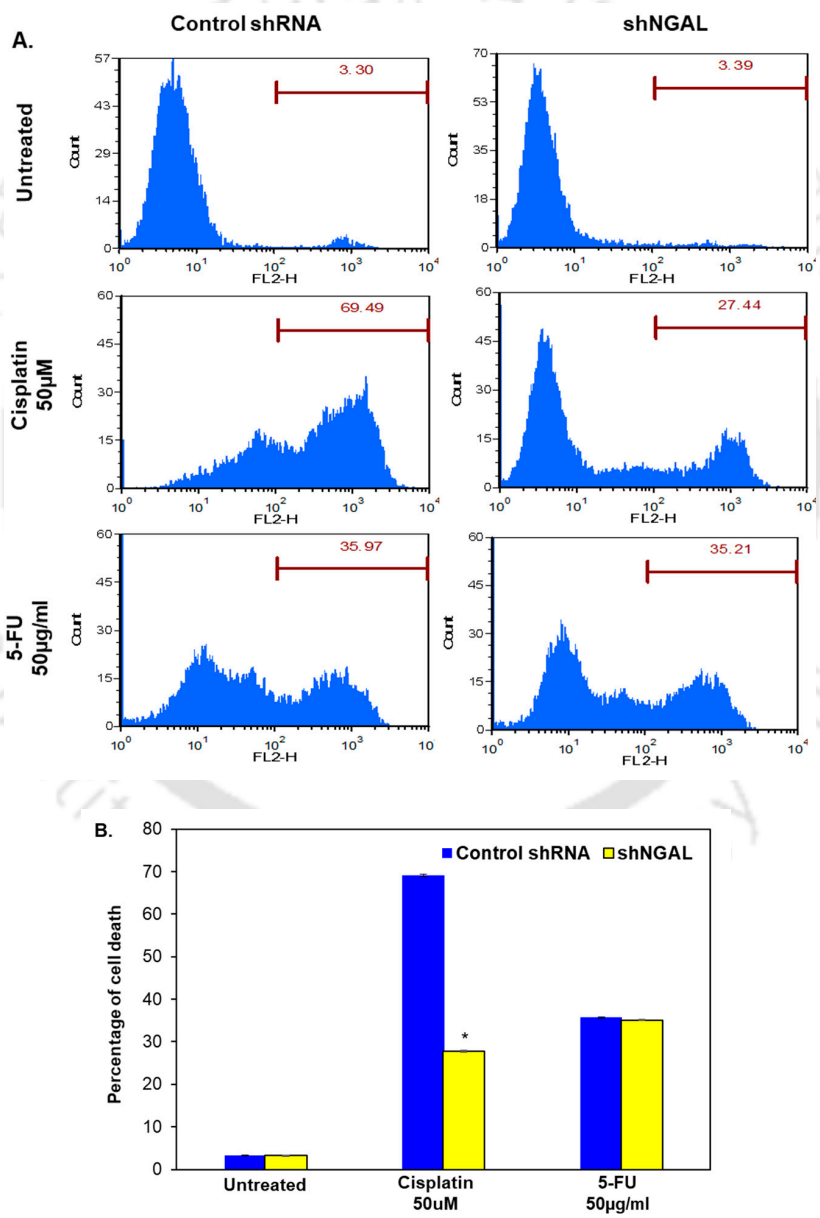

Figure 6. Silencing of NGAL selectively induces resistance against cisplatin. (**A**) Cells were treated with cisplatin and 5-FU, and percentage of cell death was measured by staining with propidium iodide on flowcytometry at 48 h. (**B**) Graphical representation of percentage of cell death (n = 3). Data are means ± SE. * = $p < 0.05$.

4. Discussion

We studied the expression of NGAL in oral cancer tissues and found that NGAL was downregulated in primary tumour and metastatic tissues. Our results were consistent with previous studies where NGAL was found to be downregulated in oral cancer tissues [17,18]. Downregulation of NGAL was found to be strongly correlated with the degree of differentiation and stage of oral cancer. Similarly, the study carried out by Hiromoto et al., 2011, showed that the downregulation of NGAL was associated with the degree of differentiation of tumours [17]. Thus, NGAL can serve as biomarker for identifying the degree of differentiation, prognosis, and severity of the disease. However, there are no reports about the expression of NGAL with respect to age, tissues, stages, grades, etc. in oral cancer. The expression of NGAL was found to be downregulated in malignant tongue, larynx, lip, cheek, gingiva, and palatal tissues of the oral cavity. Moreover, downregulation of NGAL was evident in all the stages (stage I–IV) and grades (grade I–III) of oral squamous cell carcinoma (OSCC).

Because tobacco is a well-characterized risk factor for oral cancer, we investigated whether NGAL is regulated by tobacco carcinogens. The main carcinogens characterized from tobacco smoke include benzo[a]pyrene, nicotine, NNK, NNN, dibenzo[a]pyrene, benzene, nitrobenzene, 2-toluidine, and 2-6-dimethylaniline. Upon activation, NNK and NNN induce mutations in tumour suppressor genes and oncogenes; they form DNA adducts that result in tumour initiation [33–37]. 4-NQO is a synthetic tobacco carcinogen used to induce oral cancer in mouse that mimics the oral cancer development in humans [38,39]. In our study, it was found that tobacco carcinogens NNK, NNN, and 4-NQO downregulated the expression of NGAL in a dose-dependent manner. This indicates that NGAL plays a key role in tobacco-induced carcinogenesis.

Next, we found that downregulation of NGAL induced oral cancer cell proliferation, survival, invasion, and migration. Many studies report that NGAL plays a key role in the invasion and migration of oral cancer and other cancers. Recently, Lin et al., 2016 reported that knockdown of NGAL increased in vitro cell motility and in vivo metastases [18]. However, this is the first study that has shown that knockdown of NGAL increases in vitro cell viability and survival in oral cancer. Similar to our findings, a recent study in colorectal cancer showed that knockdown of NGAL increased cell proliferation, survival and induced EMT [40]. Presently, the mechanism involved requires further study. Our study shows that knockdown of NGAL activated mTOR signalling and reduced autophagy via the LKB1-AMPK-p53-Redd1 signalling axis. Aberrant activation of mTOR is seen in OSCC and is associated with poor prognosis [41–45]. Phosphorylated S6, the downstream target of mTOR, was found to be upregulated in epithelial dysplasia and OSCC; it also can serve as a potent diagnostic biomarker for oral cancer [46]. mTOR signalling can be activated by various stimuli. During hypoxia or energy starvation, LKB1 is activated, which, in turn, phosphorylates AMPK. Thus, the activated AMPK phosphorylates TSC2, which results in switching off mTOR signalling [47,48]. It is well established that activation of mTOR signalling inhibits autophagy, and studies also suggest that Redd1 regulates autophagy [49]. A similar mechanism was observed in our study, indicating that silencing of NGAL mediates autophagy via Redd1. Moreover, as mentioned earlier, p53 was found to be downregulated in NGAL knockdown cells. Our results were similar to previous studies in which NGAL was shown to regulate the expression of p53 [50,51].

Furthermore, AMPK activates p53 during metabolic stress by phosphorylating MDMX on serine 34, resulting in inhibition of p53 ubiquitylation [28]. Moreover, in cells lacking p53, ectopic expression of p53 induced the endogenous activity of Redd1; additionally, the promoter region of Redd1 comprises of p53 binding sites, indicating that Redd1 is a direct transcriptional target of p53 [26]. Thus, our study demonstrates that knockdown of NGAL activates the mTOR pathway via the LKB1-AMPK-p53-Redd1 signalling axis. Moreover, the expression of cyclin-D1, Bcl-2, and MMP-9 were upregulated and caspase-9 was downregulated, which are the key molecules involved in oral cancer cell proliferation, survival, invasion, and migration. In addition to promoting mTOR signalling, knockdown of NGAL decreased autophagy. Activation of autophagy by many chemotherapeutic agents in HNSCC induced apoptosis and downregulated the mTOR pathway. Many small molecule tyrosine kinase inhibitors

such as gefitinib, erlotinib, and dasatinib induced autophagy and suppressed mTOR signalling, indicating that an increase in autophagy suppresses tumour growth in vitro and in vivo [52]. These studies indicate that autophagy serves as a tumour suppressor. Thus, our study clearly demonstrates that knockdown of NGAL increases oral cancer cell proliferation, survival, invasion, and migration by upregulating mTOR signalling and suppressing autophagy.

5. Conclusions

Our results suggest that NGAL is downregulated in oral cancer tissues and is strongly associated with degree of differentiation, stage of the tumour, and lymph node metastases. The tobacco components, primarily NNK, NNN, and the synthetic carcinogen 4-NQO, were implicated in the downregulation of NGAL. Mechanistic studies revealed that knockdown of NGAL augmented cell survival, invasion, and migration by activating the mTOR pathway and also downregulated autophagy via the LKB1-AMPK-p53-Redd1 signalling axis (Figure 7). This suggests the NGAL is one of the key molecule involved in oral cancer tumorigenesis. Therefore, agents that can restore the expression of NGAL would be advantageous in developing effective therapies against this dreadful disease.

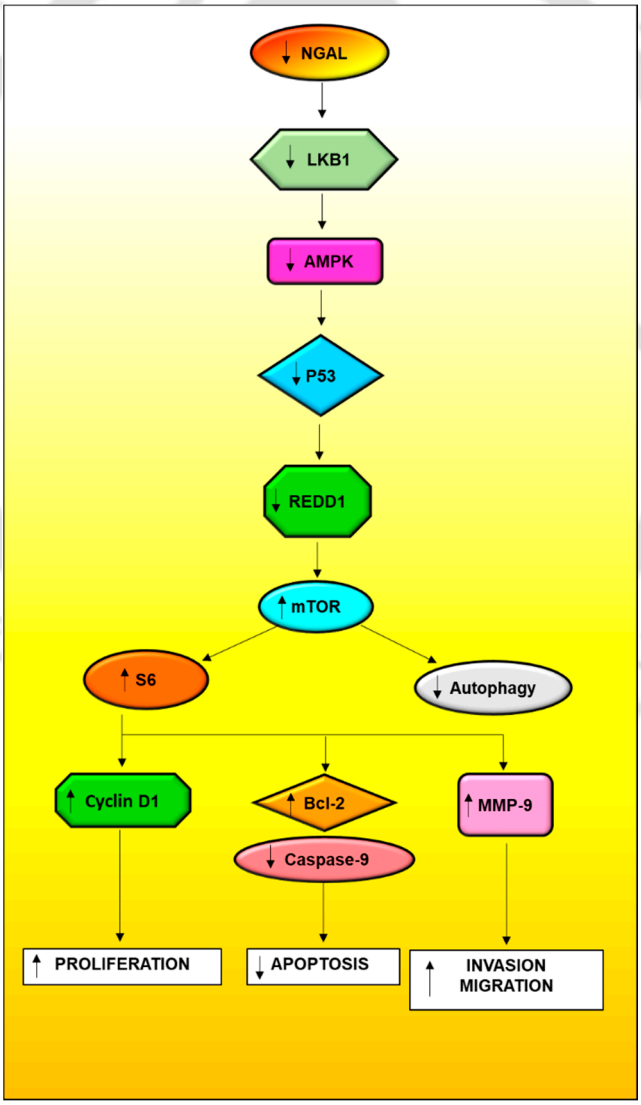

Figure 7. Downregulation of NGAL activates mTOR signaling via LKB1-AMPK-p53-Redd1 and decreases autophagy.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/10/7/228/s1, Figure S1: Expression of NGAL in different stages of the development of oral cancer. CAT: Cancer adjacent tissue. Mean composite score of NGAL levels in Normal tissues (n = 10), inflammation (n = 5), hyperplasia (n = 5), cancer adjacent tissues (n = 5), malignant tissues (n = 42), and metastatic tissues (n = 4). Data are mean ± SE. * = $p < 0.05$. Figure S2: Expression of NGAL in different types of oral cancer. Mean composite score of NGAL levels in NT-Normal tissue (n = 10), MEC-Mucoepidermoid carcinoma (n = 8), ACC-Adenoid cystic carcinoma (n = 3), BCC-Basal cell carcinoma (n = 2), SCC-Squamous cell carcinoma (n = 28), and MSCC-Metastatic squamous cell carcinoma (n = 4) of oral cancer. Data are mean ± SE. * = $p < 0.05$. Figure S3: Expression of NGAL with age and gender in oral cancer tissues. (A) Mean composite score of NGAL levels in tissues from normal male (n = 11), normal female (n = 10), malignant male (n = 100), and malignant female (n = 39) patients with oral cancer. (B) Mean composite score of NGAL levels in normal (n = 17) and malignant oral cancer patient tissues of age groups 0–30 year (n = 4), 30–45 year (n = 18), 45–60 year (n = 63), and 60–100 year (n = 52). Data are mean ± SE. * = $p < 0.05$.

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Curriculum Vitae

Dr. Javadi Monisha was born on August 10th 1988 in the city of Hyderabad (Telangana). She passed the Secondary Examination (10th Class) conducted by Board of Secondary Education, Andhra Pradesh in 2003 and Higher Secondary Examination (12th Class) conducted by Board of Intermediate Education, Andhra Pradesh in 2005. She completed B.Tech (Industrial Biotechnology) from Bharath University, Chennai, in July, 2009 and M.Tech (Biotechnology) from Sri Indu Institute of Engineering and Technology, Hyderabad (affiliated to JNTU, Hyderabad) in January 2012. She joined in the Ph.D. program in December, 2012 at Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781 039, Assam, India. She successfully completed her course work at IITG with 9/10 CPI. She received Institute Fellowship (IIT Guwahati) from December 2012 to January 2017, under the scheme run by the Ministry of Human Resource and Development (MHRD), New Delhi. She has defended her thesis on 20th August 2018. The main focus of her research is to understand the role of proteins involved in the multistep process of development of cancer that could serve as early biomarkers and therapeutic targets. Moreover, her research interest also includes elucidating the mechanisms involved in the development of chemoresistance and the possible approaches to overcome the problem. She has edited a monograph 'Cancer Cell Chemoresistance and Chemosensitization'. She is credited with over 40 scientific publications and recipient of many awards.

I was born not knowing and have had only a little time to change that here and there.

Richard P. Feynman