THE UNIVERSITY OF HULL

Expression of angiogenic growth factors in laryngeal carcinoma

Being a Thesis submitted for the Degree of MD

In the University of Hull

by

Korampalli Tirupati Srinivasu

MBBS, MRCS, DOHNS, DLO

September 2012

Contents.

Abstract

CHAPTER 1 - Introduction

CHAPTER 2

Expression of angiogenic related growth factors in laryngeal cancer

CHAPTER 3

Angiogenic growth factor expression in relation with tumour progression

CHAPTER 4

Prognostic correlation of angiogenic factor with tumour progression and treatment and tumour characteristics

CHAPTER 5

List of tables

List of Figures

Appendix

Publications from this thesis

1.Protein profiling of angiogenesis related growth factor in laryngeal carcinoma: expression in relation to tumour progression
Korampli T S, Green VL, Stafford ND, Greenman J **Int J Oncol.** 2011 Jul 8. doi: 10.3892/ijo.2011.1117

Presentations:

International

a) American Head and neck society meeting – Canada – 2012

b) American head and neck society meeting – Chicago-2011

National

a) British Academic conference of Otolaryngology – Glasgow - 2012

b) North of England meeting– Leeds -2011

c) North of England meeting -Manchester -2010

Abbreviations

AA: Acetaldehyde

AJCC: American Joint Committee on Cancer

ANG: Angiogenin

CCDN1: Cyclin D1

Chemo: Chemotherapy

Cox 2: cyclo-oxygenase2

DFS: Disease free survival

DXT: Radiotherapy

EGFR: Epidermal growth factor receptor

ELISA: Enzyme linked immunosorbant assay

VEGF: Vascular endothelial growth factor

FDA: Food drug agency

FGF: fibroblast growth factor

GERD: Gastro esophageal reflux

GM-CSF: Granulocyte macrophage colony stimulating factor

G-CSF: Granulocyte colony stimulating factor

HGF: Hepatocyte growth factor

HNSCC: Head and neck squamous cell carcinoma

HPV: Human papilloma virus

HRP: Horseradish peroxidase

IGF BP: Insulin like growth factor basic protein

IL-8/CXCL-8: Interleukin 8

L: Laryngectomy

LOH: loss of heterozygosity

LSCC: Laryngeal squamous cell carcinoma

M: Metastasis

MDSCC: Moderately differentiated squamous cell carcinoma

MVD: Micro vessel density

MMP 9: Matrix metallo proteinase 9

N: Node

ND: Neck dissection

NICE: National institute for clinical excellence

PDGF: Platelet derived growth factors

PDSCC: poorly differentiated squamous cell carcinoma

RT: Radiation therapy

SCC: Squamous cell carcinoma

SPSS: Statistical package for social sciences

Sur: Surgery

T: Tumour

TKI: Tyrosine kinase inhibitors

TMB: Tetraethyl benzoate

TIMP-1: Tissue inhibitors of metalloprotienases -1

UK: United Kingdom

VA: Veterans association

WDSCC: Well differentiated squamous cell carcinoma

Acknowledgements

I would like to thank my supervisors, Professor Nick Stafford, Professor John Greenman who have guided me through this work. In particular I would like to thank Professor Nick Stafford who introduced me to this exciting area of head and neck cancer research and opening my eyes to the world of research in clinical medicine, as well as professor John Greenman whose support has ensured a enjoyable and productive research opportunity. I am also grateful to my laboratory colleagues who have taught me new skills and helped to verify my research data. In particular Dr Vicky L Green for teaching and helping me with both antibody array and ELISA techniques, and provided support through my project work. I would like to thank Mr Jose, ENT consultant and ENT theatre staff for providing the tissue samples for the project.

Abstract

Head and Neck cancer is the sixth most common malignancy in the world and each year over 2,200 cases of carcinoma of the larynx are diagnosed in the UK. Patients have an overall five-year survival rate of 67%. Despite advances in treatment, patient prognosis is poor largely due to recurrence. The formation of new blood vessels (angiogenesis) is vital for the growth and metastasis of solid tumours, with the expression of key angiogenesis-related proteins having been shown to have prognostic significance. This study aims to identify key proteins within the tumour microenvironment, which may be involved in angiogenic processes occurring in laryngeal carcinoma, and associated metastatic nodes at different stages of disease, and to evaluate any potential therapeutic value.

Both laryngeal tumour tissue and associated metastatic nodes were obtained from seven patients undergoing surgical resection; control uvula tissue was obtained from five healthy volunteers undergoing uvulopalatopharyngoplasty. Protein was extracted from the tissue using a commercial kit (Proteojet lysis reagent, Fermentas Life Sciences, York, UK, Calbiochem/Merck, Nottingham, UK) and the expression of 55 angiogenesis-related proteins was determined using a human angiogenesis array (R&D systems). Subsequently the level of six of the 55 angiogenesis-related proteins showing the greatest level of difference were determined in the tissue lysates using ELISA (R&D Systems) in a cohort of thirty six patients diagnosed with laryngeal cancer. The patient group comprised 30 men and 6 women with a mean age of 68 years (range 51-89).

The relative expression of 32/55 angiogenic-related proteins increased in tumour tissue between stage T1 to T3-T4 and the same trend was seen for 29 of these proteins in nodal tissue; MMP9, platelet factor 4 and Serpin E1 did not show an increase until T4. Thirty-one of the 32 proteins were expressed at a higher level in T3/T4 tumour tissue compared with the corresponding node, and 29/32 proteins were expressed more highly in T1 tumour compared with T1 node. The level of expression of these 27 proteins was consistently higher in T4 tumour tissue compared with control.

Specific analysis of the six most differentially expressed angiogenic-related proteins demonstrated increased levels of angiogenin in early stage tumours (p =0.034) compared with late stage of the tumour and increased IGF BP3 in tumours compared with the matched metastatic nodes (p = 0.016). No statistically significant results were observed for VEGF, FGF, TIMP1 or IL-8.

Survival analyses revealed that none of the factors were associated with recurrence or patient's survival.

In summary this study suggests that a network of proteins present in the tumour microenvironment are likely to be involved in angiogenesis and therefore contribute to growth and metastasis of laryngeal tumours during the later stages of tumour development. Angiogenin and IGF BP3 may play a role in tumour progression of laryngeal malignancies, although they demonstrated no significant role in predicting the survival of the patients with laryngeal malignant tumours.

INTRODUCTION

1.1 Anatomy of larynx

The adult larynx is situated in the midline of the neck at the level of the third to sixth vertebrae. It extends from the laryngeal inlet, near the base of the tongue to the trachea. At its inlet, the larynx communicates with the oropharynx and laryngopharynx (hypopharynx). The laryngeal inlet is bounded anteriorly and superiorly by the epiglottis, posteriorly and inferiorly by the arytenoid cartilages and the interarytenoid region, and laterally by the aryepiglottic folds. The pharynx extends along the sides of the laryngeal inlet to form the pyriform fossae. It consists of a cartilaginous framework connected by ligaments, membranes and muscles. (Figure1a).

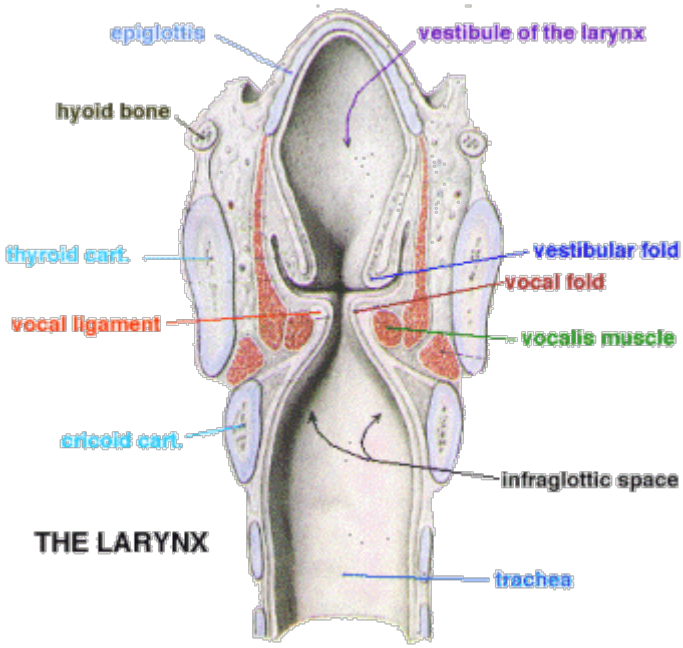


Figure 1a: Coronal section of larynx demonstrating the relation ship of the laryngeal components. (www.med.jhu.edu/voice/larynx.html.)

The larynx is lined both internally and on its outer, posterior, surface by pseudo stratified, ciliated, columnar epithelium interspersed with goblet cells. The exceptions in the normal larynx are the surface of the vocal cords and the anterior surface of the epiglottis, which are covered by stratified squamous epithelium.

The blood supply of the larynx is derived mainly from two pairs of arteries: the superior and inferior laryngeal arteries. The superior laryngeal artery supplies the larynx above the vocal cords; it is derived from superior thyroid artery, a branch of external carotid artery. Inferior laryngeal artery supplies larynx below the vocal cords, it derived from inferior thyroid artery which itself is derived from the thyrocervical trunk of the subclavian artery (Figure 1b).

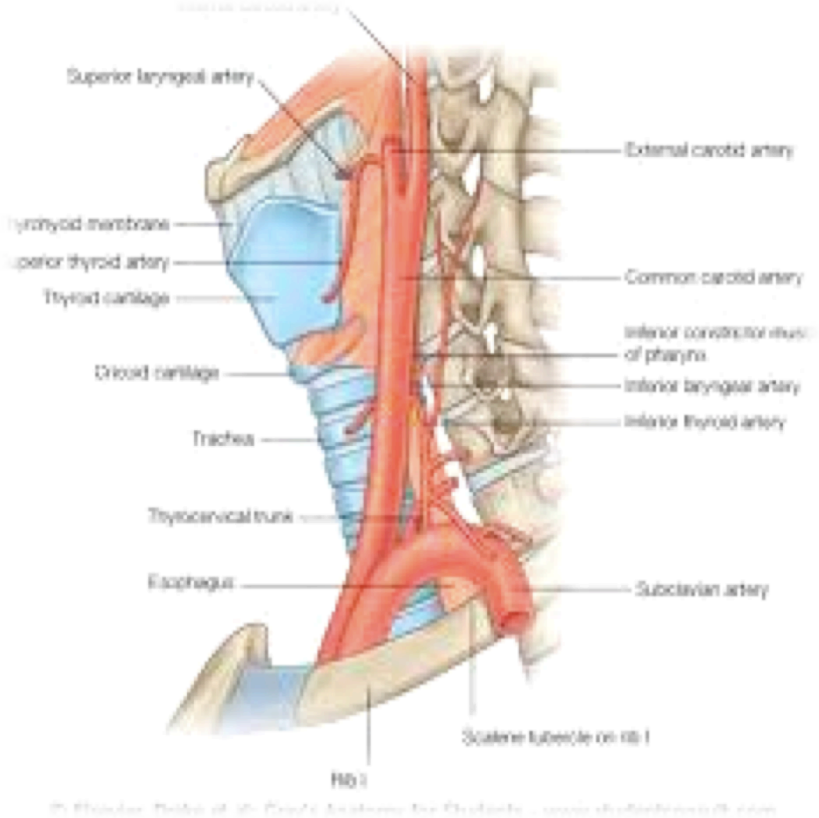


Figure 1b: Arterial supply of the larynx lateral view. (Greys Anatomy)

The vocal cords form a dividing line for both the sensory and the secretomotor innervations of the mucosa within the larynx. Above the vocal cords, the mucosa is innervated by the internal laryngeal branch of the superior laryngeal nerve. Below the vocal cords, the mucosa is supplied by the recurrent laryngeal nerve. The vocal cords themselves are innervated by both nerves. The motor supply to the intrinsic muscles of the larynx is derived mainly from the recurrent laryngeal nerve. The cricothyroid muscle however, is supplied by the external branch of the superior laryngeal nerve (Figure 1c).

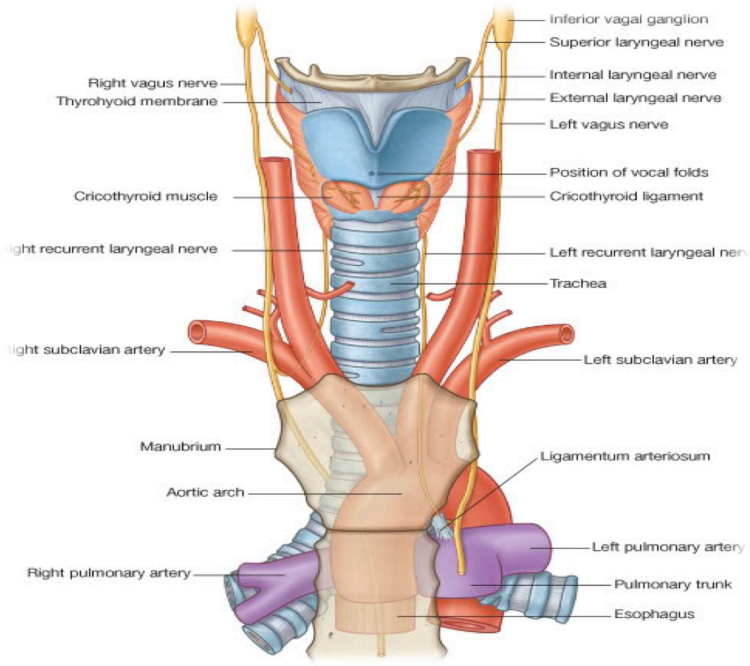


Figure 1c: Nerve supply of the larynx (Greys Anatomy)

1.1.1.Embryology

The larynx is derived from the second, third, fourth and sixth branchial arches. Its development commences during the third and fourth weeks of intrauterine life. The

supra glottis is derived from the midline bucco-pharyngeal primordium and bronchial arches 3 and 4. It is rich in lymphatics, which often drain bilaterally. The glottis is formed by the fusion of the lateral structures derived from the tracheobronchial primordium and arches 4 and 5 and is less well supplied with lymphatics, a reason why glottis cancers are rarely associated with lymph node metastasis. This difference in embryological origin can explain the difference in clinical behavior of cancers from each site.

The lymph vessels superior to the vocal folds accompany the superior laryngeal artery pierce through the thyro-hyoid membrane and drain into the upper deep cervical lymph nodes. The lymph vessels inferior to the vocal folds drain into the inferior deep cervical lymph nodes (supraclavicular nodes) through the pre-laryngeal, pre-tracheal, and para-tracheal lymph nodes.

1.1.2. Lymphatic drainage

Supraglottic Larynx

The lymphatic drainage is separated into two components, a superficial mucosal component that drains into a deep system of collecting ducts. The deep system unites with the lymphatic drainage of the inferior pharynx. The draining vessels of the unified deep system exit the larynx through the natural defect in the thyrohyoid membrane that permits passage of the superior laryngeal neurovascular bundle. At this point, one component of the lymphatic drainage extends superiorly and terminates in the ipsilateral

Level II nodes while a second component extends lateral and drains into nodes located at the junction of Levels II and III. There is occasionally a third component that drains into the nodes located in Level III nodes. Tumours involving the supraglottic larynx are at risk for crossed lymphatic drainage. However, the drainage mechanism is unclear. There appears to be cross-drainage of the superficial mucosal lymphatic's, however, no consistent direct cross-drainage of the deep collecting duct has been described (Mukherji et al., 2001).

Glottic Larynx

There is a paucity of lymphatics draining the true vocal cords (TVC). The superficial mucosal lymphatics form a continuous layer along the posterior shmlect of the larynx. However, the lymphatics draining the TVC are sparse form a natural barrier between the supraglottic and infraglottic larynx. The predominant lymphatic drainage of the advanced TVC carcinoma occurs by acquiring the lymphatic drainage that occurs by extension into the supraglottic or subglottic larynx.

Pyriform Sinus

The lymphatic drainage is divided into anterior and posterior groups. The anterior collecting system exits along with the lymphatics of the supraglottic larynx through the natural defect in the thyro-hyoid membrane described above. These vessels course

through pre-laryngeal lymph nodes and primarily drain into the Levels II, III and the Level VII nodes. Advanced disease may involve Levels IV and V.

The posterior group penetrates the superior constrictor muscle and drains into the lateral retropharyngeal lymph nodes and the internal jugular chain. Cross-lymphatic drainage occurs from the superficial lymphatics along the midline of the posterior pharyngeal wall.

As a result, pyriform sinus carcinomas have a rich drainage system. Both ipsi-lateral and contralateral lymph nodes from the skull base to the base of the neck are at risk from metastases in patients with moderately advanced tumours.

1.1.3 Functional anatomy of larynx

The larynx is the organ of phonation, and it is also important in airway maintenance. Temporary closure of the larynx occurs physiologically in three situations: speech, swallowing and just before sneezing and coughing. Phonation, the production of sound by the larynx for articulation by the palate, tongue, teeth and lips, depend on the co-ordinated movements of the abdominal and thoracic muscles of respiration are approximated and vibrate. Sound is generated in the larynx, and by stimulating exhaled air into vibration its pitch and volume can be manipulated. The intensity of the sound produced is dependent on the velocity of the expired air and disposition of the vocal cords with respect to each other.

During swallowing, the airway is protected by the sphincteric actions of the interarytenoid muscles and by the adductors of the glottis. The larynx is elevated and anteriorly displaced below the base of the tongue by the contraction of the suprahyoid muscles. The combination of these two actions produces a rotational displacement of the epiglottis, which comes to lie, to a greater or lesser extent, over the laryngeal inlet.

1.2 Lymph nodes of neck

The lymph nodes of the neck can be divided into six levels within the defined anatomic triangles (Figure 1.2). These groups and the areas that they drain are particularly important when locating and clinically assessing a "neck mass" or possible malignancy.

Rouviere developed the original classification system of cervical lymph nodes in 1938. The latest classification has been created by the American Joint Committee on Cancer and the American Academy of Otolaryngology - Head and Neck Surgery, which is summarized (Appendix 1).

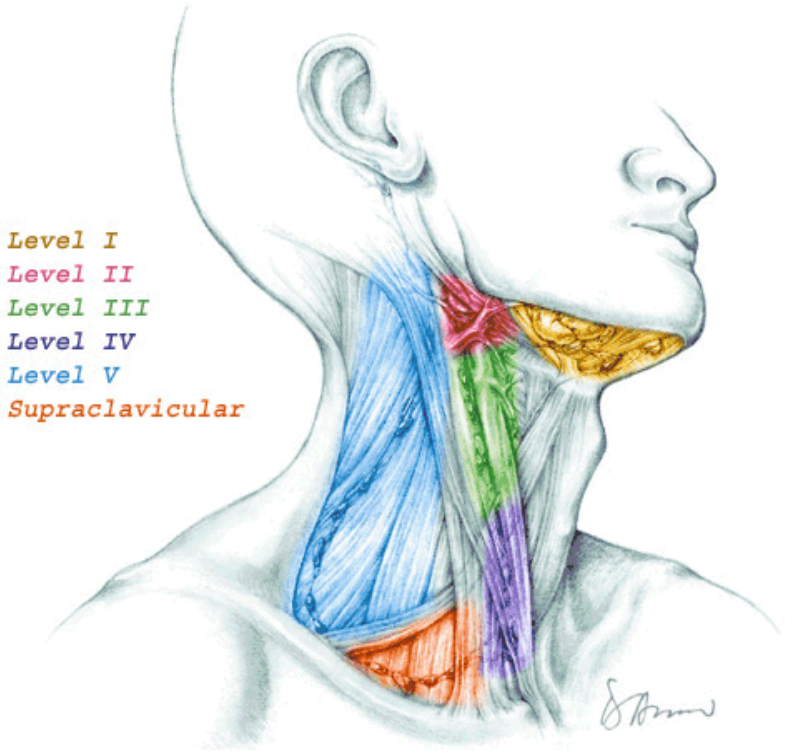


Figure 1.2**:** Schematic Illustration of the Nodal Classification

(Reprinted with permission from reference (Mukherji et al. 2001)

1.3 Epidemiology of Laryngeal Cancer

Carcinoma of the larynx accounts for 1.7% of all the newly diagnosed cancers in the world, 25% of all head and neck malignancies and in 90% of cases is squamous cell carcinoma (SCC) (Figure 1.3). Each year over 2,200 cases of carcinoma of larynx are diagnosed in the UK (Parkin et al., 1999). The incidence rate in the UK for carcinoma of larynx is 3/100,000 and the mortality rate is 1/100,000 per year (CRUK 2011) (Figure 1.4).

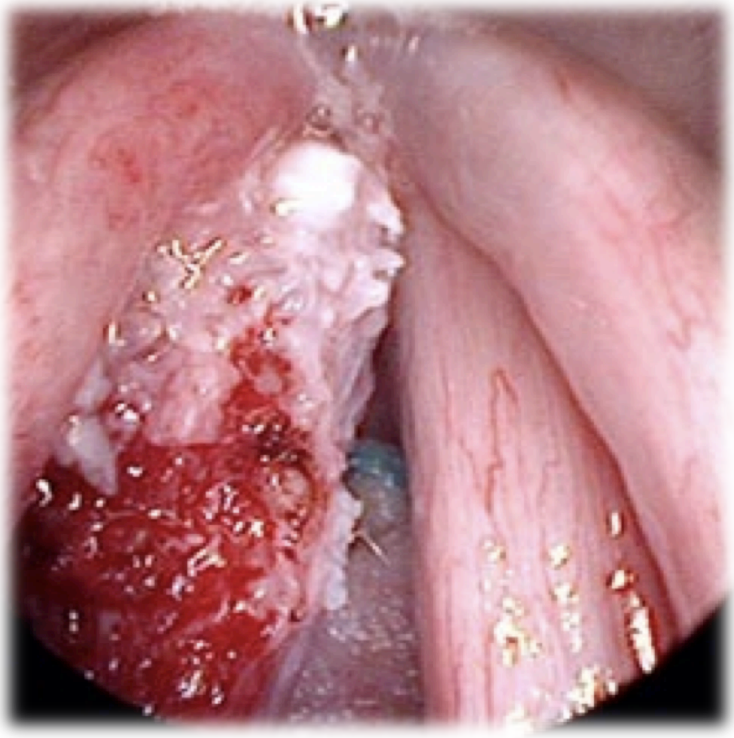


Figure 1.3**:** Cancer of left vocal cord

Source: http://stanfordhospital.org

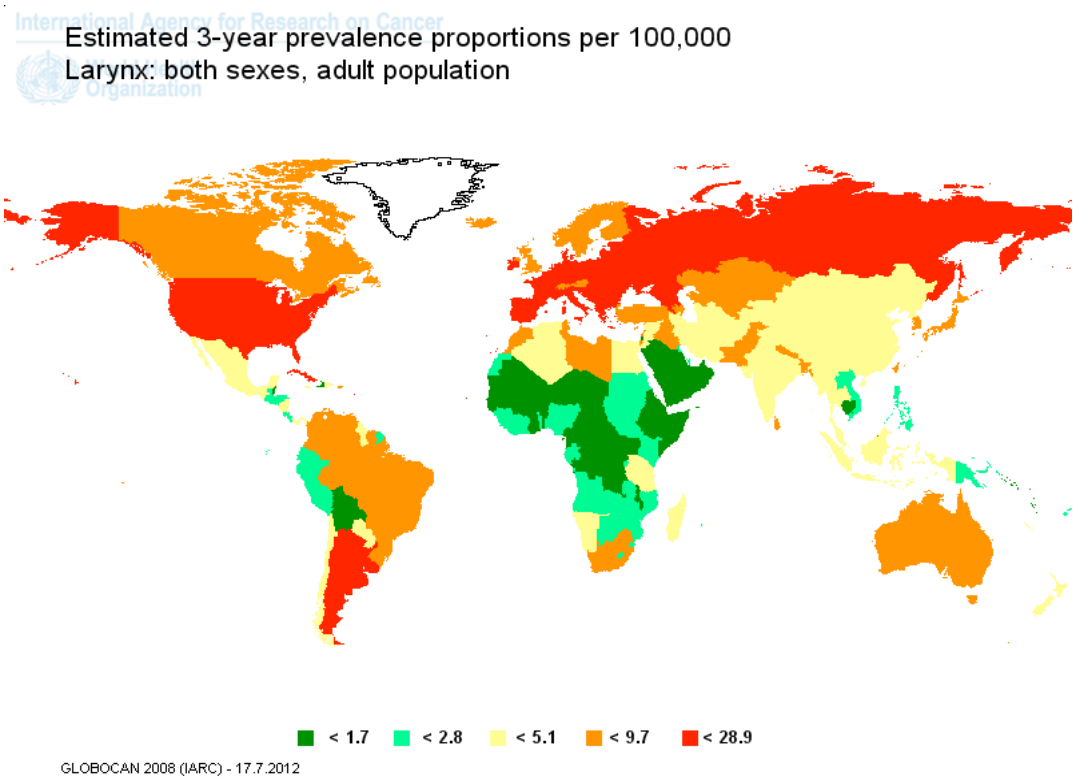


Figure 1.4: Global incidence / mortality rate of carcinoma of larynx (Globocan 2011)

LSCC accounts for more than 95% of all laryngeal malignancies and is the most common squamous cell carcinoma of the head and neck (Almadori et al., 2005; Cooper et al., 2009)**.** Laryngeal cancer is a disease mainly of middle aged and elderly men with a peak incidence in the sixth to eighth decade of life (Robin et al., 1991), women are found to be affected earlier in life than men. World wide it represents 10% of malignancies in men and 4% in women**.** The overall male to female ratio varies between 4:1 and 20:1 (Yang et al., 1989; Stephenson et al., 1991). Laryngeal cancer is the 18th most common cancer in males in the UK, with 1,890 new cases diagnosed in 2008 (Office for National Statistics, 2012) (Figure 1.5). Recent review of inequalities in laryngeal cancer survival in men and women in England and Wales showed five year survival for all laryngeal cancer combined was up to 8% lower in women than in men the study examined all the laryngeal cancer in England and Wales from 1991-2006, survival cancer of glottis has highest survival in both sexes because of early diagnosis. The substantial deprivation gap in laryngeal cancer survival in men is well known, recent review by Ellis et al suggest the this disparity in survival is primarily driven by tumours with an unspecified anatomical location, and partly by supraglottic tumours (Ellis et al., 2012)**.** The variation in incidence rates both between countries and between men and women are likely to reflect the variation in the prevalence of smoking and to a slightly lesser extent alcohol consumption as the combined effect of these two risk factors estimates to account for 89% of laryngeal cancer cases (Ferlay et al., 2010). It has been estimated that the lifetime risk of developing laryngeal cancer is 1 in 175 for men and 1 in 856 for women in the UK (Sasieni et al., 2011) and the overall five year survival for cancer of larynx in the UK is 67% (Coleman et al., 2004).

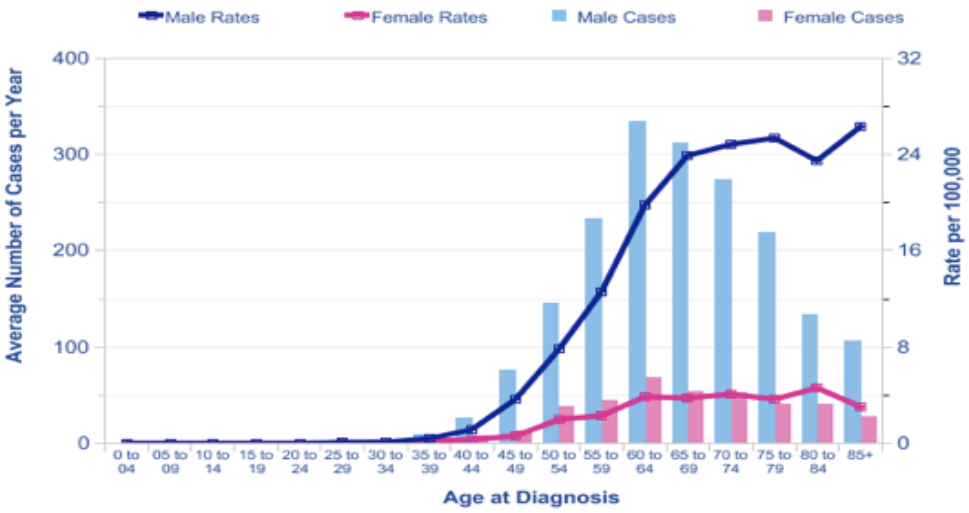


Figure 1.5: Laryngeal cancer, Average Number of New Cases per Year and Age-Specific Incidence Rates, UK, 2006-2008

The main anatomical sub sites of the larynx, as classified in the International classification of diseases for oncology (ICD-O 3rd edition), include supraglottic, glottis and sub glottis. Glottis tumours are most common, out numbering the supra-glottis by a ratio of 2:1 (Marck and Lupin 1989; Yang et al., 1989; Robin et al., 1991; Tuyns 1994). Glottic cancers predominate in men while tumours of the supraglottis tend to be more common in women (Coupland et al., 2009)

The anatomic site of occurrence of the tumour within the larynx influences the type of presenting symptoms. Glottis tumours normally present with hoarseness and are therefore typically small when detected. The supra glottis is a relatively clinically silent area, and as such, tumours at this site are often large by the time of diagnosis. Supra glottis tumours present with a change in vocal quality (muffled or hot potato voice), airway obstruction, dysphagia and/or cervical metastasis. Tumours of the pyriform sinus are usually large, typically present as odynophagia or referred otalgia.

The pathogenesis of HNSCC is a multistep process that generally begins with mucosal damage by carcinogens such as tobacco and alcohol multiplying genetic alterations. These alterations are seen in premalignant lesions, which can have a high rate of progression to malignancy (Neville and Day 2002). HNSCC, especially of the oral cavity and larynx, typically originates from a non-invasive neoplastic epithelial precursor lesion, the stages of pre-invasive proliferation, ranging from squamous hyperplasia through to carcinoma insitu are cumulative termed squamous intraepithelial lesion (SIL). Laryngeal dysplasia is a premalignant condition with between 2 and 10 lesions reported per 100 000 of the population. Recent systemic review and meta-analysis demonstrated overall malignant transformation rate is highly correlated with severity of grade of dysplasia, with the risk increasing threefold between mild/moderate dysplasia (10.6%) and severe dysplasia (30.4%) (Weller et al. 2010), with an overall transformation rate of 14%. Premalignant lesions that exhibit more advanced molecular changes, such as loss of heterozygosity, have a greater risk of progression to HNSCC (Epstein et al., 2002). As HNSCC develops from premalignant lesions in the field, cells exhibit alteration of cell surface markers; show increased dysplasia, loss of cellular organization and eventually invasion. Recent systemic review demonstrated no good evidence for the use of biomarkers in predicting the future behaviour of laryngeal dysplastic lesions (Nankivell et al., 2011).

The development of HNSCC involves the alteration in genes controlling the DNA repair, proliferation, apoptosis, invasion and angiogenesis. Specific genetic alterations were assigned to each step of the well-established adenoma – carcinoma sequence. Knowledge of the temporal relationships between genetic events has diagnostic and therapeutic implications in terms of targeted screening to detect premalignant transformation and early detection of tumour recurrence (Califano et al., 1996). These

can result in inactivation of the p53 tumour suppressor gene, inactivation of the cyclin-dependent kinase (CDK) inhibitor p16 and over expression of epidermal growth factor receptor (Hardisson 2003).

1.4 Aetiology

1.4.1 Smoking

Tobacco is the best known causative factor in laryngeal cancer, with 98% of the patients being smokers (IARC 1986 IARC 1988) (1986). The carcinogenic effect of tobacco smoke is correlated with the intensity and duration of smoking (Wynder et al., 1956; Tuyns et al., 1988). The relative risk of laryngeal cancer was 4.4 for smokers of up to ten cigarettes per day, rising to 34.4 for smokers of over 40 cigarettes per day, demonstrating dose-dependent relationship between smoking and incidence of HNSCC (Wynder et al., 1976). Tobacco smoke consists of a potent mix of more than 4000 chemicals, 43 of which have, to date been identified as carcinogens and include polycyclic hydrocarbons, nitrosamines and radioactive polonium-210. Bostara et al (1997) analysed 97 dysplastic lesions of the head and neck region, including 47 cases of laryngeal dysplasia, and found a direct relation between the degree of dysplasia, malignant transformation and amount of cigarette smoking and alcohol consumption. Gallus et al (2003) in his study, which included a large number of laryngeal cancers in women, provided definite evidence that cigarette smoking is a prominent risk factor for laryngeal cancer in women, since women accounted for 78% of cases in this population. Data on a decline in laryngeal cancer risk after cessation of smoking were presented by Vaezi et al. (2006), the authors found that increased risk might last for up to 40-45 years

after cessation of smoking, where as previous data have reported a decline in laryngeal cancer risk after 15 years of such cessation in the general population.

1.4.2 Alcohol

Alcohol is an independent risk factor for laryngeal cancer, where risk of the cancer increase fivefold with high alcohol consumption. The relationship between the occurrence of laryngeal cancer and alcohol consumption has been consistently demonstrated by several epidemiological studies (Tuyns et al., 1988). Alcohol seems to be more significant in the etiology of supraglottic than glottic cancer and its carcinogenic effect is also dose dependent. However there is no evidence that the effect is dependent on the type of alcoholic beverage (Wynder et al., 1956; Tuyns et al., 1988). Most of the studies have concluded that the joint effect of alcohol and tobacco is multiplicative (Guenel et al., 1988; Zatonski et al., 1991; Maier et al., 1992). Combined effect of the risk factors, alcohol and smoking is estimated to account for 89% of laryngeal cancer cases (Hashibe et al., 2009).

Increasing evidence has shown that acetaldehyde (AA) is responsible for the co-carcinogenic effect of ethanol. AA is formed during the metabolism of ethanol by alcohol dehydrogenase and aldehyde dehydrogenase. Numerous *in vitro* and *in vivo* experiments have shown that AA has a direct mutagenic and carcinogenic effect by inducing inflammation and metaplasia of tracheal epithelium, delaying cell cycle progression and enhancing cell injury associated with hyper-regeneration (Seitz et al.,

2001) when inhaled, AA has been shown to cause nasopharyngeal and laryngeal carcinoma (Woutersen et al., 1986).

Chronic alcohol consumption leads to an induction of cytochrome p-4502E1 (CYP2E1), which metabolizes ethanol to acetaldehyde. This cytochrome enzyme is also involved in the metabolism of various xenobiotics, including pro-carcinogens. Induction of CYP2E1 in the upper aero-digestive tract may be particularly relevant with respect to the pro-carcinogens present in tobacco smoke and the established synergism between them. Studies into its role in HNSCC have shown conflicting results, but recent meta-analysis suggested that individuals with the homozygote genotype of PstI / RsaI or Dral polymorphism might be associated with HNSCC, especially in the Asian population (Tang et al., 2010).

The type of alcoholic beverages consumed affects the risk of developing cancer. In a study population of 28,180 it was found that compared with non-drinkers, subjects who drank 7-21 units per week of beer and/or spirits but no wine had a relative risk of 3.0 of developing oropharyngeal cancer or oesophageal cancer. However, subjects who drank a similar amount but who also drank wine, which accounted for over 30 percent of their total alcoholic intake had a relative risk of 0.5. For those who consumed over 21 units per week the relative risks, including and excluding wine, were 5.2 and 1.7 respectively (Gronbaek et al., 1998). The meta-analysis looked into the risk associated with light alcohol drinking, has reported overall alcohol drinking versus non-/occasional drinking was associated with an approximately 2-fold increase in risk of laryngeal cancer (RR-1.90; 95%CI: 1.59-2.28). While light alcohol drinking (<1drink/day) did not show any significant association with risk of laryngeal cancer (RR-0.88; 95%CI: 0.71-1.08) and

heavy drinking (>4drinks/day) was associated with a 2.5-fold increased risk (RR-2.62; 95%CI: 2.13-3.23) (Islami et al., 2010)

1.4.3 HPV

The involvement of human papilloma virus (HPV) in head and neck carcinogenesis was first suggested by Syrjanen (Syrjanen et al., 1983). The overall published prevalence of HPV infection on approximately 1800 laryngeal cancer tested until December 2007 is around 24% (Syrjanen 2005; Torrente and Ojeda 2007). HPV laryngeal carcinomas harbour mainly high-risk HPV genotype with HPV-16 being the most frequent genotype (Torrente et al., 2005). High-risk oncogenic subtypes HPV-16, 18, 31, 33 and 35 have been shown to be capable of transforming oral epithelial cells through the viral oncoproteins E6 and E7 (Chow et al., 2010), which inhibits the actions of the tumour suppressor genes p53 and Rb respectively (Figure 1.6). The prevalence of HPV-induced head and neck cancers is increasing, while the prevalence of tobacco- and alcohol-induced cancers is declining (Nasman et al., 2009). In a meta-analysis, Hobbs et al. have shown a weak association between HPV-16 and laryngeal cancer (odds ratio (OR) 2.0. 95% confidence interval (CI) 1.0 4.2). In contrast, the association between HPV16 and cancer was the strongest for the pharyngeal tonsils (OR: 15.1, 95%CI 6.8 33.7) (Hobbs et al., 2006). At present the evidence linking HPV to laryngeal carcinoma is considered to be incomplete (Torrente and Ojeda 2007).

Many studies have now confirmed that HPV- positive tumours in the head and neck region have a better prognosis compared with those that are HPV negative (Fakhry et

al., 2008). In a recent paper by Lassen and colleagues, the overall 5-year survival rate was 62% in p16-positive patients compared with 26% in the p16-negative patients treated with radiotherapy (Lassen et al., 2009). This may be due to the fact that radiotherapy down regulates the E6/E7 oncogene expression releasing their inhibitors on the tumour suppressor gene thereby allowing apoptosis to occur (Betz et al., 1996)

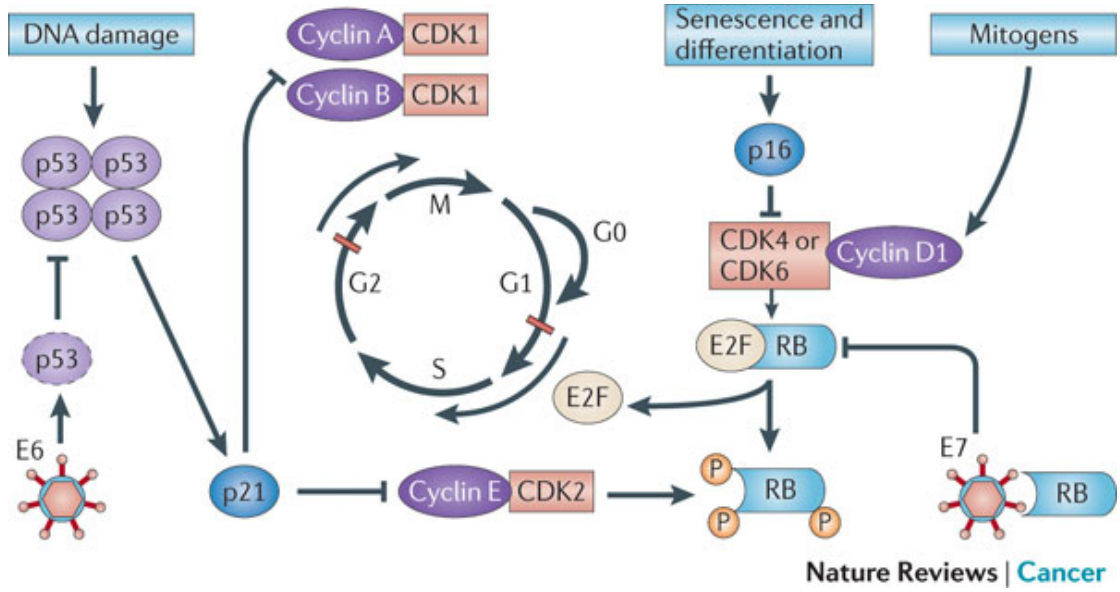


Figure 1.6: Cell cycle degranulation by human papilloma virus
(Ref: Leemans et al. 2011)

The cell cycle is regulated by complexes of cyclins and cyclin-dependent kinases (CDKs), some of which are indicated. In addition, there are various important inhibitors of these cyclin–CDK complexes. To allow cell cycle progression, cells have to pass the G1 restriction point that is controlled by the retinoblastoma pocket proteins, RB, p107 (also known asRBL1) and p130 (also known as RBL2). Only RB is indicated, but the other pocket proteins have similar activities. These normally bind to and inactivate the E2F transcription factors, which induce the expression of S phase genes. In response to a mitogenic signal, the cyclin D1–CDK4 and cyclin D1–CDK6 complexes are activated.

These phosphorylate the Rb pocket proteins, causing release (and therefore activation) of E2Fs. Induction of cyclin E by E2F and subsequent additional phosphorylation of RB by the cyclin E–CDK2complex initiates entry into S phase. The inhibitor for the cyclin D1–CDK4 and cyclin D1–CDK6 complexes is p16INK4A, which is encoded by CDKN2A, a gene in the INK4Alocus at chromosome 9p21. The expression of p16INK4A mediates senescence and differentiation. The interplay between the cyclins, CDKs and their inhibitors determines whether the restriction point can be passed, and a growth factor stimulus is usually required. A second important control mechanism of the cell cycle occurs during G2 phase, when the DNA has been replicated and replication errors are repaired. The key protein involved in the response to replication errors and other DNA damage is p53, which is usually maintained at low concentrations by MDM2-mediated degradation.

DNA-damage sensors, including ataxia-telangiectasia and ataxia-telangiectasia and Rad3-related (ATR), phosphorylate the checkpoint kinases CHK1 and CHK2, leading to increased p53 activity by phosphorylation of various downstream molecules, including p53 itself. The p53 tetramers act as a stress-induced transcription factor and induce the expression of p21CIP (also known as CDKN1A), which inhibits several cyclin–CDK complexes and halts the cell cycle. Besides its crucial role in cell cycle control, p53 is also a master regulator of apoptosis and many other stress-associated cellular functions, and is therefore one of the main targets for inactivation in many cancers.

The human papillomavirus (HPV) genome contains various early and late open reading frames and encodes two viral oncoproteins: E6 and E7. The E6 protein binds p53 and

targets the protein for degradation, whereas the E7 protein binds and inactivates the Rb pocket proteins. The molecular consequence of the expression of these viral oncoproteins. Is cell cycle entry and inhibition of p53-mediated apoptosis, which allows the virus to replicate. In a 'productive infection' the expression of E6 and E7 is confined to the differentiating layers of the squamous epithelium of the cervix and virions are produced. An oncogenic infection is associated with E6 and E7 expression in the basal layer (where the stem cells reside) and causes abrogation of the cell cycle checkpoints.

1.4.4 Gastro Esophageal Reflux Disorder

Gastro esophageal reflux (GERD) may cause laryngeal carcinogenesis (Ward and Hanson 1988; Freije et al., 1996). The association of GERD with laryngeal cancer was first suggested in 1983 by Oslon, (Oslon, 1983) who identified laryngeal cancer in a series of patients with GERD. Lewin et al (2003) then published the first study of laryngeal dysplasia and early cancer in relation to GERD. The authors additionally found a high incidence of GERD in patients with early carcinogenic changes in the larynx. A recent case control study by Tae et al (2011), performed 24 hr ambulatory double pH monitoring in 29 consecutive laryngeal cancer patients, and has identified prevalence of pathological laryngo-pharyngeal reflux was significantly higher ($p=0.049$) in laryngeal cancer patients than the control group. This study concluded that laryngo pharyngeal reflux might be a possible risk factor in the development of laryngeal cancer. Similar data from Ciani et al (2003) also showed that of 93 patients with gastric resection, seven had current precancerous lesions in the control group, in contrast, only one patient showed mild dysplasia of the vocal cord. Kaufman and Burke (1997) reported a marked increase in abnormal pH studies in the patients with early

laryngeal carcinoma. They reported 31 consecutive cases of laryngeal carcinoma in which LPR was documented in 84%, but only 58% were active smokers. There is also a subgroup of non-smokers in whom reflux appears to be the only risk factor.

Occupational exposures including asbestos, wood and cement dust all increase the risk of laryngeal cancer (Smith et al., 1990; Gustavsson et al., 1998). Recent meta-analysis found some evidence that subjects exposed to polycyclic aromatic hydrocarbons (PAHs) has significantly increased risk of developing laryngeal cancer (meta-RR 1.29; 95% CI 1.10-1.52), similar association was noted with other industrial exposure like engine exhaust (meta-RR 1.17; 95% CI 1.05-1.30), the textile industry and rubber industry (meta-RR 1.41: 95% CI 1.05-1.90), but exposure to wood dust, formaldehyde and cement dust were not significantly associated with laryngeal cancer (Paget-Bailly et al., 2012).

In summary cigarette smoking and alcohol appear to be the primary causative factors for laryngeal cancer. Prevention should be focused largely on smoking cessation and limiting alcohol consumption

1.5 Genetic alteration in cancer of larynx

Genetic factors play a significant role in the development of HNSCC in common with other tumours, with several oncogenes having been implicated in HNSCC oncogenesis. Aberrant expression of the members of the *ras* and *myc* families' *ins*-2, *hst*-1, cyclinD1 epidermal growth factor receptor, bcl-2 and bax are all believed to contribute towards

cancer development. High frequency of loss of heterozygosity involving chromosomal regions 3p, 5q, 8p, 9q, 9p, 11p and 11q have been reported in HNSCC with highest frequencies occurring on 3p, 9p and 17p (Ah-See et al., 1994) all of which are associated with the development and progression of disease (Califano et al., 1996). Patmore et al (2007) investigated the genetic profiles of 68 carcinomas and noted that -4p, +8q, +12q and -18q were significantly associated with laryngeal SCC when compared with hypopharyngeal and oropharyngeal SCC. The most frequent and relevant cellular changes in laryngeal carcinogenesis are those involving p53, cyclinD1 (CCDN1), p16 and EGFR and these are discussed below.

1.5.1 p53

Nuclear phosphoprotein p53 is one of the most widely studied molecular markers in HNSCC. It has been defined as 'the guardian of genome' (Lane 1992) because of its importance in coordinating the cells response to DNA damage, by inducing cell cycle arrest and apoptosis, thus protecting cells against somatic mutation. p53 over expression has been reported in laryngeal SCC at a frequency ranging from 38%-79% of the tumour samples analyzed (Pruneri et al., 1996; Narayana et al., 1998; Pai et al., 1998). According to Boyle and co-workers, p53 inactivation occurs in the transition from pre-invasive to invasive form. Some others argue the opposite, presenting alterations of p53 among early steps of neoplastic transformation.

Loss of heterozygosity of p53 and the presence of tobacco carcinogenin-induced inactivating mutations in the coding sequence of p53, or the accelerated destruction of

its protein product, p53, by viral oncoproteins, such as by HPV E6 represent common molecular alterations in HNSCC (Forastiere et al., 2001; Mao et al., 2004).

p53 mutations have been hypothesized to be the earliest event in the development of a genetically altered field, identifying an area of clonally related cells with a malignant potential (Braakhuis et al., 2003). p53 over expression, detected by IHC in a high percentage of laryngeal squamous cell carcinomas (Anwar et al., 1993) was hypothesized to correlate well with p53 mutation (Maestro et al., 1992). But recent studies suggested p53 gene mutation is more reliable than IHC over expression for characterization and it has been reported to predict the response to radiotherapy in LSCC patients (Taylor et al., 1999). p53 alterations have been proposed as independent predictors of the recurrence in LSCC (Narayana et al.,1998).

1.5.2 Cyclin D1

Chromosome region 11q13 has been identified as the site of several putative oncogenes, such as Bcl-1, int-2, hst-1, EMS-1 and cyclinD1/prad1. Amplification of 11q13 is detected in approximately one–third of HNSCC. But only cyclin D1 (CCDN1) has shown consistent over expression/amplification. The function of cyclin D1 is to activate Rb via phosphorylation, thus facilitating progression from the G1 phase to S phase. Despite these related, but opposite functions, the loss of p16 gene and gain of cyclinD1 are independent mechanisms leading to cell cycle dysregulation. Over expression/amplification of CCDN1 has been associated with the advanced stage of the disease and could indicate more aggressive behaviour of such HNSCC. CCDN1 is a member of the cyclin family of regulatory proteins involved in cell cycle progression,

which interacts with cyclin-dependant kinases. CCDN1 gene amplification and over expression were studied in HNSCC patients at the same time in tumour and peri-tumour tissues (Izzo et al., 1998). When CCDN1 amplification is observed, CCDN1 is always over expressed. Almadori et al (Almadori et al., 2004) hypothesise that in early phases of tumourigenesis, altered p53 gene function (Roh et al., 2000), and CCND1 gene over-expression (Roh et al., 2000) increases the genetic instability and promotes further genetic and chromosomal alteration such as CCND1 amplification (Izzo et al., 1998) which is considered by some authors key for the ultimate transforming event by the selection of a malignant sub clone from a genetically altered field (Braakhuis et al., 2003).

1.5.3 EGFR

Epidermal growth factor receptor (EGFR) is a trans-membrane glycoprotein belonging to the ErbB/HER family of receptor tyrosine kinases located on chromosome 7p12. It is composed of an extracellular ligand-binding domain, a hydrophobic trans-membrane segment, and an intracellular TK domain. Activation of EGFR leads to activation of intracellular signalling pathways that regulate cell proliferation, invasion, angiogenesis and metastases (Baba et al., 2012).

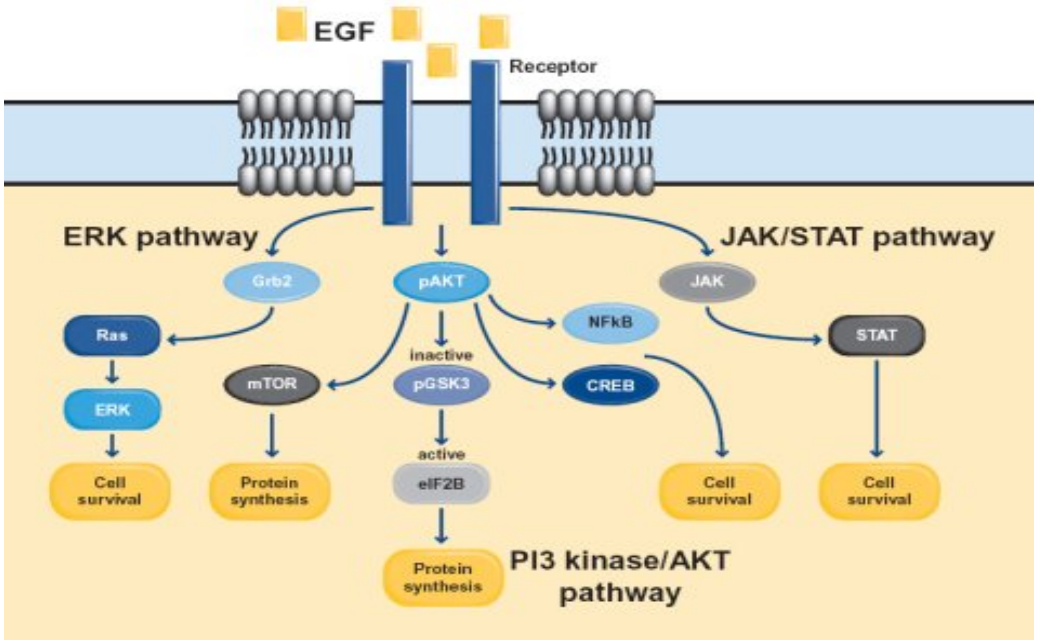


Figure 1.7: Overview of the EGF signalling pathway.
(Source: Epidermal growth factor and cancer by c cooper et al., \ 2009)

Activation of the EGF receptor results in autophosphorylation of key tyrosine residues. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains and leads to the activation of downstream signalling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway (Figure 1.7), the phosphatidylinositol 3-kinase (PI3) pathway and the Janus kinase/Signal transducer and activator of transcription (JAK/ STAT) pathway. These pathways act in a coordinated manner to promote cell survival.

Epidermal growth factor receptor promotes growth of epidermal cells and regulates cell proliferation. EGFR is often related to cancer due to mutations or over expression leading to aberrant signalling and resultant excessive proliferation (Hynes and Lane 2005; Lai and Johnson 2010). The EGFR gene is found to be amplified in 25%, and its mRNA was over expressed in 43% of laryngeal SCCs. Half of the expressed cases

occurred in the absence of detectable gene amplification. Both alterations appeared in advanced laryngeal SCC. Furthermore, over expression of EGFR protein is an early event in carcinogenesis, rising with increasing degree of epithelial abnormalities, mainly in the progression of squamous intraepithelial lesions to SCC. Up to 90% of HNSCC exhibit over-expression of EGFR leading to increased tumour proliferation. EGFR over-expression is an indicator of poor prognosis for HNSCC and the importance of EGFR has lead to the development of anti EGFR therapies, namely Cetuximab an anti-EGFR monoclonal antibody, to the treatment of locally advanced and recurrent/metastatic disease due to its critical roles in cell survival and proliferation (Burtness 2005). These molecules are reversible competitors, competing with adenosine triphosphate (ATP) for the tyrosine kinase-binding domain of EGFR. Inhibition of receptor activation inhibits downstream signalling pathways, resulting decreased cell proliferation and survival (Baba et al., 2012).

1.5.4 c-erb B-2

The human c-erb B-2 gene, located on chromosome 17 is a 185 kDa glycoprotein with tyrosine kinase activity and shares extensive structural similarity with the epidermal growth factor receptor. The oncogenic potential of c-erb B-2 can be released by point mutations, truncation of extracellular and cytoplasmic domains, and amplification and /or over expression of the normal gene (Weinstein et al., 1996; Krecicki et al., 1999; Tantawy et al., 1999). Over-expression of the c-erb-B2 protein has been found in several human cancers (breast, ovary, and pancreas). In recent years, c-erb-B2 over expression in LSCC has been reported in 34 to 55% of tumours (Weinstein et al., 1996; Krecicki et al., 1999; Tantawy et al., 1999). Prospective Study by Tantawy et al in 34 patients with laryngeal carcinoma identified over expression of c-erb B2 by IHC. Over-

expression of c-erb B2 was related to more aggressive tumours with high capability of invading cartilages, with poor prognosis in patients with laryngeal cancers.

1.6 Histology

In 1976, Fertilo proposed the classification of the laryngeal carcinoma. Squamous cell carcinoma (SCC) is by far the most common histological finding in laryngeal malignancy, accounting for more than 90% of laryngeal cancer in most studies (Krecicki et al., 1998). The second most common malignancy of the larynx is lymphoma. Non-squamous tumours comprise a small subset of laryngeal malignancies, and are more likely presenting supra glottis or sub glottis than in the glottis. Salivary gland lesions of the larynx account for approximately 1% of laryngeal malignancy. Tumours also rarely arise from the soft tissue and supporting structures of the larynx and include chondrosarcoma, fibrosarcoma and lymphoma (Nicolai et al., 1990).

Tumours are traditionally graded according to their degree of differentiation to well-, moderately and poorly differentiated SCC. Well-differentiated SCC closely resembles normal squamous epithelium. Moderately differentiated SCC contains distinct nuclear pleomorphism and mitotic activity, including abnormal mitoses. In poorly differentiated SCC, immature cells predominate with numerous typical and atypical mitoses and minimal keratinisation.

1.7 Staging

The objective of the any staging system is to aid the clinician in planning treatment, to give some indication of prognosis, to assist in evaluation of results of treatment and to

facilitate the exchange of information of between treatment centres, thus providing a method of conveying clinical experience. The staging of cancers of the head and neck is done via the tumour, node, and metastasis (TNM) system for describing the anatomical extent of disease and is based on the assessment of three components

T: extent of primary tumour

N: status of regional lymph nodes

M: presence or absence of distant metastases

The addition of number to these three components indicates the extent of malignant disease. TNM staging system of the American Joint Committee on Cancer (AJCC) (Appendix 2) Stages I and II are considered early, while stages III and IV are considered late. Thus, T1 or T2 tumours without clinical or radiologic evidence of metastasis are early stage tumours. Tumours that are T3 or T4 and/or tumours of any T stage associated with regional (N1, N2, or N3) or distant (M1) metastasis are designated as advanced staged carcinoma (Appendix 2) (Sobin et al., 1997; Hermanek et al., 1987).The limitation of T staging in carcinoma of larynx is, the poorer prognosis with increased T stage is explained by the increasing propensity towards nodal metastasis with large tumours**.** If the nodal metastases are removed as a confounding factor then T stage per se does not influence prognosis. Tumours of the larynx are classified according to the number of anatomical surfaces involved. This has led to problems e.g, a large 3cm tumour of supra glottis may still remain T1, whereas in the glottis this would almost certainly be a T3. In addition depth of invasion is not measured, but is of prognostic and therapeutic importance. Therefore the TNM system, although useful, has limitations.

1.8 Tumour progression

It is generally accepted that development of cancer is a multistep process resulting from the sequential accumulation of genetic and epigenetic defects and the clonal expansion of selected cell populations. Papers by Califano and Sidransky (Glazer et al., 2009; Ha et al., 2009) (Figure 1.8) showed that in HNSCC tumour progression involved changes in genetic alterations from dysplasia (9p21, 3p21, 17p13), to carcinoma in situ (11q13, 13q21, 14q31) and finally invasive tumours (4q26-28, 6p, 8p, 8q). Among them, loss of chromosomal region 9q21 is found in 70% of dysplastic lesions of the oral mucosa and together with the inactivation of the remaining alleles of p16 and 14 by promoter hyper-methylation, represent one of the earliest and most frequent events in HNSCC progression (Califano et al., 1996; Forastiere et al., 2001).

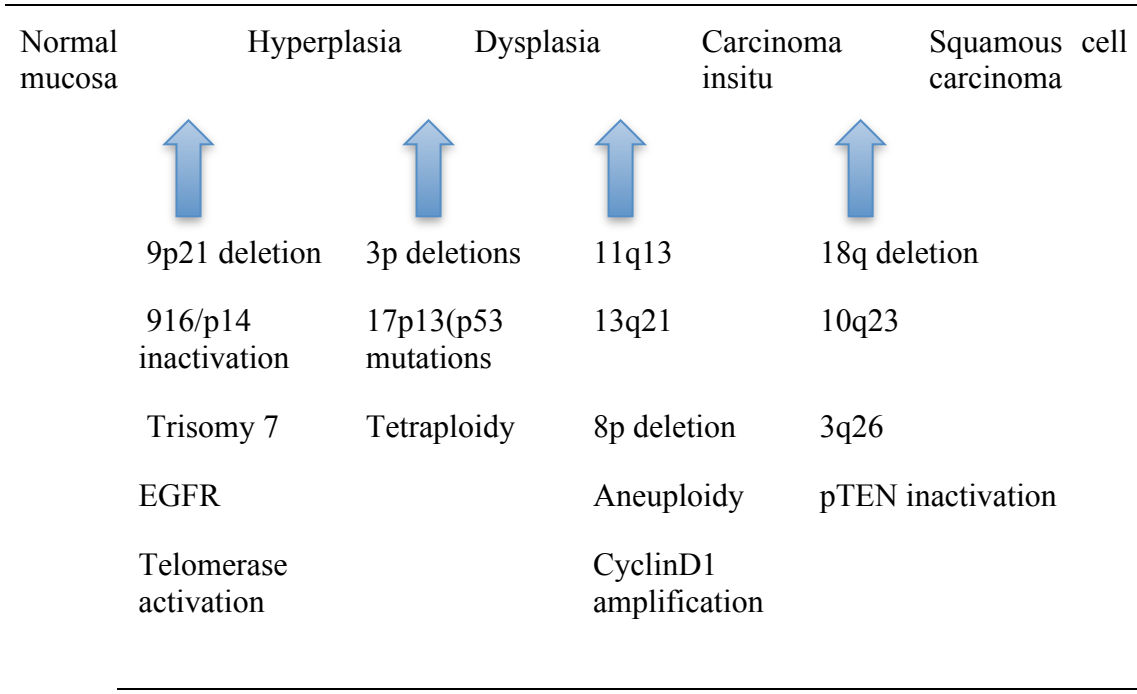


Figure 1.8: Hypothetical model for HNSCC carcinogenesis, modified from Califano et al Ref: J clin pathol, B Perez-Ordonez (2006)

Loss of chromosomal region 9p21 is found in 70-80% of cases, thus representing the most common genetic alteration seen in squamous dysplasia of the head and neck and has been found in pre-neoplastic lesions, including 30% cases of squamous hyperplasia (Yoo et al., 2004). Additional studies of microsatellite DNA allelic imbalance in laryngeal carcinogenesis have confirmed that dysplasia correlates with loss of heterozygosity (LOH) at 3p21, 5q21, 9p21 and 17q13 (Sanz-Ortega et al., 2007) (Table 1.1). The CDKN2A gene locus found in chromosome 9p21 encodes two different transcripts; p16 and $p14^{ARF}$ that are responsible for G1 cell cycle regulation and MDM2 mediated degradation of p53. P16 is often inactivated in HNSCC through homozygous deletion, by promoter methylation, and less commonly by point mutations.

LOH9P	70-80%
LOH 3P	60-70%
LOH 17P	50-60%
LOH 11q	30%
LOH 13q	30%
Inactivation of FHIT and RASSF iA P53 mutation	50-80%
Cyclin D1 amplification	30%
Inactivation of $p16i^{nk4A}$	80%

Table 1.1: Frequent molecular abnormalities in head and neck SCC

Histological progression is marked by increasing genetic instability (Voravud et al., 1993), increasingly deregulated proliferation (Shin et al., 1993) and increasingly abnormal activation of key regulatory molecules such as epidermal growth factor receptor and telomerase catalytic subunit (Shin et al., 1994; Hohaus et al., 1996).

The increased invasiveness of developing HNSCC tumours has been associated with a wide variety of cell surface receptors and transcription factor- associated alterations. HER2 and EGF receptors (O'Charoenrat et al., 2001; Uno et al., 2001) are both highly expressed on premalignant tissues of certain HNSCC tumours in comparison to normal epithelial tissue. Rosin et al showed that loss of heterozygosity at 3p and, or 9p represented an essential step in progression (Rosin et al., 2000) Over-expression of EGFR is associated with an increased degradation of the extracellular matrix by metalloprotease and cathepsin D, which plays an important role in tumour growth, invasion and metastasis, as well as tumour induced angiogenesis (Johansson and Kahari 2000; Almadori et al., 2004) and is potentially correlated with invasiveness.

1.8.1 Field Cancerization

Slaughter et al (1953) described the theory of 'field canceriztion', to explain the high propensity to develop recurrences after treatment of HNSCC and the high likelihood that multiple independent tumours will develop in the head and neck mucosa. This hypothesis proposed that long term carcinogenic exposure results in 'condemned mucosa' containing many mutated cells, from which multifocal independently arising tumours develop. The field cancerization process has been divided into three phases

(Braakhuis et al., 2003), in the initial phase, a stem cell acquires genetic alterations and forms a 'patch', a clonal unit of altered daughter cells; it could be recognized on the basis of mutation in the p53 gene. The conversion of patch into an ' expanding field' is the next critical step which requires additional genetic alteration which confers growth advantage to one or more sub clones and allows them to proliferate reaching dimensions of more than 7cm in diameter. In an expanding field, clonal divergence can lead to the development of several different 'malignant tumour' (third phase) over years (Bellacosa et al., 1996).

1.8.2 Angiogenesis

Angiogenesis is defined as an outgrowth of new endothelial cells from mature pre-existing endothelial cells. In order for the tumour to grow beyond 2-3mm, it requires the development of new vasculature a process known as angiogenesis as described in detail in section 1.11.

1.8.3 Metastasis

The metastatic process for HNSCC appears to be similar to that for other solid tumours, with a sequential process of initiation of tumour growth, invasion beyond the basement membrane into sub mucosal tissues, vascular and lymphatic invasion, systemic transport, exit from the circulatory system at a distinct organ, invasion of the tissues at this site and further growth. HNSCC lesions are generally highly vascular and have an enhanced lymphatic vasculature to facilitate drainage from the area (Beasley et al.,

2002). Furthermore, HNSCC lesions have the ability to alter immunological responses by recruiting immunosuppressive cells and secreting immunosuppressive factors in the region to evade the host's immune system defences (Young 2006). The combination of the increased immune suppression and vascularisation allows the tumour to take advantage of the increased lymphatic's to spread locally and regionally.

1.8. 4 Lymphatic spread

The evidence demonstrated that, usually, the lymphatic tumours spread to the neck follow predictable pathways. Overall prevalence of occult neck metastasis in laryngeal carcinomas is about 25%, varying with the subsite of origin and with the T stage. As reported in the literature (de Campora et al., 1994), the incidence of occult metastasis, in electively dissected neck, is about 30% for supra-glottic carcinoma and 20% for T3-T4 glottic carcinoma. Lymphatic spread is the major mode of metastasis in HNSCC. The presence of lymph node metastasis is the strongest single predictor of disease specific survival at the time of HNSCC diagnosis (Becker et al., 2004). Patients with pathologically positive nodes have a 50% lower cure rate than patients without nodal metastasis (Becker et al., 2004). An additional poor prognostic sign is the presence of extra-capsular spread of tumour outside the confines of the lymph node which occurs in >60% of nodes larger than 3cm and up to 23% of nodes smaller than 1cm (Puri et al., 2003). In a recent meta-analysis, patients with extra capsular spread were 2.7 times more likely to die from regional and distant recurrence within 5 years compared with patients without extra nodal spread (Dunne et al., 2006). PET CT provides anatomic localization of areas of increased glycolysis which may correspond to rapidly proliferating tumour cells of volumes greater than 5mm. Currently CT with intravenous

contrast is the imaging modality of choice due to greater sensitivity at identifying suspicious cervical lymph nodes compared with other imaging techniques (Puri et al., 2003).

Lymphangiogenesis is defined as outgrowth of new lymphatic endothelial cells (LEC) from mature pre-existing lymphatic endothelial cells. A variety of signals promote lymphagiogenesis include members of the VEGF family, angiopoitin 1-and 2, neuropilin-2, hepatocyte growth factor, FGF-2, and IGF1 and -2. While angiogenesis is promoted predominantly through the VEGF-A protein, VEGF-C and –D are potent inducers of LEC proliferation and migration, which may be enhanced by neuropilin-2 binding (Zeng et al., 2006).

In summary the initiation, growth, invasion and direct or lympho-vascular spread of head and neck SCC is known to result from a multistep process that may involve both tumour-specific and patient molecular, genetic, and immunological factors (Table 1.2) The progression from local tumour to regional and finally distant disease correlates with a dramatic drop in survival.

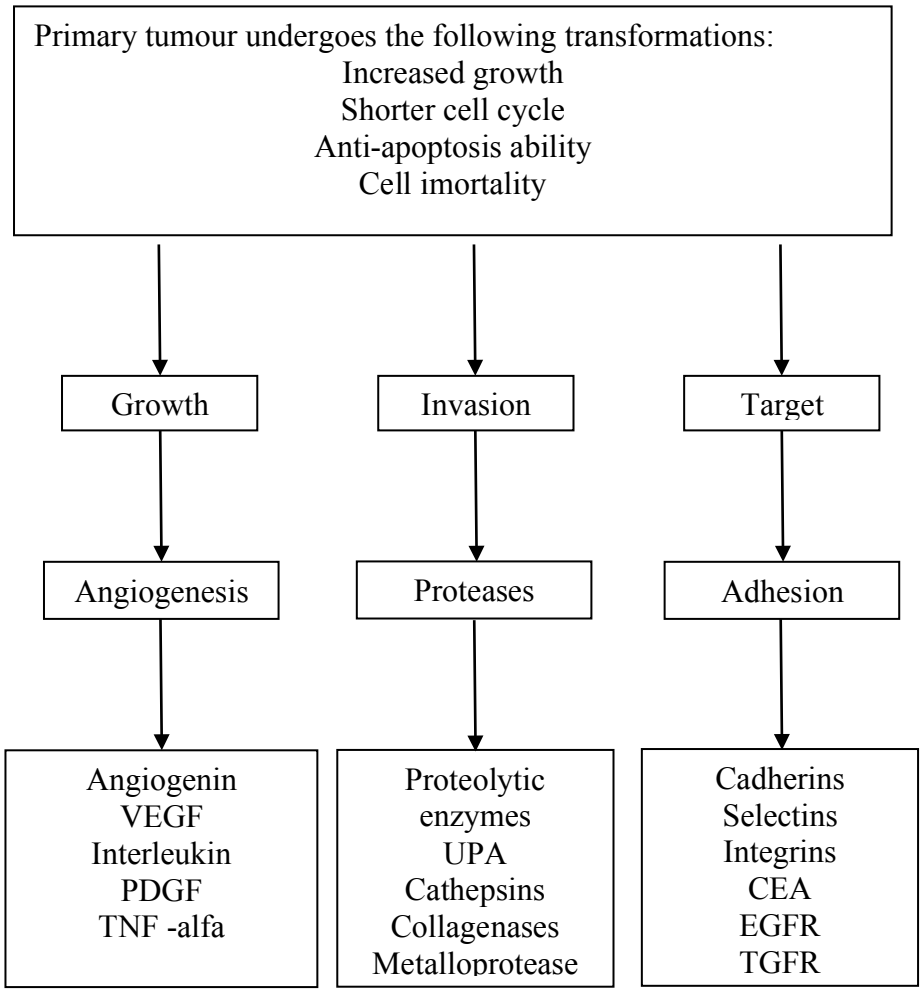


Table 1.2**:** Three primary factors associated with tumour cells undergoing invasion and metastasis.

1.9 Treatment

Treatment of laryngeal carcinoma is complex because of the crucial functions of the anatomic area. Laryngeal cancers diagnosed and treated at an early stage (stage I or II) are associated with high cure rates. Higher staged cancers (stage III or IV), on the other hand, result in poor outcomes in terms of cure and function (Beasley et al., 2002).

Early staged laryngeal cancer is usually managed with a single modality: radiotherapy, Tran's oral laser resection, or conservation laryngeal surgery. Advanced laryngeal cancer generally requires multimodality therapy; thus, different combinations of surgery, radiotherapy, and chemotherapy may be considered (Shah et al., 1997; Klem et al., 2008). The tumour characteristics that influence the choice of treatment include tumour site and tumour T stage (e.g., tumour extent, vocal cord mobility, thyroid cartilage invasion). Intent of the treatment is primarily to eradicate the tumour and secondly to preserve the function of the larynx as much as possible.

Dey et al (2002) in his recently updated (2011) systemic review comparing radiotherapy, open surgery and endolaryngeal excision for early (T1, T2a) glottis laryngeal cancer, evaluated one of the four randomized control trials comparing surgery *vs*. radiotherapy which has included substantial number of patients with glottis cancer with five year follow up has demonstrated open surgical outcomes were better whilst radiotherapy outcomes were worse. But interpretations are limited by concern about the adequacy of treatment regimens and deficiencies in the reporting of the study design and analysis. Although randomized controlled studies have not compared the treatment outcomes for early stage laryngeal cancer, organ– preservation approaches, including function–preserving partial laryngectomy, radiation, and chemo radiation, are considered acceptable treatment options for these patients (Pfister et al., 2006).

Primary surgical treatment should be considered in patients with high volume disease, patients with T4a tumours, or patients with anticipated poor functional outcome e.g. poor voice, intractable aspiration. Both induction chemotherapy followed by irradiation and concurrent chemotherapy and radiotherapy have been reported as valuable

alternatives to total laryngectomy in patients with advanced larynx cancer. A landmark study conducted within Veterans affairs (VA) hospitals, demonstrated that an organ-preservation is possible without reduction of survival in patients with locally advanced laryngeal cancer with induction chemotherapy followed by radiation (1991). Subsequent studies (Forastiere et al., 2003) by the radiation oncology group (RTOG) reported high laryngeal preservation rates with concurrent chemoradiation when comparing three different organ preservation approaches (induction chemotherapy followed by radiation, radiation alone, and concurrent chemo radiation) which yielded similar 2-year and 5-year survival rates, but concurrent chemo radiation resulted in the highest laryngeal preservation rate of 88%. These studies together with other studies (Richard et al., 1998; Lefebvre et al., 2009), have established organ preservation approaches as the standard of care for many patients with locally advanced laryngeal cancers. A recent population based study has analysed 13,808 patients who were diagnosed with localized and/or regional glottis and supra-glottic cancer and demonstrated increased use of radiation with advantage of speech preservation had no adverse impact on patients' survival with laryngeal cancer (Zhang et al., 2012).

1.9.1 Radiotherapy (RT)

Early and localized laryngeal cancers (stage I and II) are treated with definite radiotherapy. A randomized control trial which included all HNSCC, showed evidence of trend towards increasing benefit with more advanced T stage with the use of continuous, hyper fractioned accelerated radiotherapy for laryngeal cancers by comparison with conventional radiotherapy regimes (Dische et al., 1997).

Post-operative radiotherapy is delivered to the primary site and/or neck in patients at high risk of loco-regional recurrence, and can improve loco-regional control and survival. RT is recommended for pT4 laryngeal cancers of any nodal stage, pT1/T2/T3 tumours with N2-N3 nodal stage and for all patients with positive resection margins and/or extra-capsular spread: other unfavourable pathological factors, including perineural and vascular invasion, are relative indications for postoperative RT. Administration of concurrent cisplatin chemotherapy with post operative RT improves loco regional control and disease free survival compared to postoperative RT alone for locally advanced tumours.

1.9.2 Chemotherapy:

The recently updated meta–analysis of chemotherapy in head and neck cancers, which used the individual patients data of 16,845 patients included 87 randomized trials performed between 1965 and 2000, provides level one evidence of a significant benefit of the addition of chemotherapy in terms of an overall survival. The hazard ratio of death is 0.88 ($p<0.0001$) with an absolute benefit for chemotherapy of 4.5% at 5-years with no difference of treatment benefit effect is observed according to tumour site (Pignon et al., 2000; Pignon et al., 2009). But Recent meta-analysis on benefit of chemotherapy with tumour sub site in HNSCC, which included 87 RCT which used individual patient data of 16,192 patients showed benefit of the addition of chemotherapy to loco-regional treatment is consistent with in all tumour locations of HNSCC and highest benefit of concomitant schedule was demonstrated only for oro-pharyngeal ($p<0.001$) and laryngeal tumours ($p<0.05$) (Blanchard et al., 2011).

1.9.3 Management of Neck

The main predictor of survival in HNSCC is the presence, number and extra capsular spread of lymph node metastases rather than size of the primary tumour (Forastiere et al., 2003).

N0: Elective neck dissection is commonly performed for the management of node negative, T2-4 supraglottic cancer. The operation of choice has often been the modified radical neck dissection. Shah et al (1993) have set an arbitrary threshold for performing neck dissection in clinically negative necks of 20 percent and this was supported by an earlier decision analysis report (Weiss et al., 1994).

Gregor et al in his retrospective study showed loco regional control was equivocal for the N0 patients. But HPL showed better results in loco regional control for the N+ patients. The study included a total of 89 patients of supra glottis cancer, of which 26 patients were treated with horizontal partial laryngectomy (HPL), 44 by primary radiotherapy and 19 patients by total laryngectomy.

In early stage disease, the N0 neck should only be electively treated if the supraglottis is involved, as there is a 60% risk of occult disease. In N1 disease, the neck should receive the same treatment as the primary. In advanced nodal disease, if the primary is treated surgically, then a neck dissection should be performed with consideration of postoperative radiotherapy. (Mackenzie 2012, Stell and Maran's textbook of head and neck surgery and oncology).

1.9.4 Treatment of recurrent disease

Approximately half of the patients will develop local, regional or distant relapse, which usually occur within the first 2-5 years of treatment (Argiris et al., 2008). The only treatment option for recurrent HNSCC is systemic chemotherapy that has a particularly intolerable toxicity to HNSCC patients who usually have problematic lifestyles and various morbidity problems (Goon et al., 2009).

1.9.5 Future treatment trends / Targeted therapy

As the mechanism of HNSCC initiation, progression, invasion, spread, and distant metastasis are becoming unveiled, new opportunities arise for target intervention. Targeted therapies are designed to treat only the cancer cells and minimise damage to normal, healthy cells. Cancer treatments that "target" cancer cells often have fewer side effects and are more effective than traditional chemotherapy drugs and radiation treatments.

New agents that specifically target cellular pathways associated with carcinogenesis are promising candidates, because they are already successfully used in other haematological malignancies as well as in solid tumours, such as colorectal and lung cancer (Segal and Saltz 2009). HNSCC lesions are generally very vascular, and have enhanced lymphatic vasculature to facilitate drainage from these areas (Beasley et al., 2002). Therefore, one of the effective pathways to target for HNSCC therapy will be tumour angiogenesis. Two primary strategies that might have the potential to change the

clinical routine within the near future are first blocking epidermal growth factor based cellular signalling (EGFR-associated) and second, blocking the angiogenesis related cellular signalling (VEGFR –associated).

Among EGF-R targeting therapies, there are two categories of molecules: monoclonal antibodies, which recognize the ligand–binding domain and interfere with receptor activation, and tyrosine kinase inhibitors which bind to the cytoplasmic region and influence with downstream signalling events. Two of the potential EGFR targeting strategies are currently in clinical use: monoclonal antibodies (mAbs) directed at the extracellular domain of the receptor (cetuximab, matazunab), and the small molecule and ATP-competitive tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib).

FDA approved Cetuximab in combination with radiation for the treatment of loco regionally advanced HNSCC in 2006; in addition, Cetuxiamb was approved as a single agent for the treatment of patients with recurrent or metastatic HNSCC for whom platinum based therapy had failed. Multinational randomized trial (Bonner et al., 2006) comparing radiotherapy with combination of cetuximab with radiotherapy showed addition of cetuxiamb significantly increased 5 year overall survival, and benefit was more pronounced in patients with under 65 (Bonner et al., 2010). Cetuxiamb has also recently shown to improve the outcome of palliative chemotherapy in a large randomized phase III study. In this study in 442 eligible patients with untreated or metastatic HNSCC, 222 patients who received chemotherapy plus cetuxiamb showed prolonged overall survival compared to patients who received only chemotherapy (Vermorken et al., 2008).

Gefitinib, Tyrosine kinase inhibitor, is a trans-membrane glycoprotein whose intracellular domain has TK activity, a phase III trials conducted to compare survival in patients with recurrent or metastatic HNSCC treated with gefitinab or methotrexate neither gefitinab improved overall survival compared to with methotrexate (Siu et al., 2007). EGFR TKIs have limited results in patients with HNSCC for example; a phase II trial of geftinib in patients with recurrent or metastatic HNSCC showed an overall response rate of 11%. Smilarly, a study of erlotinib in patients with recurrent and /or metastatic HNSCC showed a response rate of 4%. Several mechanisms have been proposed to explain tumour resistance to EGFR TKI which include Ras mutations, epithelial-mesenchymal transition, upregulation of Cyclin D1 and p13 kinase/Akt pathway (Baba et al., 2012). A phase II trial with Elotinib, an orally, potent reversible and selective inhibitor of EGFR TK, with 150 patients with locally recurrent and metastatic HNSCC showed an overall response rate of 4% indicating that this drug as a single agent slightly effective (Soulieres et al., 2004).

Bevacizumab, a humanized monoclonal antibody directed against VEGF, was the first antiangiogenesis agent to attain approval by the USFDA. In a phase I/II study, an EGFR inhibitor, erlotinib was combined with an anti-VEGF antibody, bevacizumab, in patients with recurrent/metastatic HNSCC, seven patients had response, with four showing a complete one (Cohen et al., 2009). Cediranib is an oral, highly potent and selective inhibitor of VEGF signalling, with activity versus VEGFR-1, -2, and -3.

In summary, target therapies are more effective in treating recurrent and advanced tumours compared to conventional chemo and radiotherapy, and has shown to have less side effects.

1.10 Prognosis

Over the last 40 years, there has been no significant improvement in five and ten year over all survival rates in HNSCC. Cancer research UK showed that from 1971-2001; the five- and ten-year survival rates barely exceed 60% despite advances in surgery and chemo-radiotherapy (cancer research UK CRUK 2009). The presence of neck node metastasis is a strong predictor of poor survival and nodal status seems to be the best prognostic indicators in patients with laryngeal cancer (Cappellari 1997). Extra-capsular spread of nodal metastases is also an unfavourable prognostic factor, significantly associated with tumour recurrence and decreased survival (Stell 1990; Hirabayashi et al., 1991).

Glottis carcinoma is generally considered to have a better prognosis than supraglottic disease (Stell 1990). This seems, however, to be largely due to the more advanced stage and higher metastasing potential of supra-glottis tumours. Overall 5-year survival following treatment is 80% for glottic and 50% for supraglottic tumours, mainly because the latter present an increased incidence of nodal metastases. Nearly two-thirds of patients with supraglottic cancer have neck metastases at the time of initial treatment. Survival decreases by more than one third when clinically positive lymph nodes are present. Five-year disease free survival of patients with supra-glottic cancer is 80% for stage I-II, 70% for stage III and 40% for stage IV. Patients with glottis cancer have a better long-term prognosis. Five-year disease free survival for stage I-II is 85% - 90%, for stage III is 75% and for stage IV is 45-50% (CRUK 2009).

Second malignant neoplasia of the aero-digestive tract is common in patients with head and neck cancer. Slaughter et al proposed the field cancerization hypothesis to explain the origin of the multiple tumours (Slaughter et al., 1953). Up to 17% of patients with early glottis cancer have been found to develop a second malignancy of the upper aero-digestive tract or lung (Narayana et al., 1998).

A retrospective of 45 patients with laryngeal cancers were analysed showed 33% of patients with p53 mutations by PCR-SSCP (polymerase chain reaction single strand conformational polymorphism). The presence of mutation was associated with significant improvement in overall survival (Chomchai et al., 1999).

The prognosis for patients with laryngeal cancer is determined by patient factors, tumour factors and treatment factors. Tumour size is recognized as prognostic factor and ideally might contribute to an early identification of the different subgroups of patients with different prognosis (Pulkkinen et al., 1999). Studies indicate that larger tumour size of laryngeal carcinoma is associated with unfavorable disease free survival (Pulkkinen et al., 1999; Gallo et al., 2003).

High-grade tumours (G3), which have higher proliferation than G2, and G1 tumour, are responsible for tumour relapse, either locally or loco-regionally (Kowalski et al., 1991; Morales-Angulo et al., 1998). Tumour characteristics such as site of the primary tumour, T stage, N status, and TNM stage have been established to have prognostic significance in a number of studies (Morales-Angulo et al., 1998; Spector et al., 2001),

moreover poor prognosis has been associated with the advanced T stage of the tumour (Eiband et al., 1989; Kowalski et al., 1991).

Recent systemic review of the literature concludes that human papilloma virus (HPV) is the most important independent prognostic factor in head and neck squamous cell carcinoma (Syrjanen et al., 2011). A systemic review reported HPV prevalence is 24% in laryngeal carcinoma (Dayyani et al., 2010; Syrjanen et al., 2011). HPV positive HNSCC in general are more responsive to radiotherapy usually combined with chemotherapy and have more favorable disease specific survival than persons with HPV negative HNSCC (Gillison et al., 2000; Lowy and Munger 2010).

Micro-vessel density has been reported to be an independent prognostic indicator of outcome in a variety of human malignancies, with increased MVD correlating with malignancy progression, shorter overall survival and relapse free survival rates. Marioni et al (Marioni et al., 2006) has showed that CD105 assessed MVD may be a valuable parameter for predicting patients having an increased risk of developing laryngeal carcinoma loco-regional recurrence. His study included 43 patients with laryngeal cancers and used immunohistochemical analysis of the samples.

1.11 Angiogenesis

Angiogenesis is the formation of new blood vessels from the endothelium of the existing vasculature and is a result of a complex multistep process involving extra-

cellular matrix remodelling, endothelial cell migration and proliferation, anastamosis and finally lumen development (Folkman 1990; Folkman 1995).

Angiogenesis is important in a number of normal physiological processes including growth and development (Risau et al., 1992), wound healing (Arnold and West 1991) and reproduction (Torry and Rongish 1992). An inadequate amount of angiogenesis contributes to the ulcer formation and excessive angiogenesis contributes to the pathology of a number of condition including arthritis, Psoriasis and solid tumours (Folkman 1995).

The importance of angiogenesis was first recognised by Folkman's group in 1971 (Folkman 1971). The concept that tumour growth is 'angiogenesis dependant' (Folkman 1990) is well accepted today and most scientific reports show that angiogenesis is linked to tumour growth and that 'every increment of tumour growth' (Folkman and Klagsbrun 1987) requires an increment of vascular growth (Folkman 1990). In the absence of blood supply, a tumour can only grow to a mass of about 10^6cells, approximately 1-2mm in diameter, Folkman and colleagues demonstrated for solid tumours to grow any larger than 2-3mm in a diameter they should be able to induce their own blood supply (Folkman et al., 1971). Whether or not angiogenesis occurs in a particular tissue depends on the balance between the relative amounts of molecules that induce or inhibit angiogenesis. In order to achieve this switch, tumour cells usually decrease the amount of inhibitors they secrete. The exact mechanisms explaining how this occurs in most neoplasms has for the most part remained elusive (Figure 1.9). In some animal models, a distinct switch to the angiogenic phenotype is seen (Folkman and Hanahan 1991). However, one can easily envisage that both the activation of

oncogenes and the inactivation of tumour suppressor genes play an important role (Parangi et al., 1995; Stellmach et al., 1996).

Sprouting is one of the major mechanisms of expansion in the network of vessels in the growing tumours through filopedia and endothelial stalk cells (Gerhardt et al., 2003) (Figure 1.10). Although angiogenesis is difficult to measure directly in human tumours, it is suggested that quantification of micro-vessel density (MVD) may be a useful index (Weidner et al., 1991).

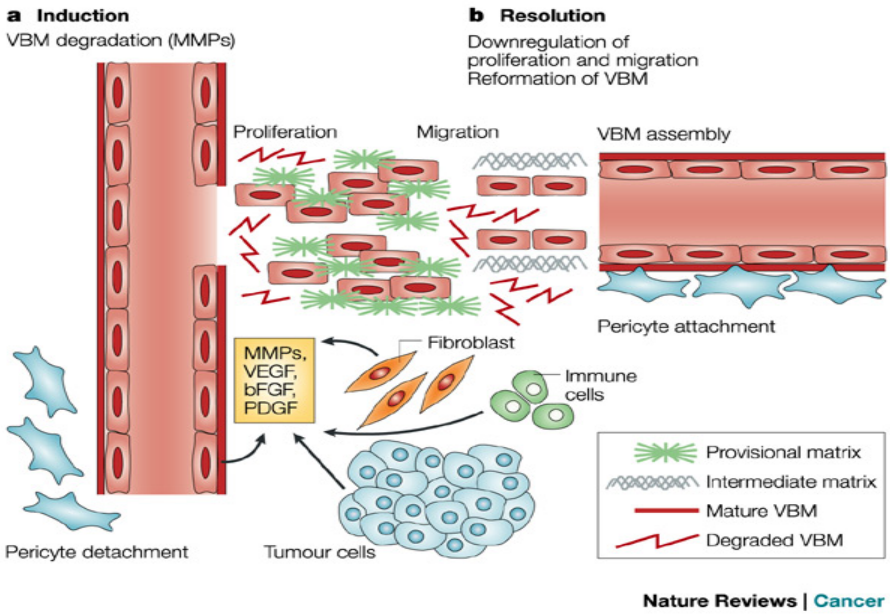


Figure 1.9 Angiogenesis is associated with degradation and reformation of the vascular basement membrane (VBM) **(**source: *Nature Reviews Cancer* 3, June 2003**)**

A | In response to growth factors and matrix metalloproteinase (MMPs), the VBM undergoes degradative and structural changes. This transition from mature VBM to provisional matrix promotes the proliferation and migration of vascular endothelial cells. Growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast

growth factor (bFGF) and platelet-derived growth factor (PDGF), are released from the BM, and are also produced by tumour cells, fibroblasts and immune cells. **B** | This induces formation of an intermediate, and then a new (mature) VBM. Together with the vascular endothelial cells and pericytes, the VBM mediates formation of a new blood vessel. The degraded VBM during this process has a crucial role in regulating angiogenesis.

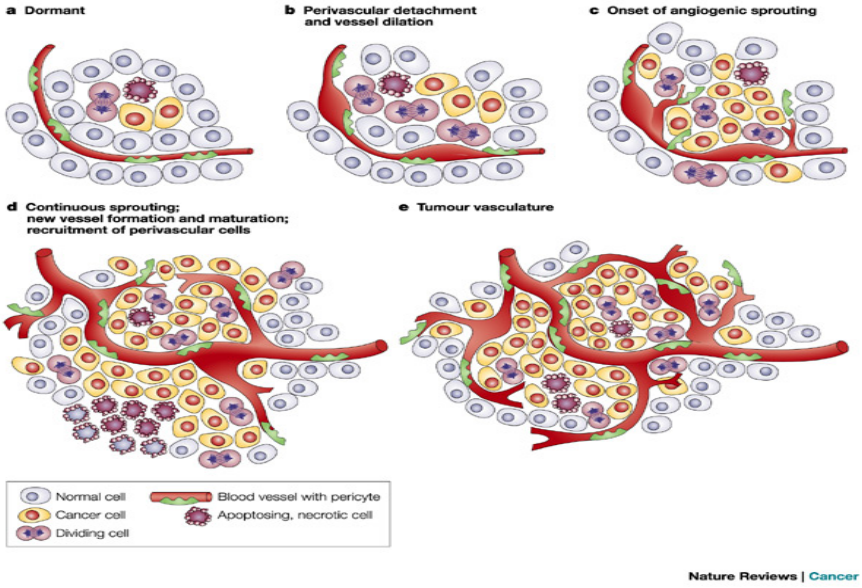


Figure 1.10**.** Angiogenic switch is a discrete step in tumour development that can occur at different stages in the tumour-progression pathway, depending on the nature of the tumour and its microenvironment.

(*Nature Reviews Cancer* 3, June 2003**)**

Most tumours start growing as vascular nodules (dormant) (**a**) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis, or the 'angiogenic switch', has to occur to ensure exponential tumor growth. The switch begins with perivascular detachment and vessel dilation (**b**), followed by angiogenic sprouting (**c**), new vessel formation and maturation, and the recruitment of perivascular cells (**d**). Blood-vessel formation will continue as long as the tumour grows, and the blood

vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen (**e**).

The process involves endothelial cell proliferation and their structural reorganization. A multitude of tumour-derived factors regulates angiogenesis, including vascular endothelial cell growth factor, basic fibroblast growth factor, prostaglandin E2, transforming growth factor beta, interleukin-8, angiopoietin-1 and 2 (Figure 1.11).

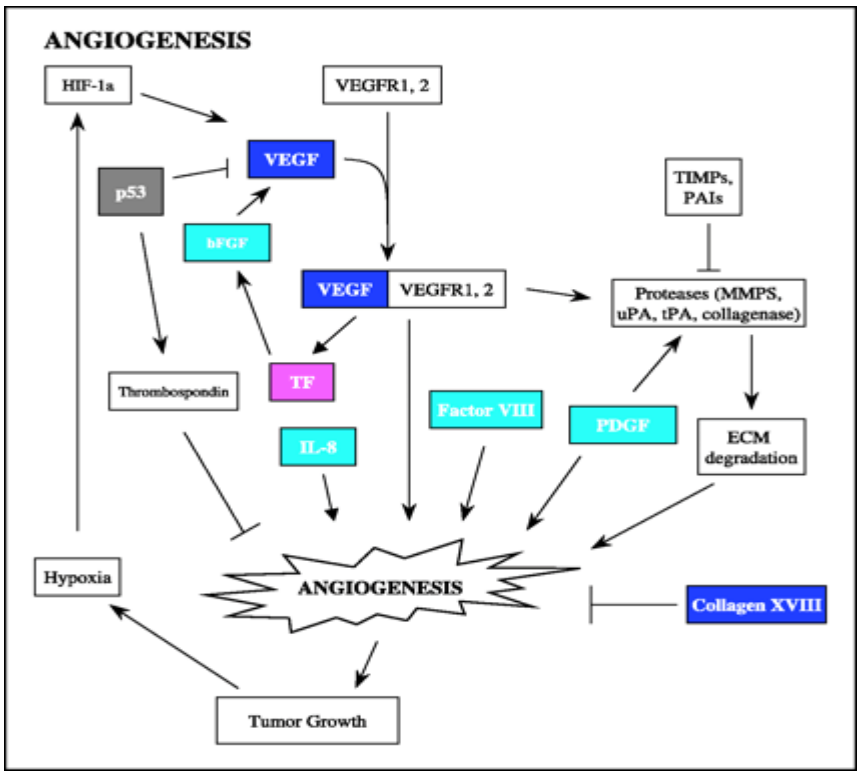


Figure 1.11 Role of different angiogenic factors in angiogenesis (Clin Cancer Res, 2005)

Angiopoietin can regulate the stability and permeability of the vasculature (Stoeltzing et al., 2003); bFGF facilitates the formation of the tube like structures. VEGF stimulates endothelial cell proliferation and motility (Roeckl et al., 1998). Studies with head and neck cancers have shown that endothelial cell motility is stimulated by HNSCC through

their release of PGE2 and TGF beta (Benefield et al., 1996). Tumour vascularisation can also occur through vasculogenesis, which differs from angiogenesis as it is the development of vessels from precursor cells that migrate to the site of vascularisation, rather than vessel formation from pre-existing mature endothelial cells (Shirakawa et al., 2002). Studies have indicated that increased tumour angiogenesis correlates with poorer outcome in several tumours (Petruzzelli 1996). Angiogenesis is linked to an increased risk of local regional recurrence, distant metastasis, and reduced survival in patients with cancer at various organs including breast, lung and ovary (Angeletti et al., 1996). Traditionally, micro-vessel density has been the parameter most often used for assessing angiogenesis; another approach involves the assessment of circulating angiogenic serum markers potentially used to assess vascular endothelial growth factor (Palka et al., 2008). The gold standard MVD measure of angiogenesis is the histological estimate of the average number of micro-vessels within a selected region. Several studies have found that intra-tumoural MVD is predictive of outcome in a number of HNSCC disease sites and could aid the identification of patients who may need aggressive or adjuvant therapy (Ito et al., 2001; Pignataro et al., 2001).

1.11.1 Angiogenensis in HNSCC

Angiogenesis is a critical element of tumour growth and metastasis, it plays an important role in head and neck SCC as in any other solid tumours (Seiwert and Cohen, 2008). The ability of the tumour to start angiogenesis is a prerequisite for metastatic spread. A variety of molecules capable of inducing angiogenesis are directly produced by HNSCC (Lingen 1999). Vascular endothelial growth factor A (VEGFA) is the best-known agent that induces angiogenesis (Christopoulos et al., 2011). It is a vascular

permeability factor that belongs to the platelet derived growth factor (PDGF) superfamily, which includes VEGF-B, -C, -E and placental growth factor (PIGF). There are many other factors involved in angiogenesis, such as epidermal growth factor (EGF) PDGF, prostaglandins, COX2, and IL-6 (Hicklin and Ellis 2005). In many studies interleukin 8 was a major angiogenic factor (Cohen et al., 1995). In addition, in the closely related bronchogenic carcinoma**,** IL-8 was the primary mediator of angiogenesis found in fresh tumour homogenates (Smith et al., 1994).

In HNSCC tumour cells attract monocytes and activate them to secrete angiogenic factors (Liss et al., 2001). Macrophages secrete number of angiogenic factors including VEGF, IL-8, fibroblast growth factor, (bFGF) (Koch et al., 1992; O'Sullivan et al., 1993). In addition, macrophages produce cytokines that act in paracrine fashion on the tumour cells, which stimulates them to produce increased levels of IL-8 and VEGF (Liss et al., 2001).

Hasina et al (2008) demonstrated that there are different mechanisms by which HNSCC induces angiogenesis. Using sample collected from patients affected by HNSCC and sample of normal and dysplastic mucosa, they conducted an immuno-histochemical analysis and gene expression profiling studies. They studied the expression of cytokines (CK) such as VEGF, IL-8/CXCL8, HGF and FGF-2 in normal, dysplastic and pathological tissue. These CK are well known mediators of HNSCC angiogenesis. The authors observed that normal mucosa generally does not express VEGF, IL-8/CXCL8, FGF2 and HGF, and the same CKs are more frequently expressed and at a higher levels in dysplastic mucosa. The incidence and intensity of expression of VEGF, IL-8/CXCL8, FGF-2, and HGF are highest in HNSCC samples. Moreover, they validated the presence

of two different clusters in relation to angiogenesis in HNSCC samples: tumours in cluster A express high levels of VEGF and FGF-2 and low levels of IL-8 and HGF and are characterised by higher levels of microvessel density than tumours in cluster B, expression on the contrary low levels of VEGF and FGF-2 and high levels of IL-8 and HGF. Factors which are expressed highly in HNSCC are VEGF, (Eisma et al., 1999) IL8 (Cohen et al., 1995), PDGF and HGF (Hasina et al., 2008).

1.11.2 Vascular endothelial growth factor (VEGF)

VEGF–A is 45-kDa heparin binding protein considered to be one of the major regulators of both physiological and pathological angiogenesis. Structurally VEGF belongs to the VEGF-PDGF super gene family. The VEGF gene has been shown to be regulated by a variety of stimuli such as hypoxia, growth factors, p-53 mutation, oestrogen, tumour promoters, e.g nitric oxide (NO). Among these most research groups have focussed on hypoxia, because of its importance and its unique transcriptional regulation (Luo and Shibuya 2001). VEGF is a member of a family that also includes VEGF-B, VEGF-C, VEGF-D and PIGF (placenta growth factor). VEGF binds two tyrosine kinase receptors, Flt-1 (Fms-like tyrosine kinases 1) and KDR (kinase insert domain-containing receptor). Flt-1 has the highest affinity to VEGF among the VEGF receptors, but its tyrosine activity is weak compared to KDR, but KDR carries weaker affinity to VEGF (Seetharam et al., 1995; Sawano et al., 1996).

Vascular endothelial growth factor (VEGF) and its receptors represent one of the best-validated signalling pathways in angiogenesis. VEGF induces the proliferation,

differentiation and migration of vascular endothelial cells (Ferrara and Davis-Smyth 1997), increases the permeability of the capillaries, and also enhances the survival of endothelial cells by preventing their apoptosis (Reinmuth et al., 2003). Current FDA approved anti VEGF -A drug, Bevacizumab, inhibits the VEGF pathway (Ferrara et al., 2004; Escudier et al., 2007; Ellis and Hicklin 2008) and two small molecule inhibitors Sorafenib and Sunitinib target VEGFR2 (Escudier et al., 2007; Ellis and Hicklin 2008).

Over-expression of VEGF in HNSCC is associated with more advanced disease, increase resistance to cytotoxic agents, and poor prognosis (Tse et al., 2007; Boonkitticharoen et al., 2008). In a meta-analysis of 12 studies including 1002 patients affected by cancer of oral cavity (70.8%), pharynx (15.2%), and larynx (14%), VEGF expression was evaluated, and its positivity was associated with a twofold higher risk of death at 2 years (Kyzas et al., 2005).

In HNSCC patients there is significant negative association of VEGF with survival; correlation was stronger in the subgroup of patients with SCC of the oral cavity and larynx (Kyzas et al., 2005). Elevated levels of VEGF are present in the HNSCC tumour microenvironment and may be related to tumour aggressiveness (Eisma et al., 1997). Benefield et al (1996) demonstrated that VEGF in supernatants from HNSCC cells mediates endothelial cell proliferation. A study by Rajesh et al showed that VEGF is an autocrine regulator of tumour cell activity in angiogenic effects on vascular endothelial cells (Lalla et al., 2003).

1.11.3 Platelet derived growth factor (PDGF)

Platelet derived growth factors are a family of disulfide-bound growth factors including PDGF-AA, -BB, -CC and -DD. Platelet-derived growth factor is a family of cationic homo- and hetero dimers of disulfide-bonded A- and B- polypeptide chains. The mature parts of the A- and B-chains of PDGF are 100 amino acid residues long and show 60% amino acid sequence identity. Eight cysteine residues are perfectly conserved between the two chains. These factors exert their effects through two tyrosine kinase receptors: the structurally related PDGF alfa and beta-receptors. Pericyte recruitment is part of development of normal functional capillaries. A number of studies has suggested the role of PDGF –BB mediated activation of PDGF beta-receptors on pericytes, signalling in recruitment of tumour pericyte. Studies demonstrated forced PDGF overproduction by tumour cells was associated with increased pericyte abundance; in two of the studies the PDGF induced pericyte abundance was also shown to lead to an increased tumour growth rate (Guo et al., 2003; Furuhashi et al., 2004).

1.11.4 Hepatocyte growth factor (HGF)

HGF is a soluble heterodimeric protein that elicits diverse responses in different tissues by activating a tyrosine kinase signalling cascade after binding to the proto-oncogenic c-MET receptor. HGF is able to induce production of the pro-angiogenic factors PDGF-AB by HNSCC cells, in addition to VEGF and IL-8 (Dong et al., 2001).

1.11.5 Interleukin (IL-8)

Interleukin 8 (CXCL8), is one of the ELR (Glu-Leu-Arg) motif-positive (ELR1) CXC-chemokines and is secreted by leukocytes and tumour cells (Xie 2001). CXCL8 has diverse functions in immune surveillance, inflammation, and angiogenesis. The biological action of CXCL-8 is mediated through binding to its receptors, CXCR1 (IL-8RA) and CXCR2 (IL-8RB) (Rollins 1997; Heidemann et al., 2003), which are members of the seven trans-membrane G-protein-coupled receptor family. It has been suggested that tumour cells produce IL-8 as an autocrine growth factor, which promotes tumor growth, tissue invasion and metastatic spread (Xie 2001). Most primary and metastatic solid tumours, such as breast, uterine, prostate, colon and pancreatic carcinomas, melanoma, and glioblastoma, constitutively express IL-8 (CXCL8) (Fasciani et al., 2000; Fujimoto et al., 2000; Li et al., 2001; Huang et al. 2002). A known function of CXCL8 in tumours is the enhancement of angiogenesis. Several studies have shown that tumor-derived CXCL8 directly modulates endothelial cell proliferation and migration, thus promoting angiogenesis (Fasciani et al., 2000; Fujimoto et al., 2000; Xie 2001).

Despite the importance of angiogenesis in head and neck cancer, few anti-angiogenic agents have shown activity in the clinical settings and have been approved for the treatment of this disease. Currently there are incomplete studies regarding this. First of all, some data suggest the existence of two different pathways in angiogenesis (Hasina et al., 2008), so it is most important to understand the pathogenesis process in order to select the correct therapeutic target in each patient. Some authors demonstrate that angiogenesis inhibition is probably not enough to completely arrest the growth of

tumours, and should consider combining this approach with cytotoxic drugs with other treatments such as radiotherapy or anti-EGFR agents (Bozec et al., 2007; Bozec et al., 2009). To date only one study with a combination of an anti-angiogenic agent and an anti-EGFR inhibitor reported a possible role of a molecular biomarker that could predict a greater possibility of response to an anti-angiogenic treatment (Cohen et al., 2009).

In summary angiogenesis plays an important role in tumour progression and metastasis of the solid tumours and recent evidence has shown that antiangiogenic therapy may play an important role in treating the advanced cancer compared to the conventional treatment with chemo-radiotherapy.

1.12 Aim of the Study

The aim of the current thesis to identify key proteins within the tumour microenvironment that may be involved in angiogenic processes occurring in laryngeal carcinoma and associated metastatic nodes. Fresh tissue lysates from tumour tissue and associated metastatic nodes were analysed by angiogenesis antibody array method, which can identify expression of 55 angiogeneic related growth factors and expression was correlated with tumour progression. ELISA method was performed subsequently for quantitative analysis of the angiogenic - related growth factors in a cohort of tumour tissue lysates of larynx and nodal tissues to confirm our initial pilot study findings. Finally, expressions of the factors were correlated for prognostic significance with tumour progression and patient and tumour characteristics.

CHAPTER 2

Expression of angiogenic related growth factors in laryngeal cancer

2.1 Objective

Angiogenesis, the process that leads to the formation of new vessels, is a hallmark of tumour progression, and its role has been studied in many cancer types including HNSCC. Factors secreted by both tumour and stroma stimulates the surrounding endothelial cells to form new blood vessels. Angiogenesis does not result from the action of a single growth factor, but is dependent on the interaction of multiple proteins with angiogenic properties. In HNSCC, like almost all other solid tumours, factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) have been shown to induce angiogenesis.

The aim of the work in this chapter was to investigate the expression of a panel of angiogenesis-related proteins in laryngeal tumour tissue, associated metastatic nodes and clinically normal mucosa from the same patient, in comparison with their expression in control uvula mucosa. The results were also correlated with tumour stage with a view to identifying potential markers of HNSCC cancer progression and metastatic spread.

2.2 Material and Methods.

2.2.1 Patients and Samples

Newly presenting patients with moderately differentiated squamous cell carcinoma of the larynx (Table 2.1) were recruited into the study. Following written informed

consent, and prior to chemotherapy and radiotherapy, fresh tissue specimens were obtained intra-operatively from the tumour (n=7) and secondary lymph node (n=7). Where possible, normal-looking mucosa was also dissected away from the resected tumour specimen (n=4). Patients were all male with a mean age of 66.4 (range 46-85) years. A sample of control uvula mucosa (n=5) was removed from healthy volunteers undergoing surgery for uvulopalatopharyngoplasty(UVPP).

Approval for the study was gained from both South Humber Research Ethics committee and Hull and East Yorkshire Hospitals R&D (06/Q1105/63 & 07/H1305/7).

Sex/Age	Stage	Histology	Treatment
			Surgery
M/85	T1N2	Mod.diff.SCC	
M/66 *	T1N2	Mod.diff.SCC	Sur+chemo+DXT
M/68 *	T3N1	Mod.diff.SCC	Sur+Chemo+DXT
M/62 *	T3N2	Mod.diff.SCC	Sur+DXT
M/46	T4N2	Mod.diff.SCC	Sur+chemo+DXT
M/68 *	T4N2	Mod.diff.SCC	Sur+Chemo+DXT
M/70	T4N2	Mod.diff.SCC	Sur+Chemo+DXT

Table 2.1: Demographics of the patients
*Patients with normal mucosa
Sur: Surgery, Chemo: chemotherapy, DXT: Radiotherapy,
Mod.diff.SCC: Moderately differentiated squamous cell carcinoma

2.2.2 Lysate preparation

Tissue was transported to the laboratory in Dulbecco's Modified Eagle's Medium (DMEM: PAA, Somerset, UK), supplemented with 10% (V/V) fetal bovine serum (FBS; Bio sera, East Sussex, UK), penicillin/streptomycin (final concentrations: 0.1 U/ml; 0.1 mg/ml respectively PAA). Tissue lysates were prepared by finely mincing

the tissue, on ice, using scalpels in ProteoJET mammalian cell lysis reagent (500µl/100mg tissue; Fermentas Life Sciences, York, UK), containing a protease inhibitor cocktail (1ml/20g tissue; Calbiochem/Merck, Nottingham, UK). The lysate was then subjected to sonication for 15 minutes on ice before centrifugation at 400x *g* for 15minutes to pellet cell debris. The resulting supernatant was aliquoted into 0.5ml lysates and stored at -80°C for antibody array processing.

2.2.3. **Protein determination and antibody array**

The protein concentration in each lysate was quantified immediately before use in the antibody array using the Coomassie blue plus assay kit (Thermo Scientific/Pierce, Rockford USA) following the manufacturer's instructions.

Briefly, a standard curve was generated by diluting the BSA stock solution in lysis buffer and protease inhibitor to give a range of standards with the blank being lysis buffer. These (10µl) along with lysate samples (10µl) were mixed in duplicate with 300µl of coomasie blue in separate wells of 96 well plate on a plate shaker for 30 seconds. Each microplate well is pipetted with 10 µl of each standard and 300µl of coomassie plus reagent is added and mixed with plate shaker for 30 seconds before incubation for 10 minutes at room temperature. Absorbance was measured at 595nm using a plate reader and the average measurements for the blank replicates were subtracted from the measurements of all of the other standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected 595

nm measurements for each protein standard vs. its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

2.3 Human Angiogenesis Array (proteome profiller™ antibody arrays)

The relative expression of fifty-five angiogenesis-related proteins (Appendix 3) was determined in each lysate using a Proteome Profiler™ human angiogenesis array kit (R&D Systems, Abingdon, UK) following the manufacturer's protocol. (Table2.2).

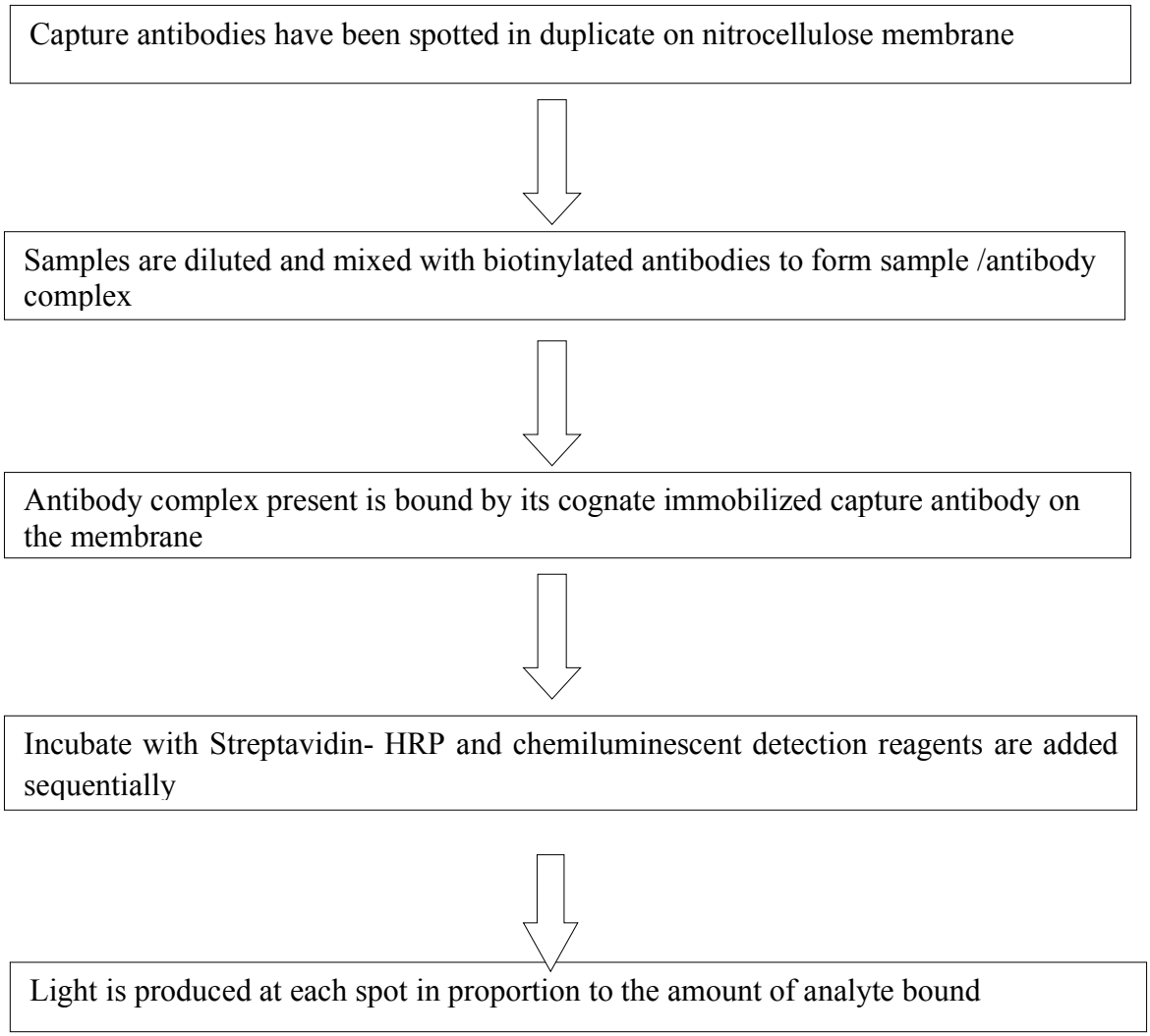


Table 2.2: Schematic representation of angiogenesis protocol

2.3.1 Principle of array (Figure 2.1)

The human angiogenesis array kit contains four nitrocellulose membranes: each membrane contains 55 antibodies; (Table 2.3) six identical positive control antibodies contain protein standards and two negative controls. All antibodies are spotted in duplicate. The membranes were handled with gloved hands and flat-tipped tweezers to avoid contamination.

Briefly antibodies for the 55 proteins of interest have been spotted in duplicate onto a nitrocellulose membrane, following mixing of the tissue lysates with a cocktail antibodies, this mixture was incubated with the nitrocellulose membrane which contained biotynylated detection duplicate spots of capture antibodies to form the antigen/antibody sandwich.

Any protein/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Streptavidin-HRP and chemiluminescent reagents were added to visualize binding. Light is proportional to the amount of antibody/antigen complex captured from the tissue lysate samples.

Detection antibody cocktail was reconstituted with 100µl of dH_20 before use. The 25x wash buffer was diluted to 1x wash buffer (40ml of 25x wash buffer was diluted with 960ml of distilled water). Array buffers, 4, 5 and 7 were ready for use. Streptavidin – HRP was diluted in 1:2000 with buffer 5. The developer and fixative were diluted with

distilled water 1:4. All assay reagents were brought to room temperature and the patients' samples were thawed and brought to room temperature before use.

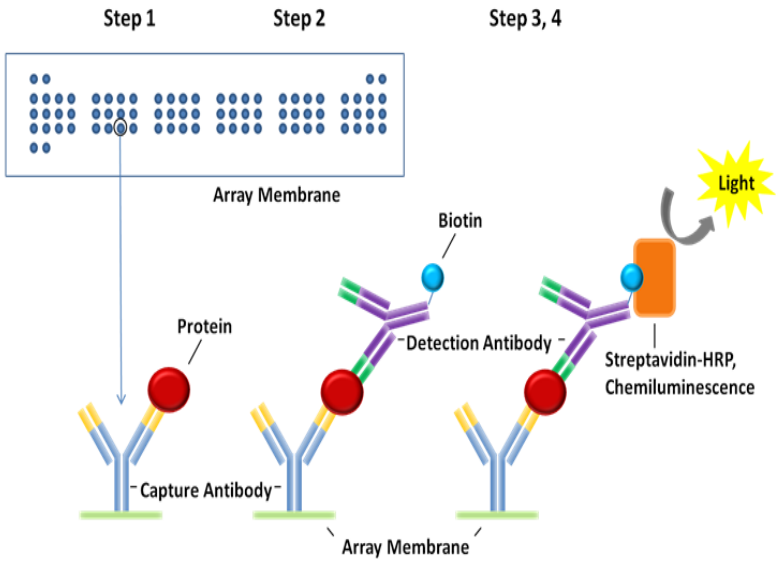


Figure 2.1: Illustration of the Proteome Profiler ™ human angiogenesis array method (reproduced from R&D Systems)

2.3.2 Method of angiogenesis array

Array buffer 7 (2ml) was added into each well of the four well multi-dishes for blocking of non-specific protein reactions and incubated for one hour on a rocking platform. At the same time, tissue lysate (100µl) was added to 0.5 ml of array buffer 4 and the volume was adjusted to 1.5ml with array buffer 5, 15µl of reconstituted antibody cocktail was added to the mixture and incubated for one hour at room temperature.

Buffer 7 was aspirated from the wells of the four well multi-dish and the sample/ antibody mixture was added to the membrane. Following covering the membrane was

incubated overnight at 4^{o}C on a rocking platform. The membrane was removed from the well to an individual petri dish and washed three times for ten minutes each with 20 ml of 1 x wash buffer.

Finally, the membranes were removed and the lower edge was gently blotted on absorbent paper to allow excess buffer to be drained off. The membranes were transferred to wash out 4 well multi dishes and were incubated with 1.5ml of diluted streptavidin-HRP for 30 minutes. The membranes were moved to individual containers and washed with wash buffer three times for ten minutes for each wash. Again the membranes were carefully removed from the individual containers and blotted on absorbent paper to remove excess buffer.

ECL reagents (3ml) (ECL western blotting detection reagent and analysis) were added to each membrane for three minutes. Each membrane was placed in a plastic wrap separately, being careful to remove air bubbles, before placing in an X ray film cassette. The membrane wass exposed to X-ray film for 1-10 minutes and the light produced at each spot is proportional to the amount of analyte bound (Figure 2.2). The average pixel density of the duplicate spots produced on the film was determined using a UVP Bio-Imaging system fitted with lab works 4.0 Image acquisition and analysis software.

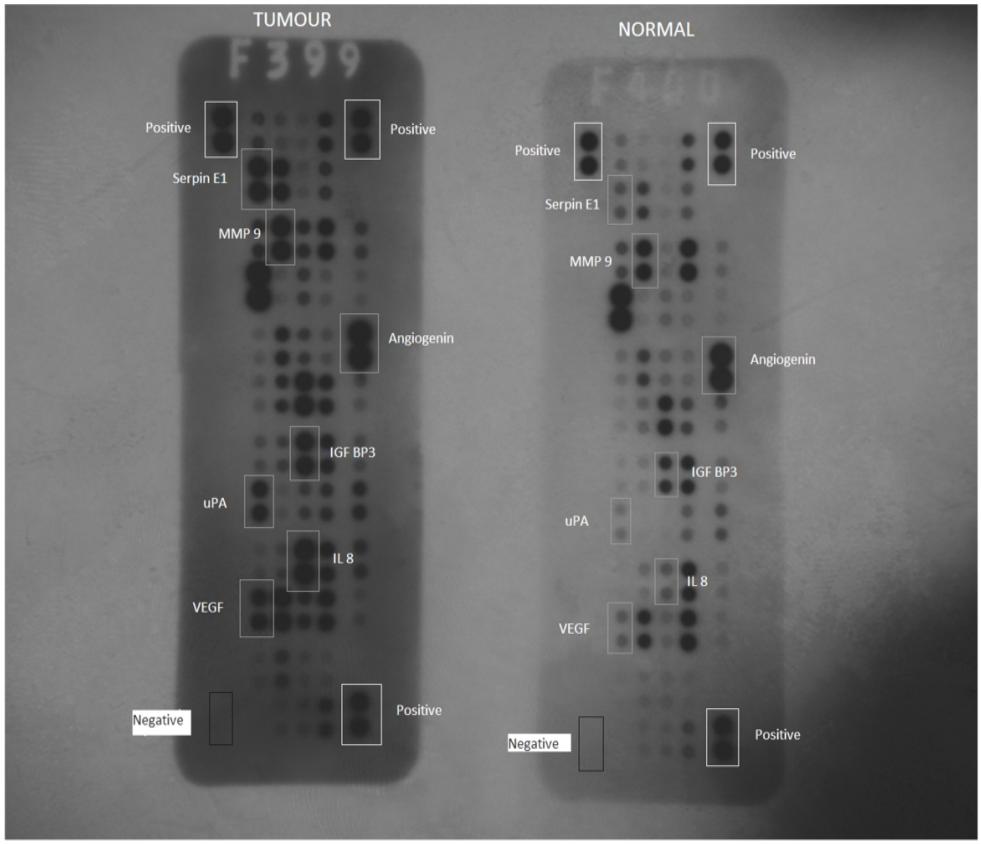


Figure 2.2: X ray film of angiogenesis antibody arrays processed to detect proteins in tumour and normal mucosal tissue taken following 10 min of exposure. [Grey boxes show examples of differential expression of proteins between tumour and normal mucosal tissue, White boxes are positive control spots and Black boxes are negative control spots].

In addition to the 55 angiogenesis-related proteins, the membrane contained three pairs of positive control and one pair of irrelevant negative control antibodies. Following subtraction of the average optical density of the negative control spots from all values, the level of the angiogenesis-related proteins were expressed as a percentage relative to the mean of the positive controls, which was assigned a value of 100%. The mean optical density and the standard deviation (SD) for the six positive control spots were determined for each of the membranes (n=7) studied and the SD was found to be approximately 20% in all cases. Therefore factors, which had an expression level within 20% of the positive control in all the T stages, were deemed not to have altered protein levels analysis (n=15, Refer Appendix 3).

Relative expression of all fifty-five angiogenesis-related proteins was compared between: i) tumour and lymph node from the same patient (n=7) and uvula control tissue (n=5); ii) clinically normal looking mucosa (n=4), and malignant tissue (tumour and node (n=4); and iii) clinically normal looking mucosa and uvula control mucosa (n=5).

2.4 Results

2.4.1 Comparison of angiogenesis-related protein expression between tumour tissue and corresponding lymph node

Fifteen of the 55 angiogenesis-related factors showed no changes in expression (within 20% of positive control) across all T stages and were not considered further. Forty of

the 55 angiogenesis-related factors investigated demonstrated a level of expression which was greater than 20% of the positive control in at least one tumour stage. Of these 40 angiogenesis-related proteins 32 showed an increase from stage T1 through to T4 (Table2.3). In most samples expression was detectable in all samples at all stages, alternatively in some cases it only became detectable in stage T3 (and then increased in T4), or was only present in T4. The same overall trend was observed for 29/32 of these factors in the nodal tissue from the same patient (excluding platelet factor 4, MMP 9 and IGF BP-1 which showed a variable expression through the stages). An example of the increasing trend is illustrated in Table 2.3.

	T1	T3	T4	T1	T3	T4
Actin A	ND	37	84	ND	23	25
ADAMTS [a]	ND	ND	ND	ND	ND	ND
Angiogenin	125	103	53	97	83	93
Angiopoietin-1[a]	ND	ND	ND	ND	ND	ND
Angiopoitin-2	ND	ND	38	ND	ND	35
Amphiregulin	ND	47	131	ND	ND	26
Antemin	ND	ND	124	ND	ND	31
Coag.factor 3	34	66	182	28	47	77
Cxcl16	ND	43	129	ND	24	44
Dppiv	68	94	182	65	74	81
EGF [a]	ND	ND	ND	ND	ND	ND
EG-VEGF [a]	ND	ND	ND	ND	ND	ND
Endoglin [c]	22	94	192	40	61	102
Endostatin	33	68	223	25	66	82
Endothelin	ND	46	233	ND	ND	99
FGF Acidic	44	64	250	ND	36	84
FGF Basic	29	58	303	ND	29	51
FGF-4[a]	ND	ND	ND	ND	ND	ND
FGF – 7	ND	31	70	ND	ND	23
GDNF [a]	ND	ND	ND	ND	ND	ND
GM-CSF [a]	ND	ND	ND	ND	ND	ND
HB-CSF	ND	55	77	ND	ND	44
HGF	ND	38	23	ND	22	37
IGFBP1[d]	28	40	56	ND	ND	ND
IGFBP2	46	90	156	40	69	98
IGFBP3	ND	109	170	ND	69	89
IL-1B	ND	45	53	ND	ND	21
IL-8	ND	103	238	ND	42	71
LAP	ND	44	129	ND	25	29
Leptin	ND	ND	30	ND	ND	ND
MCP-1	ND	22	81	ND	ND	ND
MIP-1α[a]	ND	ND	ND	ND	ND	ND
MMP-8 [b]	42	1 1	94	29	60	64
MMP-9 [d]	104	120	158	100	74	110
NRG1-B1[a]	ND	ND	ND	ND	ND	ND
Pentraxin3 [b]	ND	73	31	ND	41	32
PD-ECGF [b]	ND	56	38	ND	34	51
PDGF-AA	ND	24	36	ND	ND	ND
PDGF-BB [a]	ND	ND	ND	ND	ND	ND
Persephin [a]	ND	ND	ND	ND	ND	ND
Plasminogen	ND	52	99	ND	20	67
Platelet factor 4[c], [d]	40	95	204	79	76	109
PlGF	ND	30	145	ND	ND	26
Prolactin	ND	ND	73	ND	ND	ND
Serpin B5	ND	37	130	ND	ND	63
Serpin E1 [c]	46	124	159	62	62	110
Serpin F1	21	56	33	ND	22	45
TIMP-1	117	87	66	125	69	125
TIMP-4 [a]	ND	ND	ND	ND	ND	ND
Thrombospondin-1 [e]	ND	ND	36	ND	39	58
Thrombospondin-2 [a]	ND	ND	ND	ND	ND	ND
uPA [b]	ND	111	104	ND	52	119
Vasohibin[a]	ND	ND	ND	ND	ND	ND
VEGF	ND	58	193	ND	ND	68
VEGF-C	ND	ND	ND	ND	ND	ND

Table 2.3: Percentage expression of angiogenic factors from stage T1 to stage T4 in tumour and nodal tissue. (Values shown are percentage expression relative to positive control samples).

[a]. Angiogenic factors excluded from analysis (level of expression was less than the standard deviation of the positive control spots in all sample tested)
[b]. Factors with variable expression in tumour and node.
[c]. Factors expressed higher in nodal tissue from T1 stage compared to matched tumour.
[d]. Factors in node showing variable expression throughout tumour progression
[e]. Thrombospodin 1 is expressed higher in nodal tissue compared with tumour at T stage 3 and 4 in node compared to tumour..
ND: not detected, expression was less than 20

Tumour tissue from stage T3 and stage T4 patients had a higher relative expression of 31 (excluding thrombospondin-1) of these 32 angiogenesis-related proteins compared with the corresponding nodal tissue. Tumour tissue from stage T1 patients also showed a higher relative expression of 29 (excluding endoglin, platelet factor4, and serpinE1) of the 32 proteins compared with the corresponding node, although the level of difference was less apparent than at the advanced stages (Table 2.3).

	T1 (n=1)	**T3 (n=2)**	**T4 (n=1)**
Angiopoitin 1	ND	ND	94
Angiopoitin 2	ND	ND	105
Amphiregulin	ND	ND	125
Antemin	ND	ND	86
Dppiv	63	76	235
FGF Acidic	45	65	269
MMP9	80	96	224
Plasminogen	ND	ND	83
Platelet factor	63	77	232
Serpin F1	ND	24	128
Timp 1	87	94	167

ND: Not detected expression was less than 20%.

Table 2.4: Angiogenesis-related proteins which demonstrated increasing expression in normal mucosa with advancing tumour stage of the patient (matched samples)

Of the remaining 8 proteins, which had detectable levels, angiogenin and TIMP1, showed a consistent decrease in expression in tumour tissue from stage T1 to stage T4 (Figure 2.3). These proteins in the corresponding nodal tissue showed variable expression; with higher expression in T stage 1 and T stage 4 tumours, (Table 2.4)

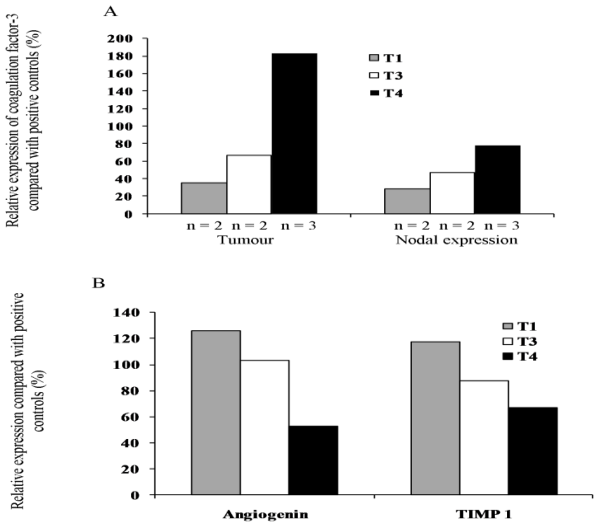


Figure 2.3: A) An example of increasing protein expression with disease stage in both tumour and node: showing percentage expression of Coagulation factor-3 relative to the mean of positive controls. B) Relative expression of angiogenin and TIMP1 in relation to positive control in HNSCC tumour tissue from different T stages

2.4.2 Comparison of angiogenesis-related protein expression between tumour patients (HNSCC primary and associated lymph nodes) and controls.

Of the 40 angiogenesis-related proteins analyzed the number of factors showing higher relative expression in tumour tissue compared with healthy controls increased from 2 in T stage 1, to 22 in T stage 3 and 33 in T stage 4. Similarly the number of angiogenic factors with a higher relative expression in the nodal tissue compared with healthy controls increased from 2 in T stage 1 to 16 in T stage 4.

2.4.3 Comparison of angiogenesis-related protein expression between normal looking mucosa, primary tumour and lymph nodes from the same patient

Macroscopically normal mucosa, tumour and metastatic nodal tissue from the same patient were obtained in four cases (T1; n=1, T3; n=2, T4; n=1). Eleven of the 40 angiogenesis-related proteins analyzed have showed an increased in relative expression in clinically normal looking mucosa with increasing tumour stage of the patient from which the normal mucosal tissue was obtained (Table 2.5).

Of the 40 angiogenesis-related factors analyzed there were only 8 which had a higher relative expression in tumour tissue from T1 patients compared to normal mucosa from the same patient, however the number of factors with a higher expression in the tumour tissue compared to the normal mucosa increased in the later stage tumours (T3; 32 and T4; 29).

	Tumour (n=4)			Node (n=4)			Normal mucosa (n=4)		
Angiogenic factor	T1	T3	T4	T1	T3	T4	T1	T3	T4
Actin [aback]	21	37	170	ND	23	40	ND	ND	79
Angiogenin[bde]	92	103	ND	74	83	81	110	99	ND
Angiopoitin-2[f]	ND	19	83	ND	ND	28	ND	ND	105
Amphiregulin [bcf]	ND	47	282	ND	ND	38	ND	ND	125
Antemin[cf]	ND	14	330	ND	ND	37	ND	ND	86
Coag.factor-3[abcdf]	68	66	390	53	47	84	63	34	272
Cxcl16[abf]	22	43	68	ND	24	69	ND	ND	100
Dppiv[abcef]	86	94	286	97	74	81	63	76	235
Endoglin[bcf]	30	94	302	64	61	93	54	38	102
Endostatin[bcdf]	66	68	222	48	66	92	69	57	142
Endothelin[bcdf]	22	46	173	ND	ND	86	28	ND	126
FGF Acidic[acdef]	85	64	282	ND	36	106	45	65	269
FGF Basic[cdef]	58	58	474	ND	29	92	78	59	242
FGF-7[bcf]	ND	31	141	ND	ND	ND	21	ND	33
HB-CSF[cd]	ND	55	175	ND	ND	75	ND	ND	ND
HGF[b]	ND	38	ND	25	22	50	23	ND	ND
IGF BP-1[abf]	54	40	ND	ND	ND	ND	ND	ND	71
IGF BP-2[bcdef]	76	90	270	43	69	146	108	89	226
IGF BP-3[bcd]	ND	109	296	ND	69	119	76	41	116
IL-IB[bd]	ND	45	ND	ND	ND	ND	46	ND	ND
IL-8[bcd]	ND	103	232	ND	42	84	101	ND	ND
LAP[bc]	ND	44	201	ND	25	47	ND	ND	ND
Leptin[c]	ND	ND	65	ND	ND	ND	ND	ND	ND
MCP-1[bc]	ND	22	133	ND	ND	ND	ND	ND	22
MMP-8[bcdf]	30	101	116	41	60	50	56	27	94
MMP-9[bcef]	80	120	261	100	74	106	80	96	224
Pentraxin3[bd]	ND	73	ND	ND	41	21	34	27	ND
PD-ECGF[b]	ND	56	31	ND	ND	54	ND	ND	50
PDGF-AA[b]	ND	24	ND	ND	ND	27	ND	ND	ND
Plasminogen[bcf]	ND	52	178	ND	ND	77	ND	ND	83
Platelet factor 4[abcef]	79	95	407	89	76	110	65	77	232
PIGF[bcd]	ND	30	310	ND	ND	37	23	ND	26
Prolactin[c]	ND	ND	122	ND	ND	ND	ND	ND	ND
Serpin B5[bcdef]	ND	37	318	ND	ND	93	41	23	227
Serpin E1[bcdf]	40	124	295	46	62	109	81	21	142
Serpin F1[abef]	37	56	59	ND	22	48	ND	24	128
TIMP-1[ef]	82	87	71	102	69	116	87	94	167
Thrombospondin-1	ND	ND	ND	ND	39	47	ND	N	ND
uPA[bcd]	ND	111	200	ND	52	133	63	ND	ND
VEGF[bcd]	ND	58	474	ND	ND	123	25	ND	42

Table 2.5:Angiogenesis-related protein expression in normal mucosa compared with matched samples of tumour and nodal tissue

[a]: Factors expressed higher in tumour tissue in stage T1 compared to normal mucosa

[b]: Factors expressed higher in stage T3 tumour tissue compared to normal mucosa

[c]: Factors expressed higher in tumour tissue in stage T4 compared to normal mucosa

[d]: Factors expressed higher in normal mucosal tissue in stage T1 compared to node

[e]: Factors expressed higher in stage T3 normal mucosal tissue compared to node

[f]: Factors expressed higher in normal mucosal tissue in stage T4 compared to node

ND: Not detected, expression was less than 20%

In contrast expression of some angiogenesis-related proteins in tumour adjacent normal tissue was higher than the nodal tissue from the same patient: Out of the 40 angiogenesis-related factors analyzed, 24 showed a higher expression in normal mucosa from T4 patients compared with the corresponding node, whereas adjacent normal mucosa from stage T3 and stage T1 biopsies had 10 and 18 factors respectively which showed a higher expression than corresponding node (Table 2.5).

2.4.4 Comparison of angiogenesis-related protein expression between normal looking mucosa and uvula control tissue

Nine of the 40 angiogenesis-related proteins analyzed had a higher relative expression in normal mucosa obtained from the tumour patient compared with control uvula tissue (Table 2.6). Of these nine factors, FGF acidic, FGF basic, IGF BP-2 and Serpin B5 were also higher in normal mucosa compared with the nodal deposits (Table 2.6).

Factor	Normal (n=4)	Control tissue (n=5)
Amphiregulin	33	27
Coagulation factor 111	103	97
Dppiv	114	91
FGF Acidic	114	84
FGF Basic	109	83
IGF BP1	36	32
IGF BP2	123	102
Platelet factor 4	112	108
TIMP-1	101	83

Table 2.6. Angiogenic factors showing higher expression higher in normal mucosa from tumour patients compared with uvula tissue from healthy donors

2.5 Discussion

A number of proteins are known to play an important role in angiogenesis, studies demonstrated the production of angiogenic proteins by HNSCC; however the majority of these have only explored the expression of a single angiogenic factor (Kyzas et al., 2005; Bran et al., 2009; Li et al., 2009). To date there have been only four studies which have looked at multiple angiogenesis-related factors in lysates of HNSCC (Petruzzelli et al., 1997; Chen et al., 1999; Ninck et al., 2003; Montag et al., 2009). Furthermore, these studies have been done with relatively small sizes of cell lines or patient samples.

Antibody array, on which antibodies are spotted, can be used to examine the expression of multiple proteins in low sample volume complex solutions by binding with specific proteins. The results of high through put methods require subsequent validation, and, much like the way quantitative PCR validates gene expression results obtained from DNA microarrays; western blot analysis is used to validate microarray data. The arrays can have good reproducibility, sensitivity, and quantitative accuracy over large concentration ranges (Haab et al., 2001). Therefore, specific hypotheses regarding the nature of molecular alterations can be tested and generated, and the observed measurements can be biologically interpreted.

A common application of antibody arrays in cancer research is the identification of biomarkers or molecules that are potentially valuable for diagnosis or prognosis or as surrogate markers of drug response. The multiplex capability of antibody arrays allows

the efficient screening of many marker candidates to reveal associations between proteins and disease states or experimental conditions. The use of combinations of proteins for disease diagnostics may produce fewer false positive and false negative results as compared with tests based on single proteins.

There are two main types of formats “label based” assays and “sandwich” assays (Figure 2.4). In label based assays, the targeted proteins are labeled with a tag that allows detection after capture by an immobilized antibody. Advantage of this method is that multiple samples can be assayed simultaneously; for example, two states could be compared by labeling each with a different colour label. The disadvantage of this method is that samples are chemically modified by the addition of the fluorescent marker: this could produce cross-reactive analyte, which may lead to false readings, and could result in high background.

Sandwich assays involve the use of two antibodies, each recognizing different epitopes of the same protein. The advantage of this method is that specificity is increased and, since it is not necessary to label the sample, the likelihood of cross-reactive analytes being produced decreases. The disadvantage of this method that two antibodies are required for detection of each analyte and it is more difficult to multiplex. Some of the challenges in the development of antibody microarrays include the availability of high-affinity/high-specificity antibodies, high-throughput antibody production and purification, inter- and intra-slide variability of protein concentration, and the stability of the arrayed antibodies. Another consideration is the type of slide used for printing.

The assay is non competitive since only one sample may be incubated on each array. Non-competitive assays results in sigmoidal binding responses with respect to analyte concentration so that a "standard curve" of known concentration of analyte required achieving accurate calibration of concentrations.

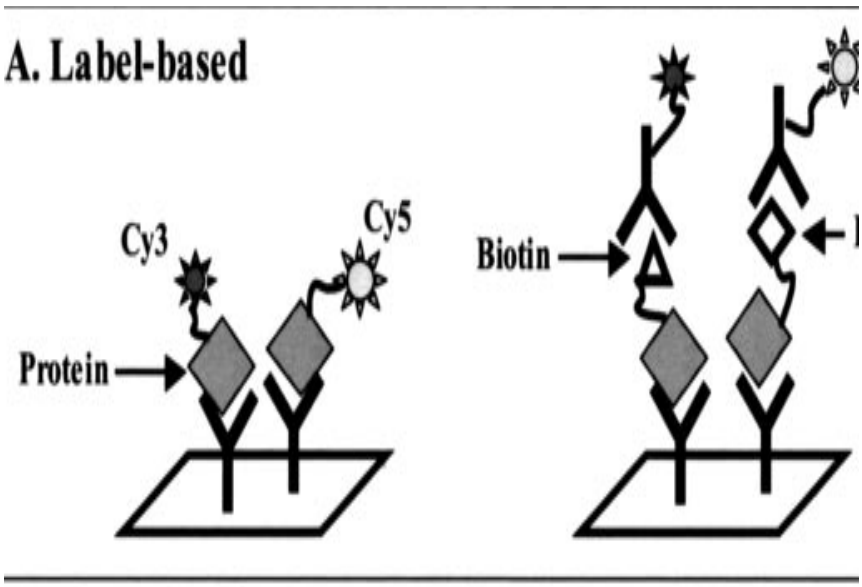
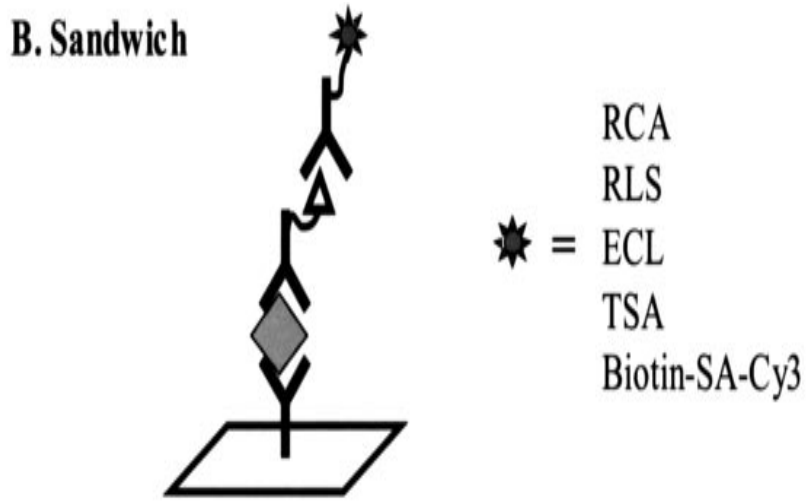


Figure 2.4: Antibody microarray assay formats.

A, Label-based assays showing detection in two colors. The **left drawing** shows a direct labeling assay in which two protein pools are respectively labeled with Cy3 or Cy5. The **right drawing** shows indirect detection in which two protein pools are respectively labeled with tags such as biotin. Labeled secondary antibodies detect the tags



B, Sandwich assay. A matched pair of antibodies binds the unlabeled analyte followed by detection by a secondary antibody. Detection is through one of the methods listed at **right**: RCA, resonance light scattering (**RLS**), enhanced chemiluminescence (**ECL**), tyramide signal amplification (**TSA**), and streptavidin (**SA**).

Source:(Haab, 2005)

Antibody arrays can be used to monitor protein abundance in the cancer cells following radiation treatment or used to identify potential biomarkers. Mathur et al., (Mathur et al., 2005) used the antibody array technique to identify DNA repair proteins that were upregulated by ischemic preconditioning in a myocardial infarction model.

Peptide microarray is another form of capture microarray, as the peptides replace the antibodies on the array surface and, like antibodies, are capable of binding proteins in a specific manner. Advantage of peptide microarray over antibody array include cost, greater resistance to denaturation, automated synthesis and purification of peptides, and the fact that peptides can be synthesized with non-natural functionalities. Function-based microarrays study biochemical properties of the proteins printed on the array, and so examine protein interactions and enzyme activities.

The measurement of levels of expression of angiogenic markers is a key step to understanding the role of these markers in angiogenesis. This was done using tissue lysates from laryngeal cancer patients using a human angiogenesis array kit. Detection of relative expression of 55 angiogenic markers simultaneously was achieved with this kit. It is a rapid and economic method when compared with other methods such as ELISA, in which the protein expression are assessed individually, however the latter would give quantitative values.

The current pilot study has investigated the expression of a panel of angiogenesis-related proteins in lysates from tumour tissue, associated lymph nodes, adjacent normal mucosa and uvula mucosa from normal controls. It was found that a large number of these proteins increase in expression in both tumour and metastatic nodes with increasing disease stage and therefore may be involved in promoting tumour progression. In addition we have shown that there is increased expression of some of these factors in the normal mucosa adjacent to the tumour compared with both the metastatic nodal tissue and uvula control tissue, suggesting that the processes may be occurring in a wider area than just the main tumour mass, potentially predisposing the patient to recurrence or the development of a second primary tumour supporting the "field of cancerization" hypothesis. The angiogenic growth factors VEGF, IL-8, bFGF, HGF are commonly found to be over expressed in HNSCC in lysates (Montag et al., 2009) and systemically (Druzgal et al., 2005; Gokhale et al., 2005). In the current study 32 of the 40 angiogenesis-related proteins analysed had an increasing expression in the tumour tissue with advancement of the tumour stage. This is in agreement with previous studies in HNSCC, which showed increased expression of VEGF (Chen et al., 1999; Sauter et al., 1999), MMP9 (P et al., 2001) HGF and FGF (Montag et al., 2009) in

tissue lysates with tumour progression. However the current study has identified many more.

In a Study which looked into multiple angiogenesis-related growth factors, Ninck et al (Ninck et al., 2003) reported an increased expression of a combination of factors in 80% of the HNSCC tumours and identified a distinct pattern of secretion which always included either VEGF or PDGF–AB, with G-CSF or GM-CSF. Montag et al (Montag et al., 2009) studied the expression of eight angiogenic factors in tissues and reported detectable expression of at least 4 factors in 90% of the tumours; with a significant association between factors VEGF-A and PDGF–BB two of the most common factors and with bFGF, HGF or G-CSF as well as PDGF–AB. Chen et al (Chen et al., 1999) investigated the expression of angiogenic factors in tumour tissue, HNSCC cell lines and serum and established that the factors IL-8, VEGF, GM-CSF and a number of other pro-inflammatory cytokines (not included in the current angiogenesis array) produced by HNSCC are consistent with the pathology of the neoplasm. Petruzzelli et al (Petruzzelli et al., 1997) studied the expression of FGFb, VEGF, TGF and PGE2 in HNSCC cell lines and reported that HNSCC produce these factor(s) to stimulate endothelial cell proliferation and that VEGF may be involved in HNSCC-induced endothelial cell mitogenesis. In the current study it has been observed that a panel of proteins increase in expression in tumour and associated nodes with the progression of tumour stage. However, further studies of large cohorts of HNSCC patients should be considered to validate the significance of these factors.

In addition we demonstrated a higher level of expression in many of angiogenic factors in both the tumour tissue and the associated nodal tissue, compared with tissue from the

healthy control group, which is in accordance with previous studies where angiogenic factor levels of MMP-9 (P et al., 2001), HGF, VEGF (Homer et al., 2000; Druzgal et al., 2005) and IL8 (Gokhale et al., 2005) were increased in patients with HNSCC compared with the control group (Druzgal et al., 2005). In contrast to the angiogenesis-related proteins, which increased with tumour progression, Angiogenin and TIMP 1 were found to decrease with disease progression. One study which supports this finding is that of Homer et al (Homer et al., 2000) who demonstrated a lower serum angiogenin expression associated with loco regional disease recurrence in HNSCC. However, Ruokolainen et al (Ruokolainen et al., 2005) reported that high levels of TIMP1 are associated with tumour progression: and showed an increased expression of TIMP 1 in serum and tumour tissue in patients with T stage 3 and 4 tumours compared with T stage 1.

Furthermore in the current study a higher expression of angiogenin and TIMP1 in tumour and associated nodal tissue compared with healthy control tissue was demonstrated, which is in agreement with the study by Charoenrat et al (P et al., 2001) who investigated levels in tumour compared with normal mucosa and also found significant correlation between levels of MMP9 and TIMP1 and advanced T stage.

A number of angiogenic factors have also been shown to differ between tumour and normal tissue, and with disease stage including urokinase plasminogen activator (uPA) and its inhibitor, cxcl16, Endothelin, IGF BP1 which all increase with tumour progression. The role of these factors have also been studied in other malignancies including breast (Homer et al., 2000), colon (Mark et al., 2010; Rinaldi et al., 2010), renal cell (Gutwein et al., 2009), ovarian (Rosano et al., 2005), gastric cancer (Li et al.,

2011). Suggesting that their role requires further elucidation as there is little evidence for their role in HNSCC.

Roesch-Ely et al (Roesch-Ely et al., 2007) have carried out proteomic analysis on both tumour and adjacent healthy mucosa and found that 72% of the healthy mucosa adjacent to the tumour had a significant association between aberrant profiles and tumour relapse events. In the current study the expression of angiogenesis-related proteins in the adjacent normal mucosa compared with that of the tumour tissue, metastatic node and healthy control tissue support the fact that although the adjacent mucosa may have a normal appearance, molecular events have occurred which are influencing protein production and could be important for subsequent tumour development.

Conclusion

In conclusion many angiogenesis-related proteins may be involved in the growth, spread and progression of laryngeal tumours and tissue that may have a clinically normal appearance is likely to be part of the affected tumour zone. Further investigation into the level of expression of specific factors in a larger cohort of patients has been carried out to validate the findings of this chapter.

CHAPTER 3

Angiogenic growth factors expression in relation with tumour progression

3.1 Introduction

Carcinoma of the larynx is one of the most frequent neoplasms in men. The highest incidence of the malignancy is observed in the fifth and sixth decades of life (Krecicki et al., 1998). It is a solid tumor and progression of the tumor is heavily dependent on angiogenesis (Folkman 1995).

Angiogenesis is a complicated multistep process involving the breakdown of the endothelial cell basement membrane, digestion of the extracellular matrix, proliferation and migration of endothelial cells upon the stimulation by angiogenic factors, and formation of new capillaries from preexisting ones. It occurs during physiological conditions such as wound healing or the menstrual cycle and is increased in many diseases, especially cancer (Folkman 1995).

The level of angiogenesis in the area of the most intensive neovascularization of a primary invasive carcinoma has been correlated with the presence of metastases, overall or relapse-free survival, as well as local recurrences in cancers of the breast (Weidner et al., 1992), lung (Angeletti et al., 1996; Lucchi et al., 1997), cervix (Bremer et al., 1996) and esophagus (Tanigawa et al., 1997). However the association of angiogenesis with prognosis has been controversial in head and neck squamous cell carcinoma.

Some of the factors known to stimulate angiogenesis positively in HNSCC are fibroblastic growth factor (FGF), vascular endothelial growth factor (VEGF), interleukin 8(IL-8), hepatocyte growth factor (HGF) and platelet derived growth factor

(PDGF), and tissue inhibitors of metalloproteinases (TIMP1) (Bergers and Benjamin 2003). Nuclear factor kB (NF–kB) is highly active in the angiogenesis of tumours. It is known that NF-kB binds to the promoter of IL-8, VEGF and cyclooxygenase 2 (COX 2) and thus increases the transcription of these angiogenic factors (Cherukuri et al., 2005).

VEGF is a heparin binding glycoprotein comprising at least four molecules; the gene is located on chromosome 6. It enhances vascular permeability and induces endothelial cell growth, proliferation, migration and differentiation (Ferrara 1996). VEGF plays a key role in tumour angiogenesis and it has been identified in many malignancies, including head and neck carcinomas (Li et al., 2005; Shang et al., 2006). Sullu et al (2010) in his study that has looked into relationship between expression of VEGF, angiogenesis and clinical pathological characteristics demonstrated that VEGF expression is closely related to tumour grade, tumour size and lymph node metastasis in laryngeal carcinoma.

FGF are heparin-binding proteins; FGF signal transduction is induced by its interactions with cell-surface associated heparin sulfate proteoglycans. One important function of FGF is the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures. They thus promote angiogenesis, the growth of new blood vessels from the pre-existing vasculature. FGF binding protein (BP) has been detected in several HNSCC cell lines and tissue (Czubayko et al., 1994). FGF BP is a secreted protein that binds to acidic and basic FGFs in a non-covalent reversible manner (Gospodarowicz et al., 1986; Wu et al., 1991). It was originally isolated from epidermoid carcinoma cells (Klagsbrun and Moses 1999). High expression of FGF BP

has a role in tumor angiogenesis; tumours utilize FGF-BP as angiogenic switch molecule (Czubayko et al., 1997). Li et al demonstrated that FGF BP is extensively expressed in primary HNSCC and is linked to the angiogenic activities of HNSCC (Li et al., 2009).

TIMP -1 is known to have at least two different functions. It inhibits the catalytic activity of MMPs and they are also able to act as growth factors (Hayakawa et al., 1992). Cell surface receptor that mediates the cell proliferation, CD63, is expressed in vascular endothelium (Harrison-Lavoie et al., 2006), the cell type where TIMP-1 was up regulated in the tumor, thereby, TIMP-1 may promote cancer progression by enhancing proliferation of endothelium and subsequent angiogenesis.

Increased expression of TIMP-1 in tumour tissue is associated with poor outcome in breast (Ree et al., 1997; Nakopoulou et al., 2002), colorectal (Zeng et al., 1995), lung (Fong et al., 1996) and gastric cancers (Mimori et al., 1997).

IL-8 is a chemokine produced by macrophages. It functions as a chemo attractant, and is also a potent angiogenic factor. Interleukin-8 (IL-8), a CXC chemokine that induces the migration and proliferation of endothelial cells and smooth muscle cells, is a potent angiogenic factor. IL-8, a CXC chemokine possessing the ELR motif, induces in-vitro endothelial cell migration and proliferation, two essential components of angiogenesis (Koch et al., 1992; Strieter et al., 1992). The angiogenic activity of IL-8 has been shown in-vitro to be equipotent to that of basic fibroblast growth factor, vascular endothelial growth factor, and other previously recognized heparin-binding proteins that promote

angiogenesis (Koch et al., 1992).

Angiogenin (ANG) is a 14-kDa 123 amino acid, member of the ribonuclease super-family, originally isolated from the conditioned medium of cultured HT-29 colon adenocarcinoma cells (Fett et al., 1985). The gene encoding angiogenin is present as a single copy per haploid genome localized on chromosome 14q11 (Gao and Xu 2008). The circulating ANG in normal plasma is produced mainly by the liver (Yoshioka et al., 2006) and it is a potent blood vessel growth inducer, it interacts with endothelial and smooth muscle cells to induce a wide range of cellular responses including cell migration, invasion, proliferation, and formation of tubular structures. ANG is not only a key angiogenic factor; it has also been implicated in tumour consolidation and proliferation (Dickson et al., 2009). Gao and Xu described the role of ANG in the angiogenic process in four areas; ribonuclease activity, basement membrane degradation, signaling transduction and nuclear translocation (Gao and Xu 2008).

The insulin like growth factor family is composed of two peptide ligands, two cell surface receptors and at least six specific IGF binding proteins. In the circulation IGFs are largely complexed with insulin growth factor binding protein (IGFBP), IGFBP-3. IGFBP-3 transports IGF in the circulation and directs it to target tissues and prevents this protects degradation of IGF and prolongs the half-lives. Independent of its binding capacity to IGFs, IGFBP3 has its own actions including regulation of growth and induction of apoptosis (Firth and Baxter 2002).

The effects of IGFBP3 on cell growth and apoptosis include sequestering IGFs from

their receptors. Apart from apoptosis, induction of IGFBP3 has a direct effect on angiogenesis. IGFBP-3 contains a highly basic heparin binding area, and specifically binds to the vascular endothelial cell monolayer (Booth et al., 1996) in a manner that may affect vascular angiogenesis; IGFBP3 inhibits VEGF mediated survival of human umbilical vein endothelial cells (HUVEC) in an IGF-independent mechanism (Zadeh and Binoux, 1997, Franklin et al., 2003) and may also effect angiogenesis

In-vivo IGFBP3 is transcriptionally up-regulated during hypoxia, a potent stimulator of angiogenesis (Diaz-Gonzalz et al., 2005) in endothelial cells (Koong et al., 2000) and IGFBP-3 mRNA is predominantly expressed in the vascular endothelial cells of human (Fraser et al., 2000) and rat (Erickson et al., 1993) which suggest a possible involvement in angiogenesis regulation, perhaps as part of a positive feedback mechanism. Oh et al (2006) identifies IGFBP3 as a fernesyl transferase inhibitor-induced negative regulator of angiogenesis in head and neck squamous cell carcinoma.

3.1.1 Objective

The present study has identified an increased expression of number angiogenic related factors with progression of the tumour in the laryngeal tissues. The aim of the present study to investigate the role of specific angiogenic factors, angiogenin, IGF-BP3, VEGF, FGF-BP, TIMP1 and IL8 with tumour progression by quantitatively measuring these proteins by ELISA technique in the laryngeal cancer tissue lysates and corresponding lymph nodes to confirm results from study results in Chapter 2.

3.2 Materials and Methods

3.2.1 Patients and Samples

A cohort of 36 patients with poor and well-differentiated squamous cell carcinoma of larynx was recruited into the study (Table 3.1). Following written informed consent, and prior to chemotherapy and radiotherapy, fresh tissue specimens were obtained intra-operatively from the tumour and secondary lymph node wherever possible. The patient group comprised males (n=30) and females (n=6) with ages ranging from 51 to 89 (median 74).

3.3 Determination of Angiogenic factors by ELISA Method

Tissue lysates were prepared using the Proteojet mammalian cell lysis reagent as described in section 2.2.2.

The protein concentration in each lysate was quantified immediately before use in the ELISA using the Coomassie Blue plus Assay kit [Thermo Scientific-/Pierce, Rockford USA] following manufacturer's instructions explained in detailed in section 2 (2.2.3).

The ELISA was not validated for tissue lysates, therefore extensive testing was undertaken. Standard curves were generated for each factor using both standards diluted in buffer and standards diluted in Proteojet lysis buffer to see if the lysis buffer in which the samples were present has any influence on the results. The standard curve which

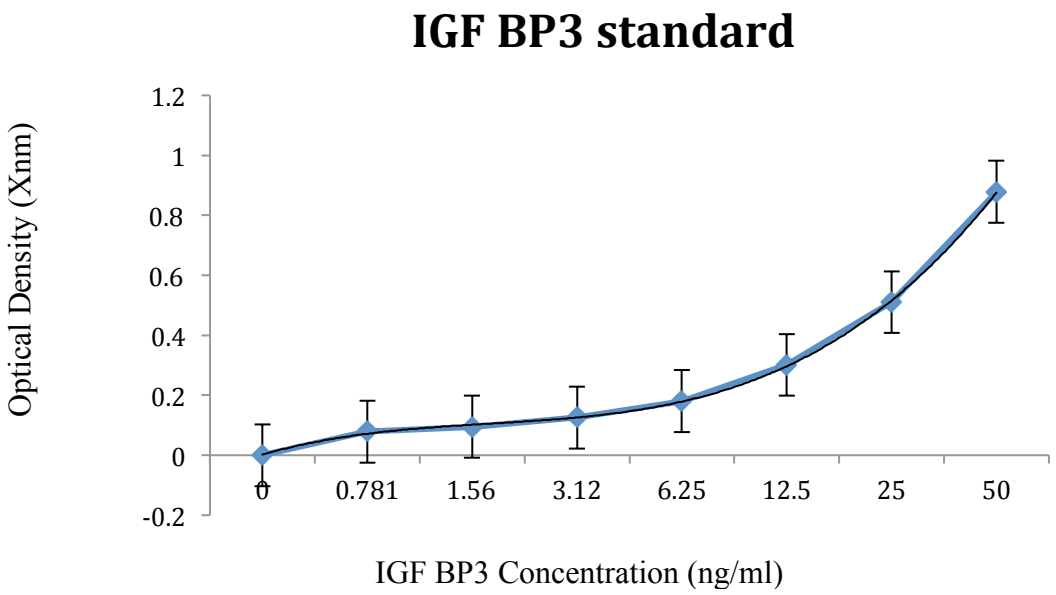
Tissue Sample	Sex/Age	Stage	Tissue Sample
Tumour Only	F/89	T1N0‡	Tumour Only
(n=27)	M/65	T1N0‡	(n=27)
	F/63	T2N0	
	M/60	T2N0	
	M/72	T2N2b	
	M/69	T3N0	
	M/60	T3N1	
	M/71	T3N2b	
	M/83	T3N2b	
	F/65	T3N2b	
	M/69	T4N0	
	F/63	T4N0	
	F/71	T4N0†	
	M/74	T4aN0	
	M/76	T4aN0	
	M/71	T4aN0	
	M/52	T4aN0	
	F/80	T4aN0	
	M/76	T4aN0	
	M/68	T4aN1	
	M/55	T4aN0	
	M/65	T4aN0	
	F/81	T4N1†	
	M/59	T4aN1	
	M/68	T4aN1	
	M/51	T4N2b	
	M/62	T4N2c	
Node only	M/69	T1N1	Node only
(n=4)	M/71	T1N0	(n=4)
	M/82	T2N2a	
	M/68	T3N2b	
Tumour and Node	M/63	T2N2c	Tumour and Node
(n=9)	M/72	T2N2b	(n=9)
	M/55	T3N0	
	F/64	T3N2	
	M/71	T4N0	

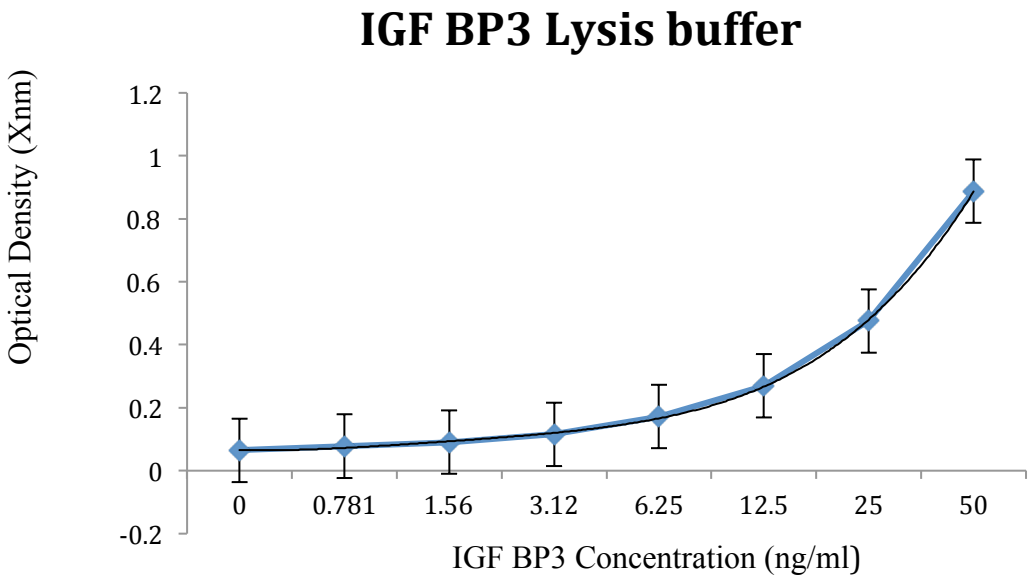
Table 3.1**:** Demographics of the patients

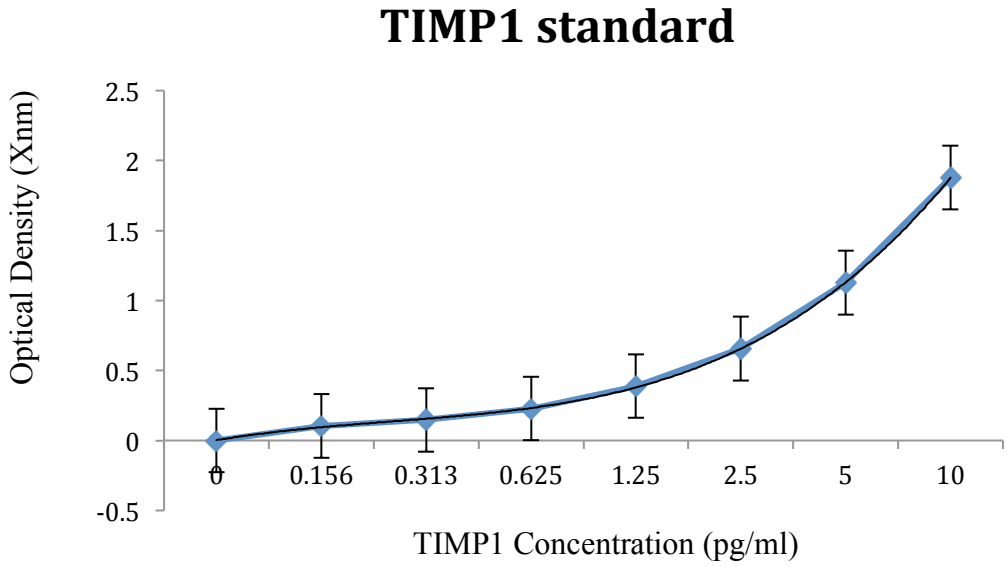
All patients were moderately differentiated squamous cell carcinoma (MDSCCC) except for:
†Partially differentiated SCC
‡ Carcinoma *in situ*

was generated with the lysis buffer for VEGF differed significantly from those diluted in proteojet lysis standard diluent. It was therefore decided that dialysis of the VEGF lysate was required to remove the lysis buffer before proceeding with the ELISA

All of the factors except VEGF generated equivalent standard curves whether the standards were diluted in normal diluent buffer or lysis buffer suggesting the ELISA was not affected by the lysis buffer (Figure 3.1).




IGF BP3 Lysis buffer
Optical Density (Xnm)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.781
1.56
3.12
6.25
12.5
25
50
IGF BP3 Concentration (ng/ml)


TIMP1 standard
Optical Density (Xnm)
2.5
2
1.5
1
0.5
0
-0.5
0
0.156
0.313
0.625
1.25
2.5
5
10
TIMP1 Concentration (pg/ml)

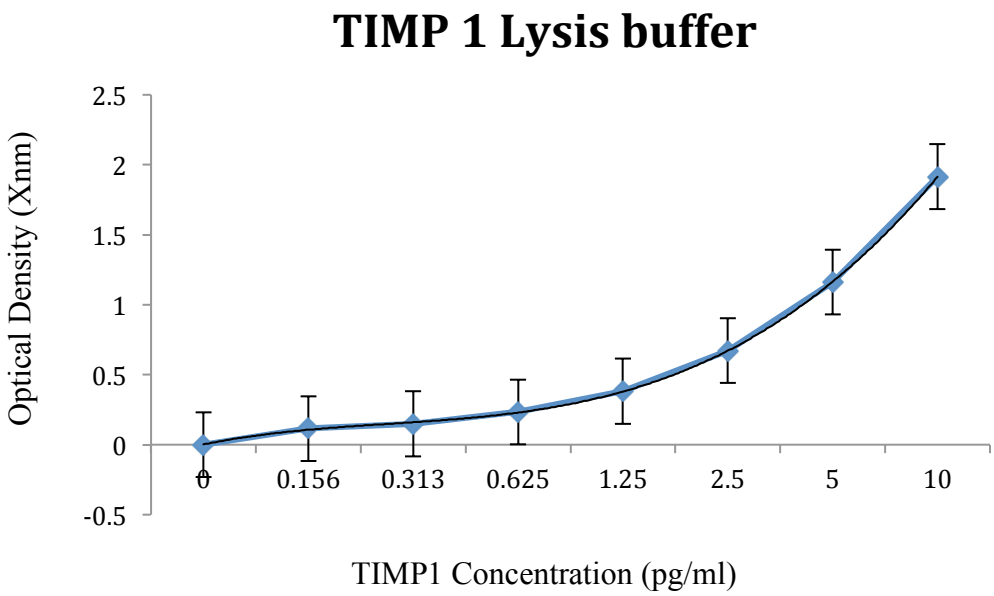


Figure 3.1**:** Examples of a four-parameter logistic standard curve showing error bars generated for IGF BP3 and TIMP1 diluted in two replicates of sample diluent or lysis buffer

3.4 Dialysis of tissue lysate

Dialysis is a separation technique that facilitates the removal of small, unwanted compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane, a process that results from the thermal, random movement of molecules in solution and leads to the net movement from areas of higher to lower concentration (until an equilibrium is reached). The rate of diffusion is directly proportional to the concentration of a molecule, while inversely proportional to its molecular weight. The rate of dialysis is also directly proportional to the surface area of the membrane and inversely proportional to its thickness. The semi permeable membrane is the key to dialysis (Figure 3.2) containing pores of a known size range that

are large enough to let small molecular weight compounds such as one in the lysis buffer pass through, but restrict the passage of large MW compounds (e.g., such as VEGF, (Molecular weight cut off) MWCO 3,500). The average or maximum pore size of a dialysis membrane determines the MWCO of the molecules that can diffuse across it.

A specialized dialysis cassette was used, a Slide-A-Lyzer G2 Dialysis Cassette (3.5K MCWO) (Thermo scientific, Rockford, USA), for the dialysis of VEGF from the tissue lysates. We chose 3.5K MCWO, as it was close to molecular weight cutoff for the VEGF and it facilitates effective removal of buffer salts and small contaminants from proteins and other macromolecules that are larger than 3,500 daltons.

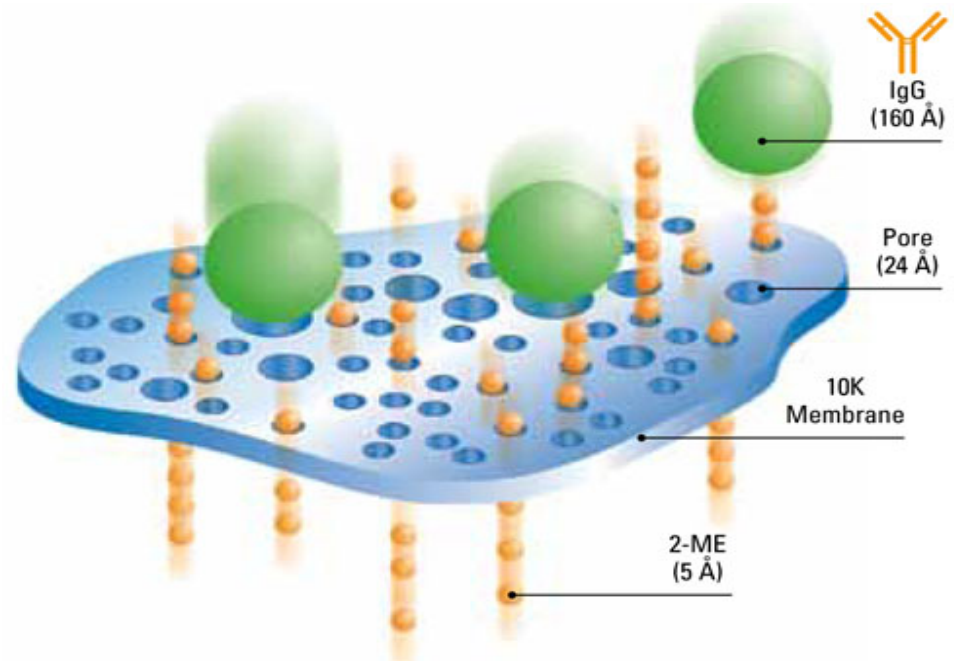


Figure 3.2**:** The dialysis membrane

A dialysis membrane is a semi-permeable film (usually a sheet of regenerated cellulose) containing various sized pores. Molecules larger than the pores cannot pass through the membrane but small molecules can do so freely. In this manner, dialysis may be used to perform purification or buffer exchange for samples containing macromolecules.

3.4.1 Method of Dialysis

The cassette was removed from its protective pouch (being careful not to touch the membrane to avoid contamination) before being immersed in dialysis buffer for 2 minutes to hydrate the membrane in the upright position to provide membrane flexibility. The cassette was then removed from the dialysis buffer and excess buffer was removed by gentle tapping of the cassette on some paper towel.

Tissue lysate (0.5-3ml) was placed into the cassette cavity using an 18-gauge one-inch beveled needle through the syringe port. Initially half of the sample was injected, avoiding foam formation, and the syringe was slowly withdrawn whilst injecting the rest of the lysate. Care was taken to avoid damage to the membrane. Once the sample was added, the gasket was resealed being careful not to trap air in the cavity; needle port was marked on the cassette.

The dialysis cassette was then placed vertically in a dialysis buffer solution with a volume 300 times the volume of the lysate. The cassette was dialysed for two hours with gentle stirring, avoiding creation of a vertex. After two hours the buffer was

changed followed by an overnight dialysis at 4^0C.

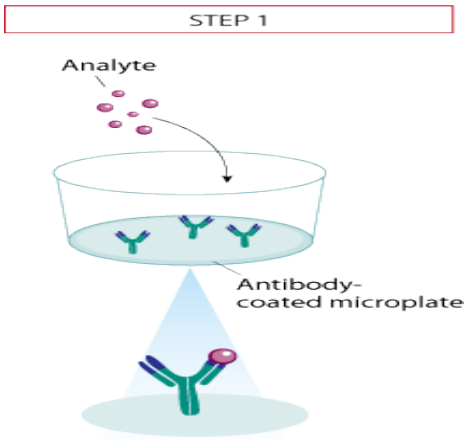
The dialysed sample was removed using the unusedport to avoid contamination of sample by slowly injecting air into the sample chamber to separate the membranes and prevent membrane puncture by the needle. The sample (3-5ml) was withdrawn slowly by turning the cassette upside down.

3.5 Method of ELISA

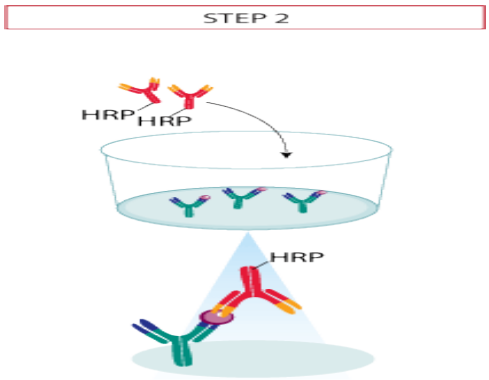
The concentrations of angiogenic factors (angiogenin, VEGF, TIMP1, FGF, IGF, IL8) present in the tissue lysates were determined using standard sandwich ELISA (Figure 3.3; R&D Systems, Minneapolis, USA) following the manufacturer's instructions.

Briefly 50µl of each standard (conc. 0-10 ng/ml (TIMP, FGF BP), 0-50ng/ml (IGF BP3), 0-2000 pg/ml (IL-8, VEGF), 0-5000 pg/ml (angiogenin)) or tissue lysate (neat or 1:10 dilution) and 100µl of assay diluents were added to separate wells of a 96 well plate in duplicate. The ELISA plate was covered with an adhesive strip and incubated for two hours at room temperature on a horizontal orbital shaker set at 500rpm to allow binding of the protein of interest to the immobilized antibody on the plate. Following incubation each well was aspirated and washed with 400µl of wash buffer three times before final aspiration using an automated plate washer (Well Wash 4 Mk 2 microplate strip Washer, Thermo Scientific, Waltham, USA). After the last wash the plate was inverted onto clean paper towels to ensure complete removal of wash buffer. A secondary antibody conjugated to horseradish peroxidase (200 µl) was then added to all wells and incubated for 1 hour at room temperature with shaking. Following further

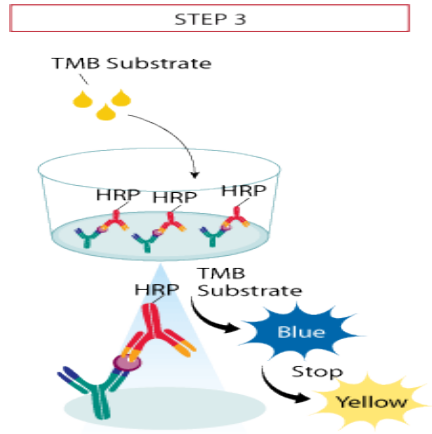
washes as described above 200µl of substrate solution was added to each well and incubated for 30 minutes protected from light. The reaction was stopped by the addition of 50µl of 2N H_2SO_4 to each well, the plate was mixed and the absorbance determined at 450nm with wavelength correction at 570nm. The plate reader performed blank subtraction and the average calculation of duplicate values automatically. Standard curves were generated using a four-parameter logistic curve fit for each factor investigated and used to determine the level of angiogenic factor present in each lysate.



Step 1: A microplate pre-coated with capture antibody is provided. Samples or standards are added and any analyte present is bound by the immobilized antibody. Unbound materials are washed away



Step 2: A second HRP-labeled antibody (detection antibody) is added and binds to the captured analyte. Unbound detection antibody is washed away



Step 3: Tetramethylbenzidine (TMB) substrate solution is added to the wells and a blue colour develops in proportion to the amount of analyte present in the sample. Colour development is stopped turning the color in the wells to yellow. The absorbance of the colour at 450 nm is measured.

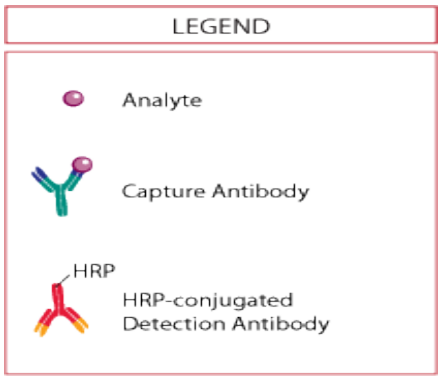


Figure 3.3: ELISA Workflow-Enzyme-linked Immunosorbant Assays:
(Source: R&D systems)

3.6 Results

Thirty-six patients with positive laryngeal cancer tissue were included in the study, from which 36 tumour samples and 14 nodal samples were available. Fourteen samples were paired lysates of tumour and node from the same patients.

The ELISA results were expressed as pg/mg of total protein for each individual tissue lysate after the converting the expressed ELISA results by the amount of the protein in each individual tissue lysate. Each lysate is multiplied by a factor of 10 before

processing for the analysis except for the factor VEGF as no dilution factor was used for processing with ELISA (Table 3.2). Figure 3.1b showing the standard curves for the factors analysed in the present study.

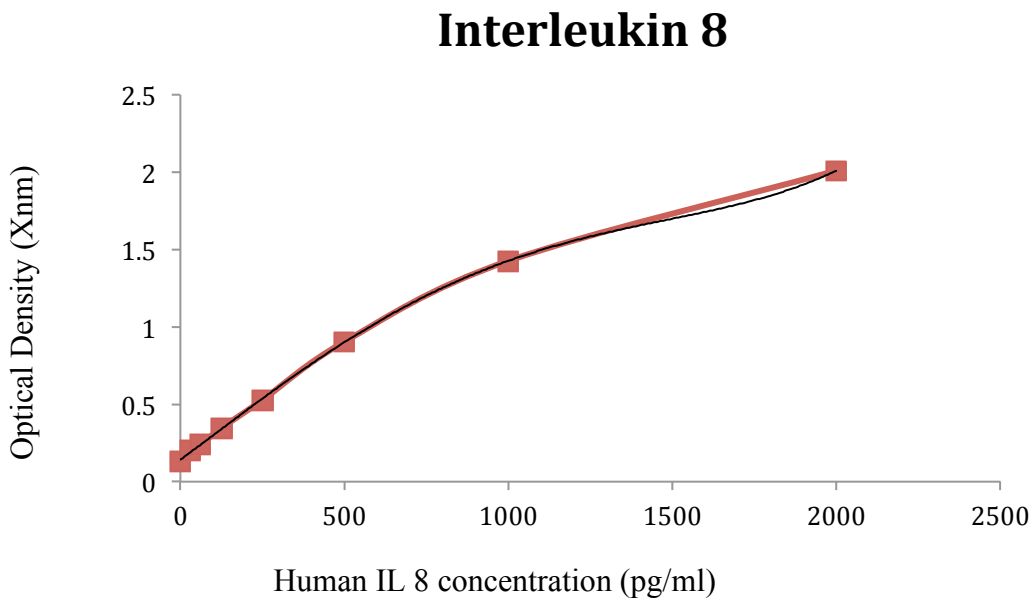


Figure 3.4a: Standard curve for Interleukin 8

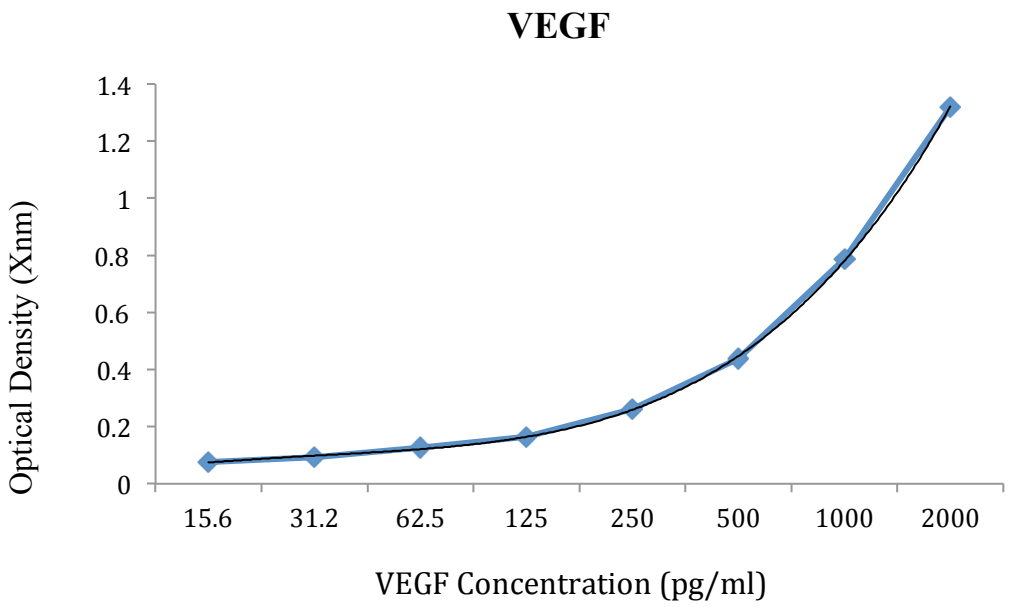


Figure 3.4b. Standard curve for the VEGF

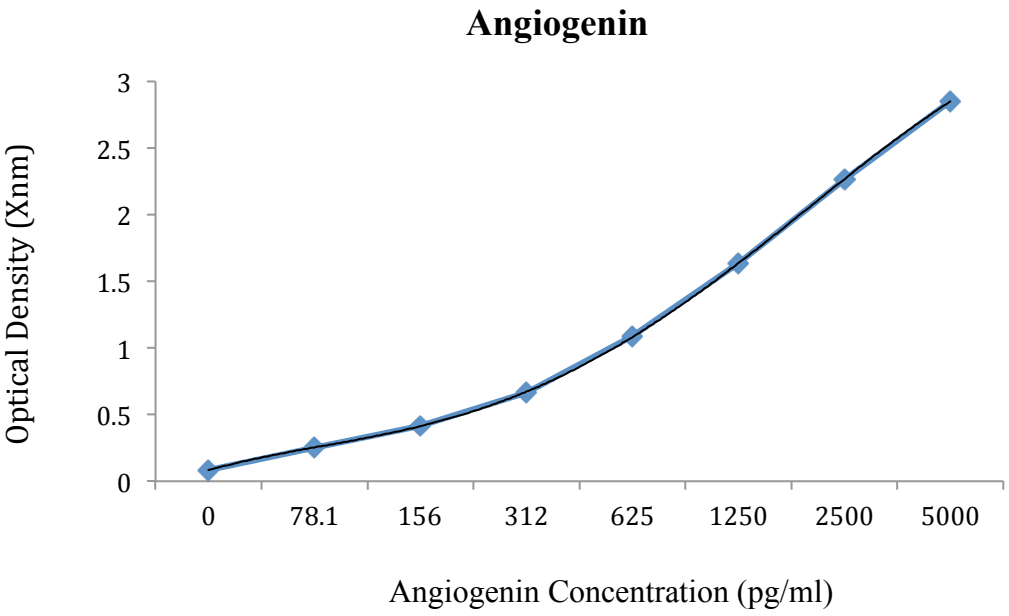


Figure 3.4c: Standard curve for Angiogenin

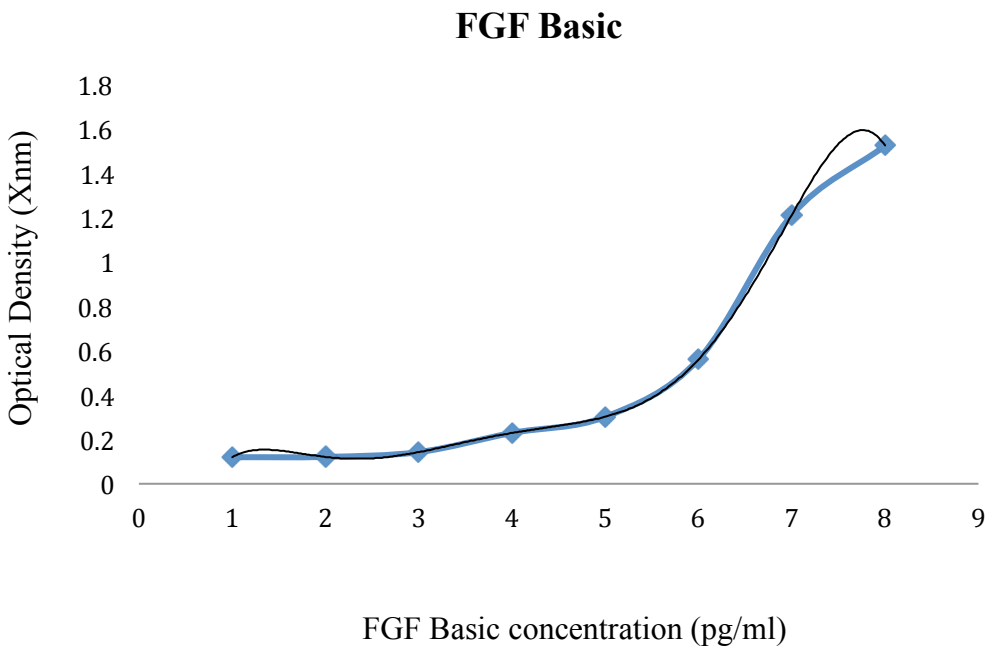


Figure 3.4d: Standard curve for FGF Basic

T	ANGIOGENIN	IL-8	IGF BP	FGF BASIC	TIMP1	VEGF
T1	8544					
	3382.9	821.6		1872.5		44.1
T2	9362.4	ND	ND	590.6	14.49	194.7
	24400	717.1	34.9	310.8	189.9	24.3
	4923.9	1520.6	ND	792.2	736.9	ND
	24399	3216.5	8.4	ND	76.9	37.2
T3	ND	ND	15.4		5.55	
	2103.9	4342.8	ND	600	942.1	88.1
	5004.3	1709.2	ND	1516.8		14.9
		817.9	768.8	ND	10.1	
	10450	497.5	9.16	346.2	0	
		ND	8.2	ND	14	11.6
		2687.9	8.6	ND	4.3	144.3
T4	9539.1	674.2	10.9	244.2	4.95	
		241.3	3	259.8	1.6	10.7
	5650	ND	2.9	150.6	11.3	
		ND				104.71
	9362.4	ND	ND	590.6	ND	
	3016.5	256.9	3.6	222.8	1.8	194.7
	6750	794.4	13.2	289.2	13.5	14.2
	8066.9	938	5.6	378.4	8.7	35.1
			10.4	736	24.1	88.3
	15814.9	281.9	23.2	554.9	21.7	
	13867		5.61	1541.2	16.3	23.6
	3382.9	821.6	ND	1872.5	ND	17.7
	17617.2	1345	ND	1048.2	7.16	44.1
	ND	1088.1	37	ND	16.5	48.9
	3900	596.2	3.2	ND	7.5	63.6
		3520	13.6	208.4	24	1.6
		1036.4	22.4	ND	17.6	
		229.3	21.1	217.4	13.5	17.6
	9936.4	710.6	7.49	414.3	22.2	
	5250	2100	5.6	209.4		40.9
		ND				19.4
	6100	522.1	7.9	780.8		
		362.1	9.9	315.9	9.7	4.4

Table 3.2**:** Results of factor expression in tumour tissue lysates (Values expressed pg/mg to total protein in each lysates)

	Angiognin	IL8	IGF BP	FGF Basic	TIMP 1	VEGF
T1	14450	293.9	9.4	1248.8	8	559.8
	ND	1337.1	16.4		4.1	141.9
T2	7050	ND	53.6	92.9	14.1	2820
	14000	ND	6.5	564.8	28.0	5600
	ND	4661.8	52.1	284.4	38.7	
T3	9300	378.6	9.3	695.3	6.1	
	ND	4066	34.4	144.3	21.6	9960
	ND	2796.6	37.1	255.8	17.1	331.2
T4	7142.8	1117.6	6.3	555	14.9	
	24850		11.3	1613	34.4	
	819.4		11.4	176.6	2.5	81.7
	18050	7220	42	739.8	36.1	7220
			8.4	113.7	4.1	

ND: Not detectable

Table 3.3: Results of factor expression in nodal tissue lysates (Values expressed pg/mg ot total protein in each lysates)

The data were analysed using the statistical package for the social sciences version 18(SPSSS Inc. Chicago, iii).

Analysis is performed by separating into two groups:

A) Early and late stage of the tumor

B) Tumor and corresponding nodal samples.

A. Analysis of expression of angiogenic factors between early and late of the tumor.

Positive angiogenic expression was identified for the most of tissue lysates, but some of the samples had not shown any positive expression/negligible expression of the angiogenic factors in the tissue lysates. All angiogenic expression was converted pg/mg before performing analysis. A total 36 samples, which have shown positive results in the study, were included, of which six samples in the early stage and thirty samples were identified in the late stage of the tumor, were included in the final analysis. Statistical analysis of angiogenic expression was carried out between early and late stage of the tumour.

The Mann Whitney U test was used to analyse expression between the early and late stages of the tumor as most of them are not paired samples and we included six different angiogenic factors for our analysis. The final stastical analysis of the six angiogenic factors identified factor angiogenin has significant results. Angiogenin was highly expressed in the early stages of the tumor compared to the late stage of tumor ($p = 0.032$) (Figure 3.5) (Table 3.4).

The remaining five angiogenic related growth factors (VEGF, FGF BP, TIMP-1, IL8 and IGF –BP3) did not show any statistical significant changes between early and late stage of the tumor (Table 3.4).

Angiogenin factor		N	Mean	Std. Deviation	Significance
Angiogenin	Early	6	12502.03	9478.49	p = 0.033
	Late	30	4527.05	5242.67	
IL8	Early	5	1255.18	1221.67	p = 0.330
	Late	30	852.46	1069.18	
IGF BP3	Early	5	8.67	15.13	p = 0.314
	Late	29	35.0717	141.37	
FGF Basic	Early	5	713.22	713.41	p = 0.269
	Late	29	430.95	500.54	
TIMP 1	Early	5	203.66	307.36	p = 0.096
	Late	28	122.48	595.15	
VEGF	Early	4	75.11	80.15	p = 0.872
	Late	30	350.25	790.96	

Table 3.4: Comparison of cytokines between early and late stage of the tumour lysate

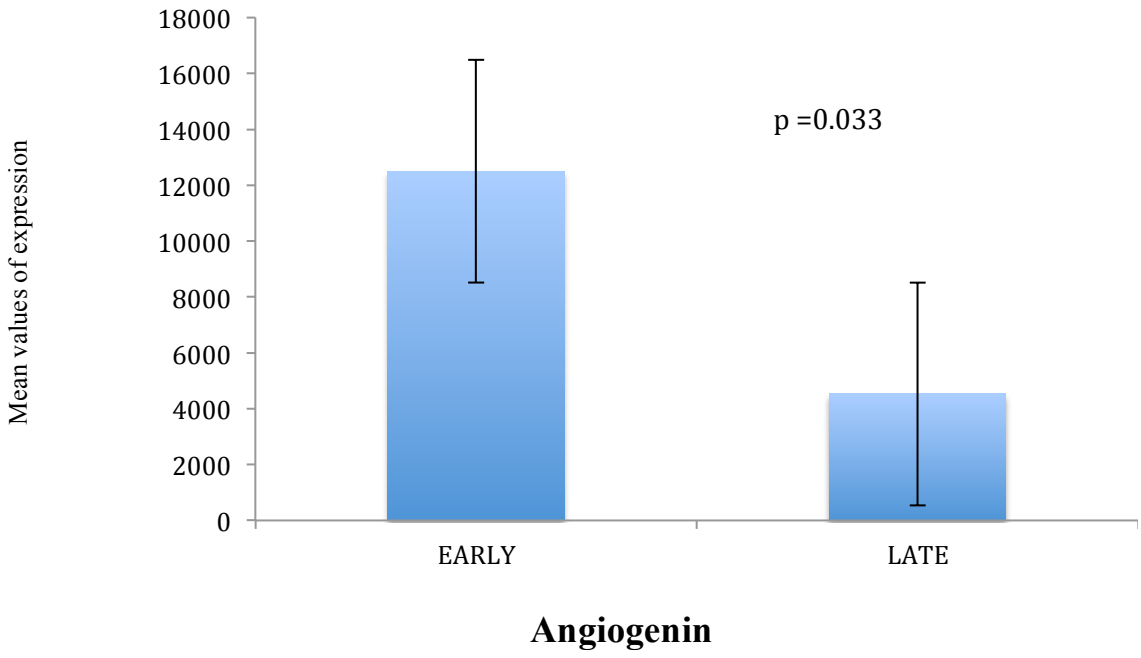


Figure 3.5**:** Increased expression of Angiogenin in early stage of the tumour compared to late stage of the tumour (p=0.033)

B) Analysis of angiogenic factor expression between tumor and node

Analysis was performed with tumor samples and corresponding metastatic node samples, which have shown positive expression in the experiment. The statistical analysis was performed analyzing the association of factors between tumor and corresponding node. A total of thirty-six tumor sample lysates and 14 nodal lysates were included for the final analysis between tumor and nodal samples

Angiogenin factor		N	Mean	Std. Deviation	Significance
Angiogenin	Tumour	36	5697.93	6661.79	p = 0.593
	Node	13	7358.63	8327.00	
IL8	Tumour	36	884.71	1077.31	p = 0.713
	Node	13	1682.43	2321.69	
IGF BP3	Tumour	35	30.54	128.79	p= 0.005
	Node	13	22.98	18.07	
FGF Basic	Tumour	34	472.46	533.45	p = 0.765
	Node	13	498.80	483.43	
TIMP 1	Tumour	33	134.78	558.16	p = 0.323
	Node	13	17.68	12.99	
VEGF	Tumour	34	317.88	747.31	p = 0.257
	Node	12	4347.89	8290.45	

Table 3.5: Statistical details of the angiogenic factor expression comparing the tumour with the corresponding node.

Mann Whitney U test was performed to analyse the data between tumor and node because of comparison of multiple factors unpaired samples. The experiment had identified the factor IGFBP3 showed statistically significant increased expression in tumor lysates compared to the nodal lysates (p value=0.005). (Figure 3.6) The remaining five (VEGF, FGF BP, TIMP-1, IL8 and angiogenin) analyzed angiogenic

factors did not show any statistically significant difference between tumor and nodal samples (Table 3.5).

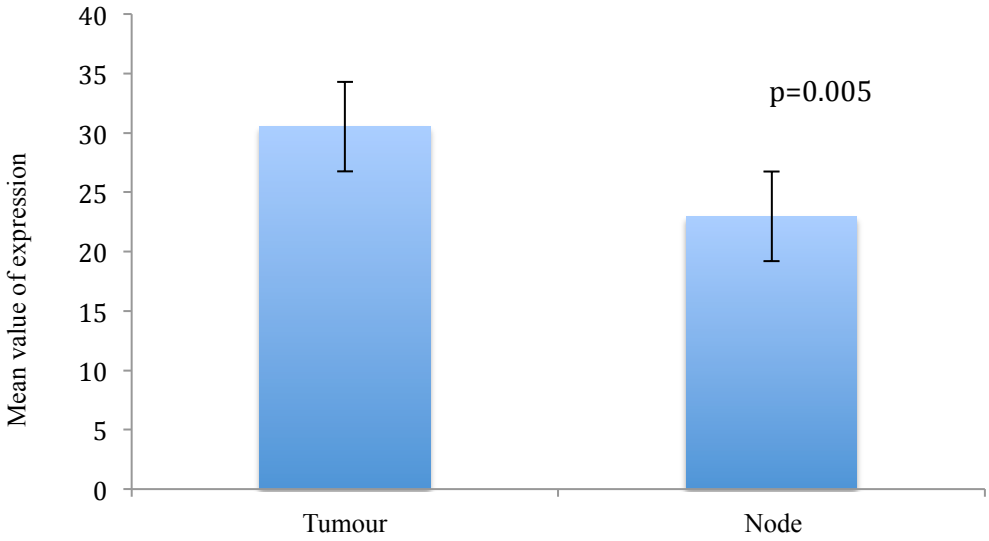


Figure 3.6: Increased expression of IGF BP3 in tumour compared with corresponding node (p=0.005)

3.7 Discussion

The current study has investigated expression of six angiogenic related growth factors in fresh tissue lysates of laryngeal malignancy, and has identified angiogenin (ANG) and IGF BP-3 as potential factors which may have a assoication in the laryngeal malignancy progression, but factors VEGF, FGF, IL-8 and TIMP-1 did not shown any statistical significant role in the current study. The reason for including six factors VEGF, IL8, FGF –BP, TIMP-1, IGF BP3 and ANG is, that these factors have showed an increased expression in pilot study with the tumour progression. There are previous studies in the literature which have looked into the role of multiple angiogenic factors, has identified factors VEGF, IL8, FGF-BP, TIMP-1 have role in head and neck cancers.

Quantitative analysis with ELISA in a cohort of tissue lysates has confirmed increased expression of ANG in the early stage of the tumour compared to the late stage of the tumour. These were revealed in the initial pilot study, and factor IGF BP3 expression was high in the tumour tissue compared with corresponding lymph nodes.

ANG expression is up regulated in various types of human cancers, including breast, cervical, gastric, hepatic, renal, prostate, pancreas, endometrial, colorectal, leukemia, lymphoma, melanoma and osteosarcoma (Gao and Xu 2008). It has been suggested that ANG expression appears to have dual role in cancer growth, involving both cancer cell proliferation and angiogenesis (Yuan et al., 2009). Moreover, in most of these cancers, prognosis is correlated with degree of angiogenin expression (Katona et al., 2005). Information on the role of ANG in head and neck malignancies is extremely sparse and limited to serum protein levels (Homer et al., 2000). Homer et al investigated the prognostic role of three angiogenic factors angiogenin, bFGF and endostatin in serum of HNSCC patients and identified sANG has significant negative association with tumour recurrence and observed similar negative trends with poorer grade and advanced stage of the tumour. The present study investigated angiogenin expression and its biological role in tissue lysates of laryngeal cancer tissue and associated nodes using ELISA method. Angiogenin expression was noted in 34/36 the tissue lysate samples of laryngeal carcinoma, and the expression was correlated with stage of the tumour, and identified an increased expression of the factor in early stage of the tumor compared with late stage of the tumor. Marioni et al (2011) investigated ANG expression in 12 head and neck baseloid SCC (HNBSCC) and compared with control group (n=24) site and stage matched conventional SCC, they have concluded that a high ANG expression in carcinoma cells was significantly associated with pathological-T stage in both the

HNBSCC (P=0.04) and the SCC (P=0.07) groups, and in HNBSCC a significant inverse correlation with endothelial expression and DFS (P=0.08). Other studies by Marioni et al who investigated ANG expression in a cohort of 108 patients both in laryngeal carcinoma cells and intra-tumoural cells identified angiogenin expression had no correlation with pT, N, stage and grading of laryngeal cancer. ANG expression was higher in laryngeal carcinoma samples of patients who had loco-regional recurrence (p=0.032) and DFS was shorter in cases with carcinoma cells showing high angiogenin expression (p=0.035). They have concluded that ANG expression in laryngeal cancer is useful to detect laryngeal SCC with a higher risk of disease recurrence (Marioni et al., 2010).

Insulin growth factors play a crucial role in regulating cell proliferation and differentiation. IGF's including IGFI and IGFII are peptide hormones with strong mitogenic effects on both normal and cancer cells (Jones and Clemmons 1995; Yu et al., 1999) as well as apoptosis suppressive effects (Dunn et al., 1997). The activities of IGFs are modulated by a family of high affinity IGFBP, of which IGFBP3 is major serum carrier protein (Stewart and Rotwein 1996; Baxter 2001). The increased expression of IGF BP3 in tumour lysates compared with the corresponding nodal lysates of the laryngeal tumours in the analysis, signify the fact it has a role in the tumor progression in the laryngeal tumours as shown in the literature (Chan et al., 1998; Han et al., 2006).

Han et al measured the serum levels of IGF-1, IGF-2 and IGF-BP3 in 77 patients with advanced non-small cell lung cancer (NSCLC) and noted higher levels of IGF BP3 and IGF-2 in NSCLS patients. They have demonstrated higher levels of IGF BP3, IGF-1

(p=0.0001 and p=0.001) were independent predictors of progression–free survival by multivariate analysis. But a study by Papadimitrakopoulou et al (2006) in HNSCC, analysed the expression of IGF BP-3 protein expression in 34 SCC of the tongue (stage II-IV) and 30 premalignant lesions of oral cavity and larynx, and showed down regulation of IGF BP3 expression in a significant fraction of patients with stage II-IV tongue SCC and in premalignant lesions. A significant inverse relationship between the loss of IGFBP3 expression and overall, disease specific, disease free survival probability was observed (p=0.0002 and <0.0001). They have concluded low IGFBP-3 expression is an unfavorable prognostic factor in patients with tongue SCC.

Sun et al (2011) evaluated intra-tumoural expression of IGF BP3 and IGF 1R in a cohort a of 131 HNSCC patients by immunohistochemistry and identified IGF BP3 positivity was associated with shorter time to progress. IGF BP3 and IFG-1R positivity were also observed in recurrent patients during a median follow up period of 53.7 months (95%ci, 19.0-90.7 months). Similarly Wu et al (2004) in a case control study compared the pre-diagnostic serum IGF BP3 and IGF-1 levels in 80 patients who subsequently developed second primary tumours (SPT) and found higher levels of IGF -1 and IGF BP3 (p=0.009 and p=0.001) in patients who developed SPT, and concluded that lower and higher levels of IFGBP3 are associated with 2.22 and 7.12 fold significant increased risk of second primary tumours.

The results of studies on VEGF expression and tumour angiogenesis and the relationship between the clinicopathological factors and prognosis in head and neck tumours, particularly in SCC of the larynx, are controversial (Tae et al., 2000; Bandoh et al., 2004). Sullu et al (2010) assessed the relationship between the expression of

VEGF and angiogenesis and clinic-pathological parameters in SCC of the larynx. This study included 140 patients with SCC of the larynx and examined immuno-histochemical expression of VEGF and CD34 in paraffin embedded tissue blocks. They have identified significant relationship between severity of VEGF expression and tumour size p=(0.006), and lymph node metastasis p=(0.048) and tumour grade p=(0.015). Kyzas et al (2005) performed a retrospective study of 69 patients with HNSCC and noted high expression of VEGF in larynx and oral cavity, and this was associated with higher clinical stage and worse survival. But they have not identified VEGF as significantly correlated with lymph node metastasis at the time of diagnosis. In contrast, Tae et al (2000) reported that there was no correlation between VEGF expression and tumour differentiation, stage, and localization in carcinomas of head and neck region. VEGF expression was high in group without lymph node metastasis. The findings in the study showed relation with VEGF expression and tumour progression and lymph node metastasis, though it was not statistically significant. The reasons for different findings in the study may be the different assessment of VEGF expression and fact that the other studies included tumour with different localizations in the head and neck region.

Other factors that are examined, factors FGF BP, TIMP-1 and IL-8 have showed increased expression in the tissue lysates, but no statistically significant role was noted with quantitive analysis with ELISA in tumour progression either between early to late stage nor between tumour and corresponding nodal tissues. Studies by Li et al (2009) who investigated expression of FGF BP in HNSCC in 35 primary (8 larynx, 11 oral cavity) and 8 metastatic specimens and 7 control tissues identified increased expression in both primary and metastatic tissue specimens but not in adjacent control tissue. They

concluded increased FGF BP expression is linked to the angiogenesis in HNSCC. Another study by Ruokolainen et al (2005) who looked into the expression of TIMP 1 both in pre-treatment serum levels (n= 68) and tumour tissue (n=74) found positive correlation between size of the tumour and circulating TIMP-1 level (p=0.035) and positive immunoreaction of TIMP-1 in tumour (p=0.039). In agreement with the current study no correlation was found with tissue TIMP-1 positivity or serum TIMP -1 levels with stage of the disease or grade of the tumour or degree of lymph node invasion. Hathaway et al. (2005) reported that the serum levels of IL-8 significantly differed by the T stage of HNSCC patients, IL-4 differed by the N stage and IL- 6 showed no significant difference by the T and N stages. Druzgal et al. (2005) reported that the serum levels of IL-6, IL-8, HGF, and VEGF were increased in patients with HNSCC as compared to the normal control group. The reasons that the current study findings differ from results of other studies could be because cohort included more numbers in late stages and have included fresh tissue lysates in our analysis and concentrated on larynx sub site, as most of the other studies in literature included multiple HNSCC sub sites.

Conclusion:

This study is the first to look at the role of multiple angiogenic factors in tissue lysates of tumour and node and have identified factor angiogenin and Insulin like growth factors as having a potential role to play in tumour development in laryngeal malignancies. In depth studies are required looking into the function of these factors offer a significant role both in prognostic and therapeutics values in the future.

CHAPTER 4

Prognostic correlation of angiogenic factor with tumour progression and treatment and tumour characteristics

4.1 Introduction

The survival prospects for patients with laryngeal cancer are moderately favorable. The most recently available data for patients in England and Wales show that for men diagnosed in 1996-99, the age standardized relative survival at one year after diagnosis was 84 percent, and at five years was 63 percent. (ONS. Cancer survival: England and Wales, 1991-2001. March 2004). The most important adverse prognostic factors for laryngeal cancers include increasing T stage and N stage. Other prognostic factors include pathologic features of the tumor, including grade and depth of invasion (Yilmaz et al., 1998); p53 expression (Gasparini et al., 1994); oncogene recognition (Weinstat-Saslow and Steeg 1994) and extent of angiogenesis (Folkman 1990).

Patients treated for laryngeal cancers are at the highest risk of recurrence in the first 2 to 3 years. Recurrences after 5 years are rare and usually represent new primary malignancies.

Angiogenesis plays a significant role in prognosis of the tumours. Dray et al demonstrated a strong correlation between high microvessel count and recurrent or metastatic disease in HNSCC (Dray et al., 1995). The most important molecules positively affecting angiogenesis in head and neck cancers are FGFb, VEGF, HGF, interleukin-8 and PDGF (Chen et al., 1999; Riedel et al., 2000; Dong et al., 2001; Ninck et al., 2003). Studies have been conducted to assess whether pretreatment serum levels of tumour associated antigen (Molina et al., 1996), pro-angiogenic (Homer et al., 2002;

Teknos et al., 2002) or pro-inflammatory cytokines, (Tartour et al., 1997; Duffy et al., 2008) growth factors (Wu et al., 2004) or hormones (Hedstrom et al., 1999) could help predict cancer recurrence, occurrence of second primary cancers (SPC), or overall survival among HNSCC patients. These studies have yielded inconsistent results.

The objective of this study is to assess the prognostic significance of expression of six angiogenic related growth factors in laryngeal tissue lysates in predicting the survival and recurrence in laryngeal cancer patients with relation to the clinical characteristics.

4.2 Material and Methods

A cohort of 36 patients diagnosed with poor to well differentiated laryngeal squamous cell carcinoma were included into the study (Table 4.1). The patient group included males (n=30) and females (n= 6) with the age ranging from 51 to 89 (median 74).

TNM staging was done in all cases according to the AJCC (American Joint Cancer Committee) which included 5, 4, 9, 18 patients in T1, T2, T3 and T4 stages respectively.

Patients were treated with curative intent, 21/36 patients were treated with combination of laryngectomy and neck dissection, 9 patients was treated with laryngectomy, three

patients treated with laser excision and 3 patients had neck dissection alone. 28/36 patients received postoperative radiotherapy because of advanced stage of the tumor, positive neck nodes and extra capsular spread of the tumor

The following statistical tests were performed: one- and two-sided t-tests, Mann–Whitney U-test. The Kaplan–Meier survival function, the log rank test and Cox regression model were applied to display and evaluate, respectively, the different disease free survival (DFS) of the patients stratified according to the selected variables. A p-value <0.05 was considered to be significant; values in the range 0.10 - 0.05 were considered as indicating a statistical trend. The SPSS version 18 (statistical package for the social sciences) was used for all evaluations.

Prognostic effects of age, gender, stage, and levels of Angiogenesis related growth factors on overall survival and DSS were evaluated using univariate and multivariate Cox proportional hazards regression models.

A cohort of 36 newly diagnosed patients with carcinoma of larynx was included in the study. It included male n= 30 and female n=6: Mean follow up for all the patients was 23.9 months and median value was 18 months.

Thirteen of 36 patients died during the follow up period. Ten were due to the primary tumor. Most of these patients were in advanced stage T4: 8: T3: 2: and T2: 1. Disease free survival and overall survival were calculated from the date of the diagnosis. Survival data was estimated between angiogenic factor expression and patient variables like age and gender and tumor characteristics T, N and M stage of the tumor.

	Sex/age	Stage	Histology	Treatment	Recurrence	Death
1	M/71	T1N0	WDSCC	ND+DXT	YES	YES
2	M/65	T1N0	ca.insitu	Laser	NO	NO
3	F/89	T1N0	ca.insitu	Laser	NO	NO
4	F/67	T1N1	MDSCC	L+ND+DXT	NO	NO
5	M/68	T1N2B	MDSCC	L+ND+DXT+C	NO	NO
6	F/63	T2N0	MDSCC	L+DXT	NO	NO
7	M/60	T2N0	MDSCC	L+DXT	YES	YES
8	M/82	T2N2A	MDSCC	ND+DXT+C	YES	NO
9	M/F72	T2N2B	MDSCC	L+ND+DXT+C	NO	NO
10	M/71	T3N0	MDSCC	L+ND	NO	NO
11	M/55	T3N0	MDSCC	L+ND+DXT	NO	NO
12	M/69	T3N0	MDSCC	L+	NO	NO
13	M/60	T3N1	MDSCC	L+ND+DXT	NO	NO
14	M/83	T3N2B	MDSCC	L+ND+DXT+C	NO	NO
15	M/71	T3N2B	MDSCC	L+ND+DXT+C	YES	YES
16	F/65	T3N2B	MDSCC	L+ND+DXT+C	YES	NO
17	M/68	T3N2B	MDSCC	L+ND+DXT+C	NO	YES
18	M/62	T3N2C	MDSCC	L+ND+DXT	NO	YES
19	M/69	T4N0	MDSCC	L+DXT+CHEMO	YES	YES
20	F/53	T4N0	PDSCC	L+DXT	YES	YES
21	M/F72	T4N0	PDSCC	L+ND+DXT	NO	NO
22	M/76	T4aN0	MDSCC	L+DXT	NO	NO
23	M/52	T4aN0	MDSCC	L+ND-DXT	NO	NO
24	M/74	T4aN0	MDSCC	L+DXT	YES	NO
25	M/55	T4aN0	MDSCC	L+DXT	YES	YES
26	M/65	T4aN0	MDSCC	L+ND+DXT	NO	NO
27	M/F71	T4aN0	MDSCC	L+ND+DXT	NO	NO
28	M/85	T4N1	PDSCC	L+ND+DXT	YES	YES
29	F/81	T4N1	PDSCC	Laser	NO	YES
30	M/68	T4aN1	MDSCC	L+DXT+C	NO	NO
31	M/59	T4aN1	MDSCC	L-DXT	YES	YES
32	M/59	T4aN2B	MDSCC	L+ND+DXT+C	NO	NO
33	M/76	T4aNX	MDSCC	L	NO	NO
34	M/51	T4N2B	MDSCC	L+ND+DXT+C	NO	NO
35	M/83	T4N2C	Mets scc	ND+DXT+C	NO	YES
36	M/63	T4aN2C	MDSCC	L+ND+DXT+C	YES	YES

L: Laryngectomy ND: Neck dissection DXT: Radiotherapy C: Chemotherapy

MDSCC: Moderately differentiated squamous cell carcinoma

PDSCC: Poorly differentiated squamous cell carcinoma

WDSCC: Well differentiated squamous cell carcinoma

Ca.insitu: carcinoma insitu

Table 4.1: Demographics of patients

4.3 Results

Angiogenic growth factor expression has not shown statistically significant results in predicting the overall survival or disease free survival with univariate or multivariate analysis (Table 4.2). Similarly we have correlated patients' variables like age and gender, TNM staging treatment with either surgery or combination of surgery and radiotherapy with survival prognostic significance has not identified any significance in predicting the survival of the patients (Table 4.2). (Figure 4.1)

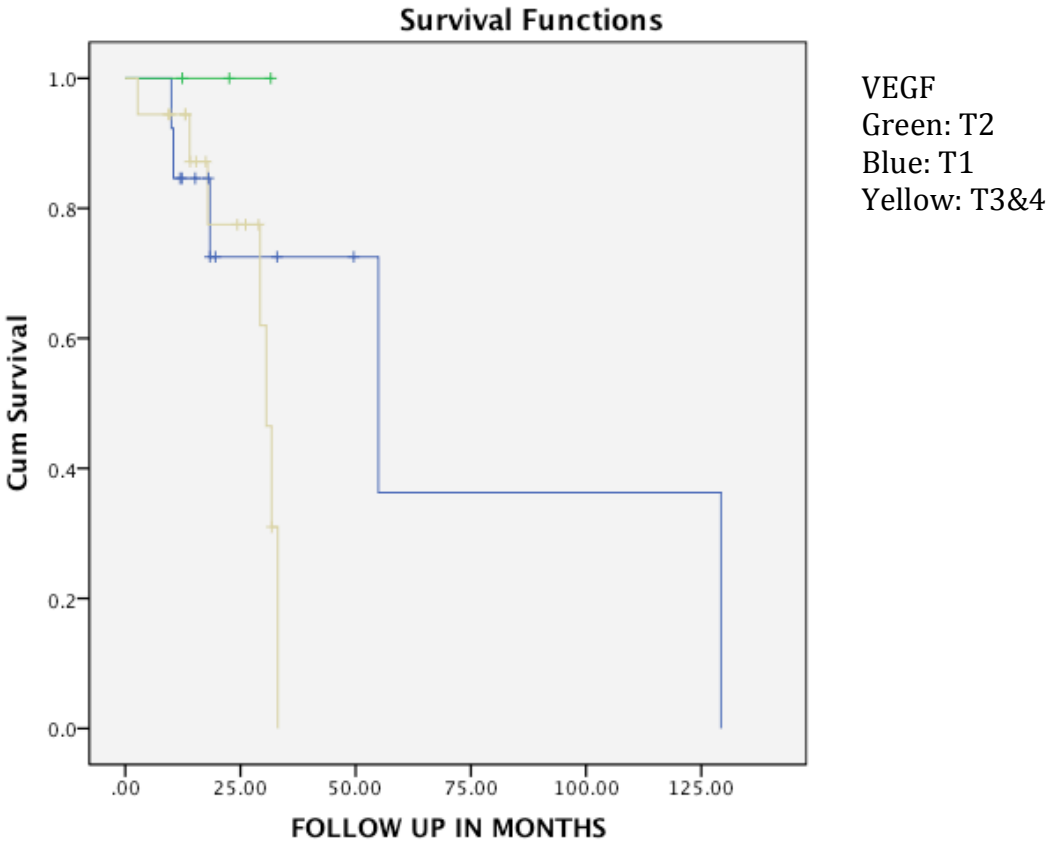


Figure 4.1a: Effect of VEGF on overall survival (log rank =0.273)

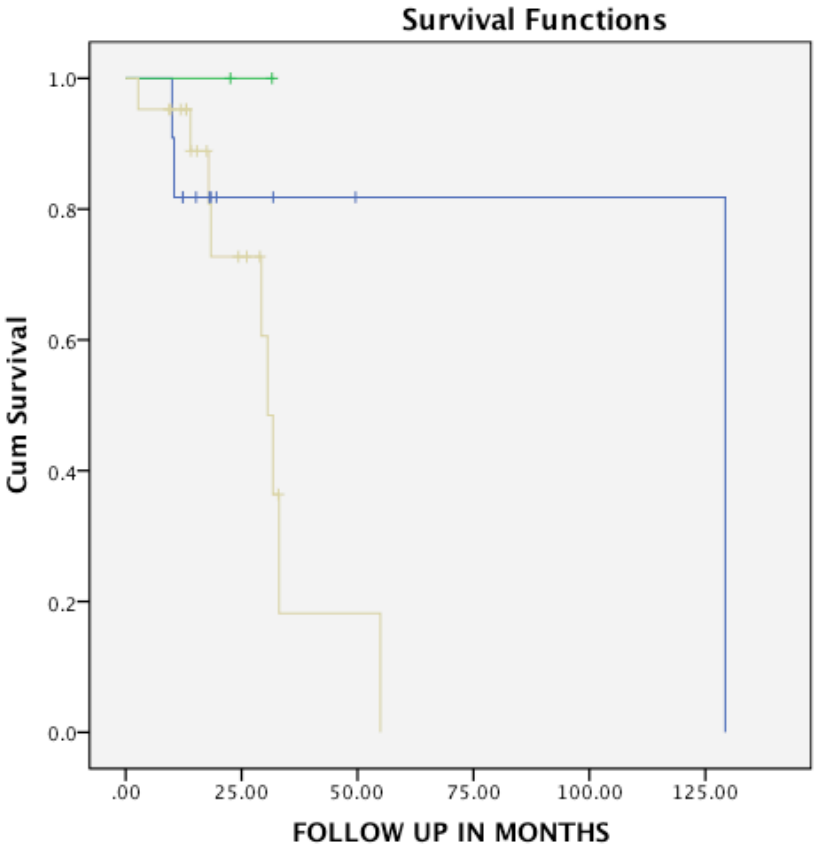
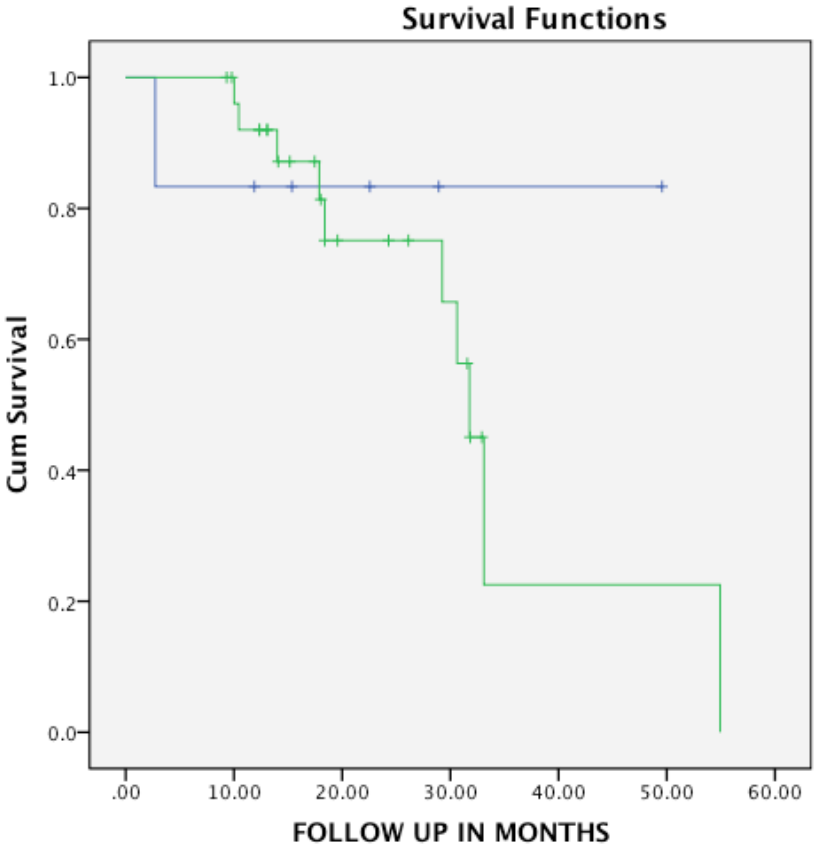


Figure 4.1b**:** Effect of TIMP 1 on overall survival (log rank = 0.367)

13(36%) patients had recurrence of the disease. Recurrence of the disease was more in advanced stage of the tumor than in early stage tumours. T stage, with stage T1: 1, stage T2: 2, stage T3: 2 and stage T4 had 7 patients with recurrence. Recurrent tumours were treated with surgery either completion of excision, radio or chemotherapy.

	OSS		OSS (Tumour/Node)		DSS		DSS(Early and late)
Chi-square	2.085				3.03		4.234
Significance	0.896				0.805		0.752
	Sign.	Mean	Tumour	Node	Sign.	Mean	Sign.
Angiogenin	0.287	5152.56	0.166	0.806	0.745	0.455	0.983
IL8	0.428	878.07	0.311	0.678	0.538	0.364	0.968
IGF	0.851	30.61	0.826	0.750	0.554	0.455	0.229
FGF	0.566	314.14	0.660	0.898	0.916	0.455	0.807
TIMP 1	0.775	37.16	0.492	0.717	0.287	0.364	0.193
VEGF	0.660	199.94	0.500	0.659	0.697	0.394	0.500

Table 4.2: Statistical values for the angiogenic factors



Blue: Radiotherapy
Green: Surgery+Radiotherapy

Figure 4.2 Overall survivals of patients treated with radiotherapy (log rank =0.512)

In this study we have identified that patients who have had treatment with radiotherapy combined with surgery have shown higher recurrence rates on univariate regression analysis (p =0.04), but not with multivariate analysis. Other variable tumor stage, age, gender and surgery have not shown prognostic significance in predicting the recurrence of the tumor either by univariate or multivariate analysis (Table 4.3).

	Tumour	Radiotherapy	Chemotherapy
Angiogenin	0.464	0.761	0.319
IL-8	0.790	0.479	0.902
IGF	0.782	0.467	0.489
FGF BP	0.687	0.163	0.337
TIMP-1	0.555	0.091	0.720
VEGF	0.649	0.909	0.482
Overall %	65.6	78.8	61.8

Table 4.3**:** Logistic regression analysis showing the recurrence rates with treatment

The present study showed that patients treated with combination of surgery and radiotherapy has identified to have role in predicting recurrence of the disease. But patient variables age, gender and tumor characteristics (T, N and M) and treatment (surgery, radiotherapy and chemotherapy) has not shown any statistical significant results in predicting either survival or recurrence of the tumour.

4.4 **Discussion**

The present study has showed radiotherapy as a prognostic factor in predicting the recurrence of laryngeal tumour. No significant role was identified in predicting survival or recurrence of tumours of the patients with laryngeal cancer, for the angiogenic related growth factors studied. Similarly, the present study has not shown any significance with patient related factors or tumor characteristics and treatment of tumours in predicting in the prognosis of the laryngeal tumour.

Studies in the literature have recognized prognostic relationship with angiogenesis in laryngeal cancer patients (Murray et al., 1997; Kupisz et al., 1999). Kupisz et al (Kupisz et al., 1999) evaluated tumour angiogenesis in 60 patients with primary laryngeal cancer by quantitating the micro vessel density with antibodies against factor VIII. They have demonstrated direct correlation between increased tumour angiogenesis and T stage and histological grade and a shorter survival rate. In other study Murray et al (Murray et al., 1997) examined the relationship of tumour angiogenesis to recurrence in 51 patients with SCC of laryngeal carcinoma, and demonstrated that angiogenesis shows strong correlation with regional recurrence and may be used as an independent prognostic indicator to determine clinically node negative patients ($p=0.025$).

Later studies in the literature have specifically looked into prognostic significance of individual angiogenic related growth factors. Most commonly studied factors in the literature in HNSCC are VEGF (Tse et al., 2007), IGF (Wu et al., 2004) MMP -9 and IL 8 (Le et al., 2012) (Hong et al., 2009) .

We have examined the correlation of expression of six angiogenic related growth factors in tissue homogenates with prognostic significance in laryngeal cancer patients. But there are only a few studies in the literature which looked into the role of multiple angiogenic related growth factors in the tissue homogenates in HNSCC. Montag et al (Montag et al., 2009) quantitatively measured expression of eight angiogenic related growth factors (HGF, bFGF, G-CSF, VEGF-A, VEGF-D, PDGF-AB, PDGF-BB and GM-CSF), in tissue homogenates of HNSCC and statistically analysed interrelation of growth factor with patient outcome. They have demonstrated that elevated protein amounts of HGF ($p=0.05$), bFGF ($p=0.008$) and G-CSF ($p=0.034$) in HNSCC tumours result in poorer prognosis. Multivariate analysis showed bFGF amounts of more than 2000pg/mg total protein were significantly related to adverse patient outcome independent of HGF and G-CSF. In other study by Ninck et al (Ninck et al., 2003), who looked into expression of multiple angiogenic related growth factors in tumour tissue of HNSCC, observed simultaneous secretion of growth factors, as 80% of tumours produced more than 2 or more factors and 60% showed more than 3 or more factors and revealed that the survival time of patients bearing tumours, which produced three or four angiogenic growth factors (mainly VEGF, PDGF-AB, G-CSF, and GM-CSF) simultaneously, was significantly poorer than that of patients bearing tumours secreting 2 or less angiogenic growth factors $p=0.013$). Our study does not confirm previous reports, supporting significant role of the angiogenic related growth factors relation with prognosis of patients in laryngeal cancer. No statistically significant prognosis results were identified in relation with expression of six angiogenic related growth factors with either survival or recurrence. It could be because the other studies have included multiple sub sites in their study and have got longer follow up. Our study included only laryngeal cancer tissues homogenates and we have very shorter follow up period of 23 months.

Other studies (Druzgal et al., 2005; Hong et al., 2009; Meyer et al., 2010) have looked into prognostic significance of expression of multiple angiogenic growth factors in serum. Meyer et al (Meyer et al., 2010) in a randomized controlled trial compared the pretreatment and post treatment levels of nine prognostic markers and identified none of the nine markers was associated with the recurrence of initial cancer. This is in agreement with our study as none of six angiogenic factors were associated with recurrence. But Meyer et al identified that high levels of IL-6 were associated with a higher SPC incidence (hazard ratio 2.68, 95%CI, 1.49-4.08).

Tumour characteristics such as site of the primary tumour, T stage, N status and TNM stage have been established to have prognostic significance in a number of previous studies (Kowalski et al., 1991; Danic et al., 2000; Spector et al., 2001). Recently Markou et al (Markou et al., 2011) studied prognostic role of six variables (age, tumour size, T stage, N status, tumour site and histological grade) in 255 patients with laryngeal carcinoma and has identified advanced T stage and supra-glottic tumour as having a statistically significant impact on disease free survival.

Among the patient factors like age, gender, tobacco and alcohol use habits and performance status have already been reported, with contrasting results (Gavilan et al., 1987; Stell 1990). Results of the present study indicate that the disease free survival (DFS) or overall survival (OS) seems to be unrelated to age and gender, but we have not looked into the affect of tobacco and alcohol on prognosis of the patients.

Marioni et al in his study, included a cohort of 108 laryngeal malignancy samples, has identified carcinoma cells showing high angiogenin expression, has shorter disease free survival and high loco-regional recurrence (Marioni et al., 2010). The reasons why current study has not shown prognostic significance with increasing expression of angiogenin with tumour progression could be, the study had a small cohort of patients with follow up of 23 months, compared to 108 patients other study with mean follow up of 26.3 months. In an other study by Marioni et al, which included a small cohort of 12 baseloid type Head and neck SCC, compared with pair matched SCC and control group noted an inverse relation between pT stage and overall survival rate and no correlation found between stage and recurrence rate, DFS and OS in SCC patients (Marioni et al., 2011).

A significant inverse relationship identified in a study by Papadimitrakopoulou et al. (2006) between the loss of IGFBP3 expression and the overall disease specific, disease free and event free survival probability. Similar prognostic role were also noted in lung cancer (Chang et al., 2002). In contrast two case control studies which have looked into the prognostic role of IGF in breast cancer by Del Giudice et al (Del Giudice et al., 1998); Wolk et al in prostate cancer (Wolk et al., 1998) showed increased risk in association with higher levels of IGFBP3.

Recurrence rates after radiotherapy for advanced stage tumours range from 25% to 50%, (Terhaard et al., 1992; Parsons et al., 1998) and for T1 tumours of the larynx ranges from 5% to 13%, and from 25 to 30% for T2 tumours (Barthel and Esclamado 2001). Present study has showed radiotherapy as predictor of the recurrence of the laryngeal tumour, patients who had surgery with radiotherapy showed significant rate of

recurrence (p=0.04). Majority of the cases require total laryngectomies in cases of recurrence. In a recent review by Agra et al. (2012) concluded that in general conservative surgery is safe effective treatment for localized recurrences after radiotherapy for early stage glottis cancer and total laryngectomy for the advanced stage tumours.

In agreement with present study results, Deganello et al (2011) in his study not identified any significant difference in the rate of neck recurrence between node positive and node negative groups between surgery only and combined therapy groups (p=0.490), as well as between selective neck dissection (SND II-IV) and modified radical neck dissection (mRND) type III groups (p-0.425). The study has evaluated the efficacy and potential pitfalls of selective neck dissection in controlling occult neck disease in clinically negative neck of patients with laryngeal carcinoma. They have included 96 patients with laryngeal carcinoma; the five-year neck recurrence rate for all patients was 4.7%.

No significant survival benefit was identified in the present study with the patients treated either with surgery or combined therapy. The meta-analysis of radiotherapy in carcinoma of head and neck data showed that altered fractionation improves five-year loco regional control by 6.4% (from 46% to 53%, p <0/0001) and also overall survival by 3.4% (from 36% to 39%, p<0.03). The meta-analysis included patients mostly oropharynx and larynx primary site, 74% of patients being in III-IV stage. The benefit in overall survival was significantly higher with hyperfactioned regimen (8% at 5 years) than with accelerated radiotherapy (2% with AF without total dose reduction and 1.7% with total dose reduction at 5 years: p=0.02) (Bourhis et al., 2006).

Conclusion

Though review of the literature has showed variable influence of angiogenic related growth factors in predicting survival and recurrence in head and neck cancer, present study has not identified any significant role for the six angiogenic related growth factors in predicting prognosis in laryngeal cancer patients. Radiotherapy is recognized as prognostic factor in predicting the recurrence of the tumor.

Chapter 5:

Concluding Remarks

The current study has looked into the role of angiogenic-related growth factors in relation to tumour progression in laryngeal cancer patients, and has identified that there is increased expression of a number of angiogenesis-related proteins from early to late stage of the tumour both in tumour and corresponding nodal tissue, when compared to the control normal tissue. Further analysis has identified angiogenic factors Angiogenin and TIMP1, showed a consistent decrease in expression in tumour tissue from stage T1 to stage T4.

Specific analysis was performed by quantitatively measuring six angiogenic-related growth factors to identify their role in tumour progression in the cancer tissue in a bigger cohort group. We have shown that the factor Angiogenin expression was high in the early stages of the tumor compared to the later stage of tumour ($p<0.032$). The remaining five angiogenic related growth factors (VEGF, FGFb, TIMP-1, IL-8 and IGF-BP3) did not show any statistically significant changes between early and late stage of the tumour. Further analysis has identified that the factor Insulin growth factor BP3 (IGFBP3) has significant expression in tumor lysates compared to nodal lysates (p value<0.005). The remaining five (VEGF, FGF BP, TIMP-1, IL-8 and angiogenin) analyzed angiogenic factors did not show any statistically significant difference between tumour and nodal samples.

The survival analysis has identified that patients treated with combination of surgery and radiotherapy had a role in predicting recurrence of the tumours. Increased expression of angiogenic-related factors has not shown role in predicting the survival of laryngeal cancer patients. Similarly, patient's variables like age, gender and tumor

characteristics (T, N and M) and treatment (surgery and chemotherapy) were not associated with either survival or recurrence of the laryngeal tumour.

Marioni et al (2010) in his study, which looked into angiogenin expression in 108 patients with laryngeal carcinoma by immunohistochemistry, identified that patients with high angiogenin expression had high loco-regional recurrence and low disease free survival. Angiogenin expression has not shown any significant role in the survival of the laryngeal tumours in the current study.

Review of the published data confirms the role of angiogenesis in the growth and progression of head and neck tumour (Hasina et al., 2008). And to date there are limited studies that have investigated the simultaneous expression of multiple angiogenic factors in HNSCC (Ninck et al., 2003, Petruzzelli et al., 1997). One study in particular by Hasina et al (2008) demonstrated that there is a different molecular mechanism by which HNSCC tumour induces angiogenesis. The authors observed VEGF, cxcl-8, HGF and FGF-2 not expressed in normal mucosa, but the same factors are more frequently expressed and at a higher levels in dysplastic mucosa. The present study demonstrated an increased expression of number of angiogenic-related growth factors from early to late stage of the tumour and were expressed highly in tumour tissue compared to adjacent normal mucosa. Which suggest a direct correlation between tumour angiogenesis and tumour progression.

Prognosis of the HNSCC has not changed much over the past two decades, and despite recent improvements in diagnostic and therapeutic techniques there has been no improvement in the 5yr survival rates for laryngeal cancer patients in the last 30 years (Jemal et al., 2006). Therefore, additional treatment options that have potential to improve outome are needed to complement presently available treatment tools in HNSCC.

An association between a greater level of angiogenesis and a higher incidence of metastases, and consequently declining survival rates, has been observed in most solid tumours (Des Guetz et al., 2006). In head and neck cancers, angiogenesis has also been studied as a predictor of a poor response to platinum based chemotherapy (Gasparini et al., 1995) or radiotherapy (Haugen et al., 2004). One of the first available reports dealing with the role of angiogenesis in laryngeal carcinoma came from Beartice et al (Beatrice et al., 1996). Since then, several neo-angiogenic biological markers have come to be considered promising prognostic parameters in laryngeal squamous cell carcinoma. Studies in the literature identified that VEGF expression correlates significantly with local recurrence and/or metastases and a shorter disease free survival, especially in loco-regionally advanced disease (Vlachtsis et al., 2005; Shigyo et al., 2007), so it also seems to be a significant predictor of complete response to induction chemotherapy. On the other hand some studies found no significant correlation between VEGF expression and LSCC prognosis (Yaylaci et al., 2006) (Marioni et al., 2008). Our results showed no correlation of angiogenic expression and prognosis of laryngeal cancer patients. Also there was no significant correlation between tumour and patient characteristics with survival.

Understanding the molecular biology of cancer has fundamentally changed the search for new therapies. Recent research has focused on targeted molecular therapy, immunotherapy, and latent/residual tumour cell ablation. Molecular targeted therapy, with the potential for increased selectivity and fewer adverse effects, holds promise in the treatment of HNSCC. Agents that specifically target these cellular and molecular pathways associated with HNSCC are promising candidates as they are already successfully used in other neoplasia such as colorectal cancer, lung cancer, breast cancer and hematological malignancies (Segal and Saltz 2009). Targeted agents for HNSCC, expected to improve the effectiveness of current therapy, include HER family, Src-family kinase, cell cycle, COX inhibitors, anti-angiogenesis (Ferreira et al., 2012). The latest literature has shown great interest in vascular target molecules such as therapy based on neoplastic antigens**.** Despite many promising therapies being tested only EGFR therapy had been approved in recent years for SCCs of the head and neck by NICE in UK for locally advanced HNSCC and unresponsive tumours in combination with chemotherapy.

HNSCC lesions are generally very vascular, and have enhanced vasculature to facilitate drainage from this area (Beasley et al., 2002). Therefore, one of the effective pathways to target for HNSCC therapy is likely to be tumour angiogenesis**.**

The importance of blood perfusion for the tumour growth makes neoplastic vessels an obvious target of modern oncological therapy and every step in the angiogenic process could be potentially targeted. The feasibility of taking an anti-angiogenic approach to laryngeal cancer was first hypothesized by Olszewski in 1976 (Olszewski, 1976). There are at least two main ways to approach the vascular profile of solid malignancies, i.e.

we can inhibit new vessel formation (anti–angiogenic therapy) or we can selectively damage existing neoplastic vessel (vascular targeting therapy) (Carmeliet and Jain, 2000). Both kinds of therapy are characterized by a high specificity for the active endothelial cells, a low toxicity, efficacy at low doses, and a relative lack of resistance. The actions of the former agents include receptor antagonism, down regulation of the angiogenic signal, and up-regulation of the endogenous angiogenesis inhibitors (Carmeliet and Jain, 2000).

VEGF is the most widely studied angiogenic factor. It increases vascular permeability and is the most potent, direct acting, angiogenic protein known (Mosch et al., 2010). In 2007, Shiryo et al (Shigyo et al., 2007) studied VEGF expression in laryngeal lesions, finding that it increased with progression from mild to moderate, to severe dysplasia and to carcinoma.

Activation of EGFR stimulates various intracellular signaling pathways. An increased EGFR expression is associated with aggressive tumour behavior (Rafferty et al., 2008) and this receptor has been found to regulate angiogenesis, development and progression of HNSCC, including laryngeal cancers (Coupland et al., 2009) While some studies ruled out any direct link between EGFR and LSCC prognosis, most authors claim that EGFR correlated with tumour progression (Resnick et al., 1995; Jiang and Yang 2009; Marioni et al., 2010).

In head and neck carcinoma, recent clinical trials showed that a combination of vinorelbine (microtubule inhibitors) and cetuximab (monoclonal antibody inhibitor) was

feasible and effective for recurrent and/ or metastatic SCCs with a good safety profile. Combinations of cisplatin or 5FU with cetuximab have also been used to treat recurrent and/or metastatic head and neck SCC. The results are promise and no compound toxicity has come to light (Massa, Dessi et al., 2010). There are currently only a few reports on the selective efficacy of anti-angiogenic therapy for treating LSCC. Recent evidence indicated that primary treatment for advanced LSCC could consist of platinum–containing chemotherapy combined with radiotherapy, the benefits of which could be intensified by adding the EGFR inhibitor cetuxiamb with a view to improve clinical terms of local control/overall survival (Habl et al., 2010). The high frequency and stability of EGFR expression in primary carcinomas and corresponding metastases is encouraging efforts to use EGFR-targeting agents (e.g. Iressa, Tarceva, Erbitux, or radiolabeled antibodies) to treat LSCC (Wei et al., 2008).

The second main anti-angiogenic approach is based on evidence that targeting the tumour vasculature to the reduce oxygen and nutrient flow through the tumour mass and the consequent ischemic necrosis. Two main VTA families have been described, i.e. 1) small molecule VTAs and 2) ligand directed VTAs. The small molecule VTAs include microtubule destabilizing agents (such as conbrestatin A-4 disodium phosphate), while ligand directed VTAs bind agents compete in occluding vessels selectively to tumour blood vessel components. The ligand directed VTA family includes monoclonal antibodies conjugated with cytokines or toxins (e.g.Ricin A), and fusion proteins (e.g. VEGF conjugated with gelonin or diphtheria toxin) (Thorpe 2004). Several in vivo studies found anti-CD105 antibodies conjugated with toxins effective as antineoplastic agents, leading to destruction of the tumour vascular network (Fonsatti et al., 2001). Experimental findings suggest that anti-CD105 monoclonal antibodies effectively

inhibit tumour angiogenesis, growth, and metastasis. Inhibiting or suppressing angiogenin might not only reduce the malignancy's angiogenesis, but also directly inhibit the tumour's growth, in LSCC (Marioni et al., 2010).

One of the most recently considered angiogenic markers, angiogenin, is a member of the ribonuclease super family involved in regulating angiogenesis in both physiological and pathological conditions. In 2012 Marioni et al (Marioni et al., 2010) found that angiogenin expression correlated with recurrence and disease free survival rates in a consecutive series of LSCC. The present study has shown that angiogenin was significantly expressed in the early stages of the tumour progression.

Recent advancement has pointed out that rRNA transcription in endothelial cells upon stimulation of various angiogenic factors is mediated by angiogenin (ANG) (Kishimoto et al., 2005; Tsuji et al., 2005). ANG-mediated rRNA transcription has been shown to be a general requirement for angiogenesis, which is a crossroads in the process of angiogenesis for a variety of angiogenic factors (Kishimoto et al., 2005). ANG inhibitors have recently been in the spotlight for anti-angiogenesis research as they inhibit angiogenesis regardless of the nature of stimuli.

Several animal models have been established to examine the anti-angiogenesis and for subsequent anti-angiogenesis activity of ANG antagonists most of the previous efforts have been focused on prostate cancer, breast cancer, and colorectal cancer (Olson et al., 2001; Ibaragi et al., 2009). The role of ANG is a less explored area, however several reasons suggest that ANG inhibitors play an important role in HNSCC. First, ANG

expression is significantly elevated in HNSCC (Homer et al., 2002). Second, there is profuse tumour angiogenesis in HNSCC tissues (Beasley et al., 2002) and VEGF another prominent angiogenic factor, is also highly up regulated in HNSCC (Matsuura et al., 2009; Okada et al., 2010). Third, ANG is a permissive factor for other angiogenic factors to induce angiogenesis (Kishimoto et al., 2005). Thus, ANG inhibitors also inhibit VEGF induced angiogenesis.

The cell surface receptors of ANG have not yet been identified. However, blockage of nuclear receptor of ANG seems to be a promising approach to inhibit the function of ANG, as biological function of ANG is related to r RNA transcription (Xu et al., 2002). Targeting nuclear translocation avoids high plasma concentration, with less side effects (Yoshioka et al., 2006). Neomycin, an aminoglycoside antibiotic, was discovered to block nuclear translocation of ANG and to inhibit ANG–induced cell proliferation and angiogenesis (Xu et al., 2002). Neomycin has been shown to inhibit xenograft growth of human cancer cells in athymic mice (Yoshioka et al., 2006) as well as AKT-induced prostate intraepithelial neoplasia (PIN) in AKT transgenic mice (Ibaragi et al., 2009). Neamine is nontoxic derivative of neomycin equally effective in inhibiting angiogenesis and tumour growth induced by ANG as well as by other angiogenic factors (Ibaragi et al., 2009).

Perspective

Angiogenesis inhibitors were declared as the fourth modality for cancer treatment when FDA approved Bevacizumab, an anti-VEGF monoclonal antibody, in 2004 for the

treatment of advanced colorectal cancer. In 2006 two anti-angiogenesis drugs were approved by FDA, Sunitinib, a multi-tyrosinase kinase inhibitor was approved for the treatment of advanced renal cancer and gastrointestinal stromal cancer: and Ranibizumab, a fragment of the bevacizumab molecule was approved for the treatment age related macular degeneration. ANG inhibitors hold particular promise owing to the essential role of ANG mediated rRNA transcription in angiogenesis in general. ANG inhibitors will be effective in inhibiting angiogenesis induced not only by ANG but also by other angiogenic factors. Moreover, the unique property of HNSCC, such as propensity of multiple primary tumours, high vascular nature of the tumour, makes HNSCC an appropriate cancer type with which anti ANG agents can be tested and developed into clinical therapy.

Conclusion

Conservative approaches to laryngeal cancer need to be made more effective, and anti-angiogenic therapy could have a central role in this field. A conservative approach that targets antigens of active vascular endothelium could have an adjuvant role in carcinoma treatment, in combination with conventional chemotherapy. Research should investigate angiogenic markers with a view to finding other, more selective vascular targets for use in cancer therapy. Clinical trials involving anti-angiogenic or antivascular agents might be able to demonstrate effectiveness of associating this kind of targeted therapy with conventional cytotoxic agents in treatment of LSC

Appendix 1: Schematic Illustration of the Nodal Classification

Level I - all nodes above hyoid bone, below mylohyoid muscle, and anterior to posterior edge of submandibular gland

Level IA - all nodes between medial margins of anterior digastric muscles, above hyoid bone, below mylohyoid muscle

Level IB - all nodes below mylohyoid muscle, above hyoid bone, posterior and lateral to medial anterior digastric muscle and anterior to submandibular gland

Level II - all nodes below skull base at jugular fossa to hyoid bone, anterior to posterior edge of sternocleidomastoid muscle and posterior to submandibular gland

Level IIA - all nodes that lie posterior to internal jugular vein and are in separable from the vein or lie anterior, lateral or medial to the vein

Level IIB - all nodes that lie posterior to internal jugular vein and have a fat plane separating the nodes and the vein

Level III - all nodes between hyoid bone and cricoid cartilage arch and anterior to posterior sternoclediomastoid muscle, and lateral to the internal carotid artery

Level IV - all nodes between cricoid cartilage arch and clavicle, anterior to posterior sternocleidomastoid muscle and poster lateral to anterior scalene muscle and lateral to common carotid artery

Level V - all nodes from skull base posterior down to posterior border of sternocleidomastoid muscle to level of clavicle, anterior to trapezius muscle

Level VA - all nodes between skull base and cricoid cartilage arch, behind posterior edge of sternocleidomastoid muscle

Level VB - all nodes between cricoid cartilage arch and clavicle, behind sternoclediomastoid muscle

Level VI - all nodes inferior to hyoid bone and above top of manubrium, between medial margins of bilateral common carotid and internal carotid arteries

Level VII - all nodes behind the manubrium between medial margins of common carotid arteries bilaterally, extending inferiorly to level of innominate vein

Appendix 2: Classification of primary tumour (T) for carcinoma of the larynx according to UICC and AICC

	Primary Tumour (T)
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma insitu
Supra glottis	
T1	Tumour limited to one subsite of supraglottis with normal vocal cord mobility
T2	Tumour invades mucosa of more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis (eg.,mucosa of base of tongue, vallecula, medial wall of pyriform sinus) without fixation of the larynx
T3	Tumour limited to larynx with vocal cord fixation and/or invades any of the following: postcricoid area, pre-epiglottic space, para glottic space, and/or inner cortex of thyroid cartilage
T4a	Moderately advanced local disease Tumour invades through the thyroid cartilage and/or invades tissues beyond the larynx(e.g., trachea, soft tissue of neck including deep extrinsic muscle of the tongue, strap muscle, thyroid, or oesophagus
T4b	Very advanced local disease Tumour invades prevertebral space, encases carotid artery or invades mediastinal structures
Glottis	
T1	Tumour limited to the vocal cord(s) (may involve anterior or posterior commissure)with normal mobility
T1a	Tumour limited to one vocal cord
T1b	Tumour involves both vocal cords
T2	Tumour extends to supraglottis and /or subglottis and/or with impaired vocal cord mobility
T3	Tumour limited to the larynx with vocal cord fixation and/or invasion of paraglottic space and /or inner cortex of the thyroid cartilage
T4a	Moderately advanced local disease Tumour invades through the outer cortex of the thyroid cartilage and /or invades tissues beyond the larynx(e.g., trachea, soft tissues of neck including deep extrinsic muscle tongue, strap muscle, thyroid or oesophagus
T4b	Very advanced local disease Tumour invades pre-vertebral space, encases carotid artery or invades mediastianal structures
Sub glottis	
T1	Tumour limited to the sub glottis
T2	Tumour extends to vocal cord(s) with normal or impaired mobility
T3	Tumour limited to larynx with vocal cord fixation
T4a	Moderately advanced local disease Tumour invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of the neck including deep extrinsic muscles of the tongue, strap muscle, thyroid, or oesophagus)
T4b	Very advanced local disease Tumour invades pre-vertebral space, encases carotid artery, or invades mediastinal structures

REGIONAL LYMPH NODES – (N)	
NO	Regional lymph node cannot be assessed
Nx	No regional lymph node metastasis
N1	Metastasis in single ipsilateral lymph node <3cm in greatest diameter
N2	Metastasis in single ipsilateral lymph node >3cm but <6cm in greatest dimensions, or i multiple ipsilateral lymph nodes none >6cm or in bilateral or contra lateral lymph nodes none >6cm in greatest dimension
N2a	Metastasis in single ipsilateral lymph node >3cm but <6cm in greatest diameter
N2b	Metastasis in multiple ipsilateral nodes none >6cm in greatest diameter
N2c	Metastasis in bilateral or contra lateral lymph nodes, none >6cm in greatest diameter
N3	Metastasis in lymph node(s), >6cm in diameter

DISTANT METASTASIS (M)	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Source: Edge SB, Byrd DR, Compton CC, et al., eds. AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 57-67.

Stage Grouping			
0	Tis	NO	M0
I	T1	NO	M0
2	T2	N0	M0
111	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T4b	Any N	M0
	Any T	N3	M0
IVC	Any T	Any N	M1

Source: Edge SB, Byrd DR, Compton CC, et al., eds. AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 57-67.

Appendix 3: Showing angiogenesis related factors in the antibody array

Angiogenic related proteins				
Actin A	EGF *	HB-EGF	MMP-8	Serpin B5
ADAMTS *	EG-VEGF *	HGF	MMP-9	Serpin E1
Angiogenin	Endoglin	IGFBP-1	NRG1-B1 *	Serpin F1
Angiopoietin-1 *	Endostatin	IGFBP-2	Pentraxin3	TIMP-1
Angiopoietin-2	Endothelin-1	IGFBP-3	PD-ECGF	TIMP-4 *
Plasminogen	FGF-Acidic	IL-1b	PDGF-AA	Thrombospondin-1
Amphiregulin	FGF-Basic	IL-8	PDGF-BB *	Thrombospondin-2 *
Antemin	FGF-4 *	LAP	Persephin *	uPA
Coagulation factor 111	FGF-7	Leptin	Platelet factor-4	Vasohibin *
CXCL16	GDNF *	MCP-1	PiGF	VEGF
DPPIV	GM-CSF *	MIP-1Alfa *	Prolactin	VEGF-C *

* Factors excluded from the analysis as expression was within 20% of positive control of all the T stages.

References

(1986). "IARC Monographs programme on the evaluation of the carcinogenic risk of chemicals to humans. Preamble." IARC Monogr Eval Carcinog Risk Chem Hum **39**: 13-32.

(1991). "Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. The Department of Veterans Affairs Laryngeal Cancer Study Group." The New England journal of medicine **324**(24): 1685-1690.

Agra, I. M., A. Ferlito, et al. (2012). "Diagnosis and treatment of recurrent laryngeal cancer following initial nonsurgical therapy." Head & neck **34**(5): 727-735.

Ah-See, K. W., T. G. Cooke, et al. (1994). "An allelotype of squamous carcinoma of the head and neck using microsatellite markers." Cancer research **54**(7): 1617-1621.

Almadori, G., F. Bussu, et al. (2005). "Molecular markers in laryngeal squamous cell carcinoma: towards an integrated clinicobiological approach." European journal of cancer **41**(5): 683-693.

Almadori, G., F. Bussu, et al. (2004). "Multistep laryngeal carcinogenesis helps our understanding of the field cancerisation phenomenon: a review." Eur J Cancer **40**(16): 2383-2388.

Angeletti, C. A., M. Lucchi, et al. (1996). "Prognostic significance of tumoral angiogenesis in completely resected late stage lung carcinoma (stage IIIA-N2). Impact of adjuvant therapies in a subset of patients at high risk of recurrence." Cancer **78**(3): 409-415.

Anwar, K., K. Nakakuki, et al. (1993). "Over-expression of p53 protein in human laryngeal carcinoma." Int J Cancer **53**(6): 952-956.

Argiris, A., M. V. Karamouzis, et al. (2008). "Head and neck cancer." Lancet **371**(9625): 1695-1709.

Arnold, F. and D. C. West (1991). "Angiogenesis in wound healing." Pharmacol Ther **52**(3): 407-422.

Baba, Y., M. Fujii, et al. (2012). "Present and Future of EGFR Inhibitors for Head and Neck Squamous Cell Cancer." Journal of oncology **2012**: 986725.

Bandoh, N., T. Hayashi, et al. (2004). "VEGF and bFGF expression and microvessel density of maxillary sinus squamous cell carcinoma in relation to p53 status, spontaneous apoptosis and prognosis." Cancer letters **208**(2): 215-225.

Barthel, S. W. and R. M. Esclamado (2001). "Primary radiation therapy for early glottic cancer." Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery **124**(1): 35-39.

Baxter, R. C. (2001). "Signalling pathways involved in antiproliferative effects of IGFBP-3: a review." Molecular pathology : MP **54**(3): 145-148.

Beasley, N. J., R. Prevo, et al. (2002). "Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer." Cancer research **62**(5): 1315-1320.

Beasley, N. J., R. Prevo, et al. (2002). "Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer." Cancer Res **62**(5): 1315-1320.

Beatrice, F., G. Valente, et al. (1996). "[Laryngeal cancer and angiogenesis]." Acta otorhinolaryngologica Italica : organo ufficiale della Societa italiana di otorinolaringologia e chirurgia cervico-facciale **16**(4): 355-362.

Becker, M. T., C. G. Shores, et al. (2004). "Molecular assay to detect metastatic head and neck squamous cell carcinoma." Arch Otolaryngol Head Neck Surg **130**(1): 21-27.

Bellacosa, A., G. Almadori, et al. (1996). "Cyclin D1 gene amplification in human laryngeal squamous cell carcinomas: prognostic significance and clinical implications." Clin Cancer Res **2**(1): 175-180.

Benefield, J., G. J. Petruzzelli, et al. (1996). "Regulation of the steps of angiogenesis by human head and neck squamous cell carcinomas." Invasion Metastasis **16**(6): 291-301.

Bergers, G. and L. E. Benjamin (2003). "Tumorigenesis and the angiogenic switch." Nature reviews. Cancer **3**(6): 401-410.

Blanchard, P., B. Baujat, et al. (2011). "Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): a comprehensive analysis by tumour site." Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology **100**(1): 33-40.

Bonner, J. A., P. M. Harari, et al. (2006). "Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck." The New England journal of medicine **354**(6): 567-578.

Bonner, J. A., P. M. Harari, et al. (2010). "Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival." The lancet oncology **11**(1): 21-28.

Boonkitticharoen, V., B. Kulapaditharom, et al. (2008). "Vascular endothelial growth factor a and proliferation marker in prediction of lymph node metastasis in oral and pharyngeal squamous cell carcinoma." Archives of otolaryngology--head & neck surgery **134**(12): 1305-1311.

Bosatra, A., R. Bussani, et al. (1997). "From epithelial dysplasia to squamous carcinoma in the head and neck region: an epidemiological assessment." Acta oto-laryngologica. Supplementum **527**: 47-48.

Bourhis, J., J. Overgaard, et al. (2006). "Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis." Lancet **368**(9538): 843-854.

Bozec, A., P. Formento, et al. (2007). "Dual inhibition of EGFR and VEGFR pathways in combination with irradiation: antitumour supra-additive effects on human head and neck cancer xenografts." British journal of cancer **97**(1): 65-72.

Bozec, A., A. Sudaka, et al. (2009). "Combination of sunitinib, cetuximab and irradiation in an orthotopic head and neck cancer model." Annals of oncology : official journal of the European Society for Medical Oncology / ESMO **20**(10): 1703-1707.

Braakhuis, B. J., M. P. Tabor, et al. (2003). "A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications." Cancer Res **63**(8): 1727-1730.

Bran, B., G. Bran, et al. (2009). "The platelet-derived growth factor receptor as a target for vascular endothelial growth factor-mediated anti-angiogenetic therapy in head and neck cancer." International journal of oncology **34**(1): 255-261.

Bremer, G. L., A. T. Tiebosch, et al. (1996). "Tumor angiogenesis: an independent prognostic parameter in cervical cancer." American journal of obstetrics and gynecology **174**(1 Pt 1): 126-131.

Burtness, B. (2005). "The role of cetuximab in the treatment of squamous cell cancer of the head and neck." Expert opinion on biological therapy **5**(8): 1085-1093.

Califano, J., P. van der Riet, et al. (1996). "Genetic progression model for head and neck cancer: implications for field cancerization." Cancer research **56**(11): 2488-2492.

Califano, J., P. van der Riet, et al. (1996). "Genetic progression model for head and neck cancer: implications for field cancerization." Cancer Res **56**(11): 2488-2492.

Cappellari, J. O. (1997). "Histopathology and pathologic prognostic indicators." Otolaryngol Clin North Am **30**(2): 251-268.

Carmeliet, P. and R. K. Jain (2000). "Angiogenesis in cancer and other diseases." Nature **407**(6801): 249-257.

Chan, J. M., M. J. Stampfer, et al. (1998). "Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study." Science **279**(5350): 563-566.

Chang, Y. S., G. Kong, et al. (2002). "Clinical significance of insulin-like growth factor-binding protein-3 expression in stage I non-small cell lung cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **8**(12): 3796-3802.

Chen, Z., P. S. Malhotra, et al. (1999). "Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **5**(6): 1369-1379.

Cherukuri, D. P., A. C. Goulet, et al. (2005). "Selenomethionine regulates cyclooxygenase-2 (COX-2) expression through nuclear factor-kappa B (NF-kappaB) in colon cancer cells." Cancer biology & therapy **4**(2): 175-180.

Chomchai, J. S., W. Du, et al. (1999). "Prognostic significance of p53 gene mutations in laryngeal cancer." The Laryngoscope **109**(3): 455-459.

Chow, L. T., T. R. Broker, et al. (2010). "The natural history of human papillomavirus infections of the mucosal epithelia." APMIS **118**(6-7): 422-449.

Christopoulos, A., S. M. Ahn, et al. (2011). "Biology of vascular endothelial growth factor and its receptors in head and neck cancer: beyond angiogenesis." Head & neck **33**(8): 1220-1229.

Cianci, R., J. Galli, et al. (2003). "Gastric surgery as a long-term risk factor for malignant lesions of the larynx." Archives of surgery **138**(7): 751-754; discussion 755.

Cohen, E. E., D. W. Davis, et al. (2009). "Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study." The lancet oncology **10**(3): 247-257.

Cohen, R. F., J. Contrino, et al. (1995). "Interleukin-8 expression by head and neck squamous cell carcinoma." Arch Otolaryngol Head Neck Surg **121**(2): 202-209.

Coleman, M. P., B. Rachet, et al. (2004). "Trends and socioeconomic inequalities in cancer survival in England and Wales up to 2001." Br J Cancer **90**(7): 1367-1373.

Cooper, J. S., K. Porter, et al. (2009). "National Cancer Database report on cancer of the head and neck: 10-year update." Head & neck **31**(6): 748-758.

Coupland, V. H., P. Chapman, et al. (2009). "Trends in the epidemiology of larynx and lung cancer in south-east England, 1985-2004." British journal of cancer **100**(1): 167-169.

CRUK (2011).Cancer Incidence UK. Cancer Research UK

Czubayko, F., E. D. Liaudet-Coopman, et al. (1997). "A secreted FGF-binding protein can serve as the angiogenic switch in human cancer." Nature medicine **3**(10): 1137-1140.

Czubayko, F., R. V. Smith, et al. (1994). "Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors." The Journal of biological chemistry **269**(45): 28243-28248.

Danic, D., M. Maruic, et al. (2000). "Prognostic factors in squamous cell carcinoma of the larynx." ORL; journal for oto-rhino-laryngology and its related specialties **62**(3): 143-148.

Dayyani, F., C. J. Etzel, et al. (2010). "Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC)." Head & neck oncology **2**: 15.

de Campora, E., M. Radici, et al. (1994). "Clinical experiences with surgical therapy of cervical metastases from head and neck cancer." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies **251**(6): 335-341.

Deganello, A., G. Gitti, et al. (2011). "Effectiveness and pitfalls of elective neck dissection in N0 laryngeal cancer." Acta otorhinolaryngologica Italica : organo ufficiale della Societa italiana di otorinolaringologia e chirurgia cervico-facciale **31**(4): 216-221.

Del Giudice, M. E., I. G. Fantus, et al. (1998). "Insulin and related factors in premenopausal breast cancer risk." Breast cancer research and treatment **47**(2): 111-120.

Des Guetz, G., B. Uzzan, et al. (2006). "Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature." British journal of cancer **94**(12): 1823-1832.

Dey, P., D. Arnold, et al. (2002). "Radiotherapy versus open surgery versus endolaryngeal surgery (with or without laser) for early laryngeal squamous cell cancer." Cochrane database of systematic reviews(2): CD002027.

Dickson, K. A., D. K. Kang, et al. (2009). "Ribonuclease inhibitor regulates neovascularization by human angiogenin." Biochemistry **48**(18): 3804-3806.

Dische, S., M. Saunders, et al. (1997). "A randomised multicentre trial of CHART versus conventional radiotherapy in head and neck cancer." Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology **44**(2): 123-136.

Dong, G., Z. Chen, et al. (2001). "Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma." Cancer research **61**(15): 5911-5918.

Dong, G., Z. Chen, et al. (2001). "Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma." Cancer Res **61**(15): 5911-5918.

Dray, T. G., N. J. Hardin, et al. (1995). "Angiogenesis as a prognostic marker in early head and neck cancer." The Annals of otology, rhinology, and laryngology **104**(9 Pt 1): 724-729.

Druzgal, C. H., Z. Chen, et al. (2005). "A pilot study of longitudinal serum cytokine and angiogenesis factor levels as markers of therapeutic response and survival in patients with head and neck squamous cell carcinoma." Head & neck **27**(9): 771-784.

Duffy, S. A., J. M. Taylor, et al. (2008). "Interleukin-6 predicts recurrence and survival among head and neck cancer patients." Cancer **113**(4): 750-757.

Dunn, S. E., R. A. Hardman, et al. (1997). "Insulin-like growth factor 1 (IGF-1) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs." Cancer research **57**(13): 2687-2693.

Dunne, A. A., H. H. Muller, et al. (2006). "Meta-analysis of the prognostic significance of perinodal spread in head and neck squamous cell carcinomas (HNSCC) patients." Eur J Cancer **42**(12): 1863-1868.

Eiband, J. D., E. G. Elias, et al. (1989). "Prognostic factors in squamous cell carcinoma of the larynx." American journal of surgery **158**(4): 314-317.

Eisma, R. J., J. D. Spiro, et al. (1997). "Vascular endothelial growth factor expression in head and neck squamous cell carcinoma." Am J Surg **174**(5): 513-517.

Eisma, R. J., J. D. Spiro, et al. (1999). "Role of angiogenic factors: coexpression of interleukin-8 and vascular endothelial growth factor in patients with head and neck squamous carcinoma." Laryngoscope **109**(5): 687-693.

Ellis, L., B. Rachet, et al. (2012). "Trends and inequalities in laryngeal cancer survival in men and women: England and Wales 1991-2006." Oral oncology **48**(3): 284-289.

Ellis, L. M. and D. J. Hicklin (2008). "VEGF-targeted therapy: mechanisms of anti-tumour activity." Nat Rev Cancer **8**(8): 579-591.

Epstein, J. B., L. Zhang, et al. (2002). "Advances in the diagnosis of oral premalignant and malignant lesions." J Can Dent Assoc **68**(10): 617-621.

Escudier, B., T. Eisen, et al. (2007). "Sorafenib in advanced clear-cell renal-cell carcinoma." N Engl J Med **356**(2): 125-134.

Fakhry, C., W. H. Westra, et al. (2008). "Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a

prospective clinical trial." Journal of the National Cancer Institute **100**(4): 261-269.

Fasciani, A., G. D'Ambrogio, et al. (2000). "High concentrations of the vascular endothelial growth factor and interleukin-8 in ovarian endometriomata." Mol Hum Reprod **6**(1): 50-54.

Ferlay, J., D. M. Parkin, et al. (2010). "Estimates of cancer incidence and mortality in Europe in 2008." European journal of cancer **46**(4): 765-781.

Ferrara, N. (1996). "Vascular endothelial growth factor." European journal of cancer **32A**(14): 2413-2422.

Ferrara, N. and T. Davis-Smyth (1997). "The biology of vascular endothelial growth factor." Endocr Rev **18**(1): 4-25.

Ferrara, N., K. J. Hillan, et al. (2004). "Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer." Nat Rev Drug Discov **3**(5): 391-400.

Ferreira, M. B., J. P. Lima, et al. (2012). "Novel targeted therapies in head and neck cancer." Expert opinion on investigational drugs **21**(3): 281-295.

Fett, J. W., D. J. Strydom, et al. (1985). "Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells." Biochemistry **24**(20): 5480-5486.

Firth, S. M. and R. C. Baxter (2002). "Cellular actions of the insulin-like growth factor binding proteins." Endocrine reviews **23**(6): 824-854.

Folkman, J. (1971). "Tumor angiogenesis: therapeutic implications." N Engl J Med **285**(21): 1182-1186.

Folkman, J. (1990). "Endothelial cells and angiogenic growth factors in cancer growth and metastasis. Introduction." Cancer Metastasis Rev **9**(3): 171-174.

Folkman, J. (1990). "What is the evidence that tumors are angiogenesis dependent?" Journal of the National Cancer Institute **82**(1): 4-6.

Folkman, J. (1990). "What is the evidence that tumors are angiogenesis dependent?" J Natl Cancer Inst **82**(1): 4-6.

Folkman, J. (1995). "Angiogenesis in cancer, vascular, rheumatoid and other disease." Nature medicine **1**(1): 27-31.

Folkman, J. (1995). "Angiogenesis in cancer, vascular, rheumatoid and other disease." Nat Med **1**(1): 27-31.

Folkman, J. and D. Hanahan (1991). "Switch to the angiogenic phenotype during tumorigenesis." Princess Takamatsu Symp **22**: 339-347.

Folkman, J. and M. Klagsbrun (1987). "Angiogenic factors." Science **235**(4787): 442-447.

Folkman, J., E. Merler, et al. (1971). "Isolation of a tumor factor responsible for angiogenesis." J Exp Med **133**(2): 275-288.

Fong, K. M., Y. Kida, et al. (1996). "TIMP1 and adverse prognosis in non-small cell lung cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **2**(8): 1369-1372.

Fonsatti, E., L. Del Vecchio, et al. (2001). "Endoglin: An accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies." Journal of cellular physiology **188**(1): 1-7.

Forastiere, A., W. Koch, et al. (2001). "Head and neck cancer." N Engl J Med **345**(26): 1890-1900.

Forastiere, A. A., H. Goepfert, et al. (2003). "Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer." The New England journal of medicine **349**(22): 2091-2098.

Freije, J. E., T. W. Beatty, et al. (1996). "Carcinoma of the larynx in patients with gastroesophageal reflux." Am J Otolaryngol **17**(6): 386-390.

Fujimoto, J., H. Sakaguchi, et al. (2000). "Clinical implications of expression of interleukin 8 related to angiogenesis in uterine cervical cancers." Cancer Res **60**(10): 2632-2635.

Furuhashi, M., T. Sjoblom, et al. (2004). "Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate." Cancer Res **64**(8): 2725-2733.

Gallo, O., A. Sarno, et al. (2003). "Multivariate analysis of prognostic factors in T3 N0 laryngeal carcinoma treated with total laryngectomy." Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery **128**(5): 654-662.

Gallus, S., C. Bosetti, et al. (2003). "Laryngeal cancer in women: tobacco, alcohol, nutritional, and hormonal factors." Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research,

cosponsored by the American Society of Preventive Oncology **12**(6): 514-517.

Gao, X. and Z. Xu (2008). "Mechanisms of action of angiogenin." Acta biochimica et biophysica Sinica **40**(7): 619-624.

Gasparini, G., P. Bevilacqua, et al. (1995). "Predictive and prognostic markers in a series of patients with head and neck squamous cell invasive carcinoma treated with concurrent chemoradiation therapy." Clinical cancer research : an official journal of the American Association for Cancer Research **1**(11): 1375-1383.

Gasparini, G., N. Weidner, et al. (1994). "Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **12**(3): 454-466.

Gavilan, J., C. Gavilan, et al. (1987). "Discriminant analysis in predicting survival of patients with cancer of the larynx or hypopharynx." Clinical otolaryngology and allied sciences **12**(5): 331-335.

Gerhardt, H., M. Golding, et al. (2003). "VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia." J Cell Biol **161**(6): 1163-1177.

Gillison, M. L., W. M. Koch, et al. (2000). "Evidence for a causal association between human papillomavirus and a subset of head and neck cancers." Journal of the National Cancer Institute **92**(9): 709-720.

Glazer, C. A., S. S. Chang, et al. (2009). "Applying the molecular biology and epigenetics of head and neck cancer in everyday clinical practice." Oral Oncol **45**(4-5): 440-446.

Gokhale, A. S., R. I. Haddad, et al. (2005). "Serum concentrations of interleukin-8, vascular endothelial growth factor, and epidermal growth factor receptor in patients with squamous cell cancer of the head and neck." Oral oncology **41**(1): 70-76.

Goon, P. K., M. A. Stanley, et al. (2009). "HPV & head and neck cancer: a descriptive update." Head & neck oncology **1**: 36.

Gospodarowicz, D., G. Neufeld, et al. (1986). "Fibroblast growth factor." Molecular and cellular endocrinology **46**(3): 187-204.

Gronbaek, M., U. Becker, et al. (1998). "Population based cohort study of the association between alcohol intake and cancer of the upper digestive tract." BMJ **317**(7162): 844-847.

Guenel, P., J. F. Chastang, et al. (1988). "A study of the interaction of alcohol drinking and tobacco smoking among French cases of laryngeal cancer." J Epidemiol Community Health **42**(4): 350-354.

Guo, P., B. Hu, et al. (2003). "Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment." Am J Pathol **162**(4): 1083-1093.

Gustavsson, P., R. Jakobsson, et al. (1998). "Occupational exposures and squamous cell carcinoma of the oral cavity, pharynx, larynx, and oesophagus: a case-control study in Sweden." Occup Environ Med **55**(6): 393-400.

Gutwein, P., A. Schramme, et al. (2009). "Tumoural CXCL16 expression is a novel prognostic marker of longer survival times in renal cell cancer patients." European journal of cancer **45**(3): 478-489.

Ha, P. K., S. S. Chang, et al. (2009). "Molecular techniques and genetic alterations in head and neck cancer." Oral Oncol **45**(4-5): 335-339.

Haab, B. B. (2005). "Antibody arrays in cancer research." Molecular & cellular proteomics : MCP **4**(4): 377-383.

Haab, B. B., M. J. Dunham, et al. (2001). "Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions." Genome biology **2**(2): RESEARCH0004.

Habl, G., A. D. Jensen, et al. (2010). "Treatment of locally advanced carcinomas of head and neck with intensity-modulated radiation therapy (IMRT) in combination with cetuximab and chemotherapy: the REACH protocol." BMC cancer **10**: 651.

Han, J. Y., B. G. Choi, et al. (2006). "The prognostic significance of pretreatment plasma levels of insulin-like growth factor (IGF)-1, IGF-2, and IGF binding protein-3 in patients with advanced non-small cell lung cancer." Lung cancer **54**(2): 227-234.

Hardisson, D. (2003). "Molecular pathogenesis of head and neck squamous cell carcinoma." Eur Arch Otorhinolaryngol **260**(9): 502-508.

Harrison-Lavoie, K. J., G. Michaux, et al. (2006). "P-selectin and CD63 use different mechanisms for delivery to Weibel-Palade bodies." Traffic **7**(6): 647-662.

Hashibe, M., P. Brennan, et al. (2009). "Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium." Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology **18**(2): 541-550.

Hasina, R., M. E. Whipple, et al. (2008). "Angiogenic heterogeneity in head and neck squamous cell carcinoma: biological and therapeutic implications." Laboratory investigation; a journal of technical methods and pathology **88**(4): 342-353.

Hathaway, B., D. P. Landsittel, et al. (2005). "Multiplexed analysis of serum cytokines as biomarkers in squamous cell carcinoma of the head and neck patients." The Laryngoscope **115**(3): 522-527.

Haugen, H., B. Magnusson, et al. (2004). "Preradiotherapy hemoglobin level but not microvessel density predicts locoregional control and survival in laryngeal cancer treated with primary radical radiotherapy." Clinical cancer research : an official journal of the American Association for Cancer Research **10**(23): 7941-7949.

Hayakawa, T., K. Yamashita, et al. (1992). "Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum." FEBS letters **298**(1): 29-32.

Hedstrom, J., R. Grenman, et al. (1999). "Concentration of free hCGbeta subunit in serum as a prognostic marker for squamous-cell carcinoma of the oral cavity and oropharynx." International journal of cancer. Journal international du cancer **84**(5): 525-528.

Heidemann, J., H. Ogawa, et al. (2003). "Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2." J Biol Chem **278**(10): 8508-8515.

Hermanek, P., L. H. Sobin, et al. (1987). TNM classification of malignant tumours. Berlin ; New York, Springer-Verlag.

Hicklin, D. J. and L. M. Ellis (2005). "Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis." Journal of clinical oncology :

official journal of the American Society of Clinical Oncology **23**(5): 1011-1027.

Hirabayashi, H., K. Koshii, et al. (1991). "Extracapsular spread of squamous cell carcinoma in neck lymph nodes: prognostic factor of laryngeal cancer." Laryngoscope **101**(5): 502-506.

Hobbs, C. G., J. A. Sterne, et al. (2006). "Human papillomavirus and head and neck cancer: a systematic review and meta-analysis." Clin Otolaryngol **31**(4): 259-266.

Hohaus, S., S. Cavallo, et al. (1996). "Telomerase activity in human laryngeal squamous cell carcinomas." Clin Cancer Res **2**(11): 1895-1900.

Homer, J. J., J. Greenman, et al. (2000). "Angiogenesis in head and neck squamous cell carcinoma." Clinical otolaryngology and allied sciences **25**(3): 169-180.

Homer, J. J., J. Greenman, et al. (2000). "Angiogenic cytokines in serum and plasma of patients with head and neck squamous cell carcimona." Clinical otolaryngology and allied sciences **25**(6): 570-576.

Homer, J. J., J. Greenman, et al. (2002). "Circulating angiogenic cytokines as tumour markers and prognostic factors in head and neck squamous cell carcinoma." Clinical otolaryngology and allied sciences **27**(1): 32-37.

Hong, D. Y., B. J. Lee, et al. (2009). "Expression of VEGF, HGF, IL-6, IL-8, MMP-9, Telomerase in Peripheral Blood of Patients with Head and Neck Squamous Cell Carcinoma." Clinical and experimental otorhinolaryngology **2**(4): 186-192.

Huang, S., L. Mills, et al. (2002). "Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma." Am J Pathol **161**(1): 125-134.

Hynes, N. E. and H. A. Lane (2005). "ERBB receptors and cancer: the complexity of targeted inhibitors." Nature reviews. Cancer **5**(5): 341-354.

Ibaragi, S., N. Yoshioka, et al. (2009). "Angiogenin-stimulated rRNA transcription is essential for initiation and survival of AKT-induced prostate intraepithelial neoplasia." Molecular cancer research : MCR **7**(3): 415-424.

Ibaragi, S., N. Yoshioka, et al. (2009). "Neamine inhibits prostate cancer growth by suppressing angiogenin-mediated rRNA transcription." Clinical cancer research : an official journal of the American Association for Cancer Research **15**(6): 1981-1988.

Islami, F., I Tramacere, et. al. (2010). "Alocohol drinking and laryngeal cancer:overall and dose-risk relation- a systemic review and meta-analysis." Oral oncology **46**(11): 802-810.

Ito, Y., T. Kamijo, et al. (2001). "Microvessel density predicts the radiosensitivity of metastatic head and neck squamous cell carcinoma in cervical lymph nodes." International journal of oncology **19**(6): 1127-1132.

Izzo, J. G., V. A. Papadimitrakopoulou, et al. (1998). "Dysregulated cyclin D1 expression early in head and neck tumorigenesis: in vivo evidence for an association with subsequent gene amplification." Oncogene **17**(18): 2313-2322.

Jemal, A., R. Siegel, et al. (2006). "Cancer statistics, 2006." CA: a cancer journal for clinicians **56**(2): 106-130.

Jiang, H. and B. B. Yang (2009). "p53, epidermal growth factor receptor and proliferating cell nuclear antigen in laryngeal squamous cell carcinoma are not predictive markers for the effect of adjuvant radiotherapy." Acta oto-laryngologica **129**(1): 101-107.

Johansson, N. and V. M. Kahari (2000). "Matrix metalloproteinases in squamous cell carcinoma." Histol Histopathol **15**(1): 225-237.

Jones, J. I. and D. R. Clemmons (1995). "Insulin-like growth factors and their binding proteins: biological actions." Endocrine reviews **16**(1): 3-34.

Katona, T. M., B. L. Neubauer, et al. (2005). "Elevated expression of angiogenin in prostate cancer and its precursors." Clinical cancer research : an official journal of the American Association for Cancer Research **11**(23): 8358-8363.

Kishimoto, K., S. Liu, et al. (2005). "Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis." Oncogene **24**(3): 445-456.

Klagsbrun, M. and M. A. Moses (1999). "Molecular angiogenesis." Chemistry & biology **6**(8): R217-224.

Klem, M. L., J. G. Mechalakos, et al. (2008). "Intensity-modulated radiotherapy for head and neck cancer of unknown primary: toxicity and preliminary efficacy." International journal of radiation oncology, biology, physics **70**(4): 1100-1107.

Koch, A. E., P. J. Polverini, et al. (1992). "Interleukin-8 as a macrophage-derived mediator of angiogenesis." Science **258**(5089): 1798-1801.

Koufman, J. A. and A. J. Burke (1997). "The etiology and pathogenesis of laryngeal carcinoma." Otolaryngologic clinics of North America **30**(1): 1-19.

Kowalski, L. P., E. L. Franco, et al. (1991). "Prognostic factors in laryngeal cancer patients submitted to surgical treatment." Journal of surgical oncology **48**(2): 87-95.

Krecicki, T., M. Jelen, et al. (1999). "C-erbB-2 immunostaining in laryngeal cancer." Acta Otolaryngol **119**(3): 392-395.

Krecicki, T., M. Zalesska-Krecicka, et al. (1998). "Laryngeal cancer in Lower Silesia: descriptive analysis of 501 cases." Oral oncology **34**(5): 377-380.

Krecicki, T., M. Zalesska-Krecicka, et al. (1998). "Laryngeal cancer in Lower Silesia: descriptive analysis of 501 cases." Oral Oncol **34**(5): 377-380.

Kupisz, K., D. Chibowski, et al. (1999). "Tumor angiogenesis in patients with laryngeal cancer." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies **256**(6): 303-305.

Kyzas, P. A., I. W. Cunha, et al. (2005). "Prognostic significance of vascular endothelial growth factor immunohistochemical expression in head and neck squamous cell carcinoma: a meta-analysis." Clinical cancer research : an official journal of the American Association for Cancer Research **11**(4): 1434-1440.

Kyzas, P. A., D. Stefanou, et al. (2005). "Prognostic significance of VEGF immunohistochemical expression and tumor angiogenesis in head and neck squamous cell carcinoma." J Cancer Res Clin Oncol **131**(9): 624-630.

Kyzas, P. A., D. Stefanou, et al. (2005). "Prognostic significance of VEGF immunohistochemical expression and tumor angiogenesis in head and neck squamous cell carcinoma." Journal of cancer research and clinical oncology **131**(9): 624-630.

Lai, S. Y. and F. M. Johnson (2010). "Defining the role of the JAK-STAT pathway in head and neck and thoracic malignancies: implications for future therapeutic approaches." Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy **13**(3): 67-78.

Lalla, R. V., D. S. Boisoneau, et al. (2003). "Expression of vascular endothelial growth factor receptors on tumor cells in head and neck squamous cell carcinoma." Arch Otolaryngol Head Neck Surg **129**(8): 882-888.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-16.

Lassen, P., J. G. Eriksen, et al. (2009). "Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck." J Clin Oncol **27**(12): 1992-1998.

Le, Q. T., R. Fisher, et al. (2012). "Prognostic and predictive significance of plasma HGF and IL-8 in a phase III trial of chemoradiation with or without tirapazamine in locoregionally advanced head and neck cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **18**(6): 1798-1807.

Leemans, C R., B. J. braakhuis, et. al. (2011). "The molecular biology of head and neck cancer." Nature reviews. Cancer **11**(1): 9-22

Lefebvre, J. L., F. Rolland, et al. (2009). "Phase 3 randomized trial on larynx preservation comparing sequential vs alternating chemotherapy and radiotherapy." Journal of the National Cancer Institute **101**(3): 142-152.

Lewin, J. S., A. M. Gillenwater, et al. (2003). "Characterization of laryngopharyngeal reflux in patients with premalignant or early carcinomas of the larynx." Cancer **97**(4): 1010-1014.

Li, A., M. L. Varney, et al. (2001). "Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials." Clin Cancer Res **7**(10): 3298-3304.

Li, C., S. Shintani, et al. (2005). "Microvessel density and expression of vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived endothelial growth factor in oral squamous cell carcinomas." International journal of oral and maxillofacial surgery **34**(5): 559-565.

Li, H., Y. Adachi, et al. (2011). "Insulin-like growth factor-I receptor blockade reduces tumor angiogenesis and enhances the effects of bevacizumab for a human gastric cancer cell line, MKN45." Cancer **117**(14): 3135-3147.

Li, W., C. Wang, et al. (2009). "Expression of fibroblast growth factor binding protein in head and neck cancer." Archives of otolaryngology--head & neck surgery **135**(9): 896-901.

Lingen, M. W. (1999). "Angiogenesis in the development of head and neck cancer and its inhibition by chemopreventive agents." Crit Rev Oral Biol Med **10**(2): 153-164.

Liss, C., M. J. Fekete, et al. (2001). "Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages." Int J Cancer **93**(6): 781-785.

Lowy, D. R. and K. Munger (2010). "Prognostic implications of HPV in oropharyngeal cancer." The New England journal of medicine **363**(1): 82-84.

Lucchi, M., G. Fontanini, et al. (1997). "Tumor angiogenesis and biologic markers in resected stage I NSCLC." European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery **12**(4): 535-541.

Luo, J. C. and M. Shibuya (2001). "A variant of nuclear localization signal of bipartite-type is required for the nuclear translocation of hypoxia inducible factors (1alpha, 2alpha and 3alpha)." Oncogene **20**(12): 1435-1444.

Maestro, R., R. Dolcetti, et al. (1992). "High frequency of p53 gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx." Oncogene **7**(6): 1159-1166.

Maier, H., A. Dietz, et al. (1992). "Tobacco and alcohol and the risk of head and neck cancer." Clin Investig **70**(3-4): 320-327.

Mao, L., W. K. Hong, et al. (2004). "Focus on head and neck cancer." Cancer Cell **5**(4): 311-316.

Marasa, L. and R. M. Tomasino (1983). "[Cutaneous melanoma. II. Proposal of a new prognostic parameter: evaluation of neoplastic angiogenesis]." Pathologica **75**(1037): 353-365.

Marck, P. A. and A. J. Lupin (1989). "Cancer of the larynx: the northern Alberta experience." J Otolaryngol **18**(7): 344-349.

Marioni, G., L. Giacomelli, et al. (2008). "Laryngeal carcinoma recurrence rate and disease-free interval are related to CD105 expression but not to vascular endothelial growth factor 2 (Flk-1/Kdr) expression." Anticancer research **28**(1B): 551-557.

Marioni, G., F. Marino, et al. (2010). "Neoangiogenesis in laryngeal carcinoma: angiogenin and CD105 expression is related to carcinoma recurrence rate and disease-free survival." Histopathology **57**(4): 535-543.

Marioni, G., G. Ottaviano, et al. (2006). "CD105-assessed micro-vessel density is associated with malignancy recurrence in laryngeal squamous cell carcinoma." European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology **32**(10): 1149-1153.

Marioni, G., A. Staffieri, et al. (2010). "Laryngeal carcinoma lymph node metastasis and disease-free survival correlate with MASPIN nuclear expression but not with EGFR expression: a series of 108 cases." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies **267**(7): 1103-1110.

Marioni, G., A. Staffieri, et al. (2011). "Angiogenin expression in head and neck basaloid and conventional squamous cell carcinoma: a site- and stage-matched comparison." Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology **40**(1): 55-60.

Markl, B., I. Renk, et al. (2010). "Tumour budding, uPA and PAI-1 are associated with aggressive behaviour in colon cancer." Journal of surgical oncology **102**(3): 235-241.

Markou, K., J. Goudakos, et al. (2011). "The role of tumor size and patient's age as prognostic factors in laryngeal cancer." Hippokratia **15**(1): 75-80.

Massa, E., M. Dessi, et al. (2010). "Phase II study of vinorelbine/cetuximab in patients with recurrent/metastatic squamous cell carcinoma of the head and neck progressing after at least two chemotherapy regimens." Oral oncology **46**(11): 818-821.

Mathur, P., S. Kaga, et al. (2005). "Antibody-array technique reveals overexpression of important DNA-repair proteins during cardiac ischemic preconditioning." Journal of molecular and cellular cardiology **38**(1): 99-102.

Matsuura, M., M. Onimaru, et al. (2009). "Autocrine loop between vascular endothelial growth factor (VEGF)-C and VEGF receptor-3 positively regulates tumor-associated lymphangiogenesis in oral squamoid cancer cells." The American journal of pathology **175**(4): 1709-1721.

Meyer, F., E. Samson, et al. (2010). "Serum prognostic markers in head and neck cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **16**(3): 1008-1015.

Mimori, K., M. Mori, et al. (1997). "Clinical significance of tissue inhibitor of metalloproteinase expression in gastric carcinoma." British journal of cancer **76**(4): 531-536.

Molina, R., M. D. Torres, et al. (1996). "Prognostic significance of SCC antigen in the serum of patients with head and neck cancer." Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine **17**(2): 81-89.

Montag, M., G. Dyckhoff, et al. (2009). "Angiogenic growth factors in tissue homogenates of HNSCC: expression pattern, prognostic relevance, and interrelationships." Cancer science **100**(7): 1210-1218.

Moon, C., Y. K. Chae, et al. (2010). "Targeting epidermal growth factor receptor in head and neck cancer: lessons learned from cetuximab." Experimental biology and medicine **235**(8): 907-920.

Morales-Angulo, C., F. Val-Bernal, et al. (1998). "Prognostic factors in supraglottic laryngeal carcinoma." Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery **119**(5): 548-553.

Mosch, B., B. Reissenweber, et al. (2010). "Eph receptors and ephrin ligands: important players in angiogenesis and tumor angiogenesis." Journal of oncology **2010**: 135285.

Mukherji, S. K., D. Armao, et al. (2001). "Cervical nodal metastases in squamous cell carcinoma of the head and neck: what to expect." Head & neck **23**(11): 995-1005.

Murray, J. D., G. W. Carlson, et al. (1997). "Tumor angiogenesis as a prognostic factor in laryngeal cancer." American journal of surgery **174**(5): 523-526.

Nakopoulou, L., I. Giannopoulou, et al. (2002). "Enhanced mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in breast carcinomas is correlated with adverse prognosis." The Journal of pathology **197**(3): 307-313.

Nankivell, P., M. Weller, et al. (2011). "Biomarkers in laryngeal dysplasia: a systematic review." Head & neck **33**(8): 1170-1176.

Narayana, A., A. T. Vaughan, et al. (1998). "Second primary tumors in laryngeal cancer: results of long-term follow-up." Int J Radiat Oncol Biol Phys **42**(3): 557-562.

Narayana, A., A. T. Vaughan, et al. (1998). "Is p53 an independent prognostic factor in patients with laryngeal carcinoma?" Cancer **82**(2): 286-291.

Nasman, A., P. Attner, et al. (2009). "Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma?" Int J Cancer **125**(2): 362-366.

Neville, B. W. and T. A. Day (2002). "Oral cancer and precancerous lesions." CA Cancer J Clin **52**(4): 195-215.

Nicolai, P., A. Ferlito, et al. (1990). "Laryngeal chondrosarcoma: incidence, pathology, biological behavior, and treatment." Ann Otol Rhinol Laryngol **99**(7 Pt 1): 515-523.

Ninck, S., C. Reisser, et al. (2003). "Expression profiles of angiogenic growth factors in squamous cell carcinomas of the head and neck." International journal of cancer. Journal international du cancer **106**(1): 34-44.

O'Sullivan, C., C. E. Lewis, et al. (1993). "Secretion of epidermal growth factor by macrophages associated with breast carcinoma." Lancet **342**(8864): 148-149.

Oh, S. H., W. Y. Kim, et al. (2006). "Identification of insulin-like growth factor binding protein-3 as a farnesyl transferase inhibitor SCH66336-induced negative regulator of angiogenesis in head and neck squamous cell carcinoma." Clinical cancer research : an official journal of the American Association for Cancer Research **12**(2): 653-661.

Okada, Y., H. Ueno, et al. (2010). "Experimental study of antiangiogenic gene therapy targeting VEGF in oral cancer." Odontology / the Society of the Nippon Dental University **98**(1): 52-59.

Olson, K. A., H. R. Byers, et al. (2001). "Prevention of human prostate tumor metastasis in athymic mice by antisense targeting of human angiogenin." Clinical cancer research : an official journal of the American Association for Cancer Research **7**(11): 3598-3605.

Olszewski, E. (1976). "Blood vascular system in cancer of the larynx." Archives of otolaryngology **102**(2): 65-70.

Olson NR. Effects of stomach acid on the larynx. Proc Am Laryngol Assoc. 1983; 104:108–112)

P, O. c., P. Rhys-Evans, et al. (2001). "Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis." Cancer **92**(3): 556-568.

P, O. C., P. H. Rhys-Evans, et al. (2001). "Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck." Archives of otolaryngology--head & neck surgery **127**(7): 813-820.

Paget-Bailly, S., D. Cyr, et al. (2012). "Occupational exposures and cancer of the larynx-systematic review and meta-analysis." Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine **54**(1): 71-84.

Pai, H. H., L. Rochon, et al. (1998). "Overexpression of p53 protein does not predict local-regional control or survival in patients with early-stage squamous cell carcinoma of the glottic larynx treated with radiotherapy." Int J Radiat Oncol Biol Phys **41**(1): 37-42.

Palka, K. T., R. J. Slebos, et al. (2008). "Update on molecular diagnostic tests in head and neck cancer." Seminars in oncology **35**(3): 198-210.

Papadimitrakopoulou, V. A., E. N. Brown, et al. (2006). "The prognostic role of loss of insulin-like growth factor-binding protein-3 expression in head and neck carcinogenesis." Cancer letters **239**(1): 136-143.

Parangi, S., W. Dietrich, et al. (1995). "Tumor suppressor loci on mouse chromosomes 9 and 16 are lost at distinct stages of tumorigenesis in a transgenic model of islet cell carcinoma." Cancer Res **55**(24): 6071-6076.

Parkin, D. M., P. Pisani, et al. (1999). "Estimates of the worldwide incidence of 25 major cancers in 1990." Int J Cancer **80**(6): 827-841.

Parsons, J. T., W. M. Mendenhall, et al. (1998). "T4 laryngeal carcinoma: radiotherapy alone with surgery reserved for salvage." International journal of radiation oncology, biology, physics **40**(3): 549-552.

Patmore, H. S., J. N. Ashman, et. al. (2007). " Genetic analysis of head and neck squamous cell carcinoma using comparative genomic hybridisation identified specific aberrations associated with laryngeal origin." Cancer letters **258**(1): 55-62

Petruzzelli, G. J. (1996). "Tumor angiogenesis." Head Neck **18**(3): 283-291.

Petruzzelli, G. J., J. Benefield, et al. (1997). "Heparin-binding growth factor(s) derived from head and neck squamous cell carcinomas induce endothelial cell proliferations." Head & neck **19**(7): 576-582.

Pfister, D. G., S. A. Laurie, et al. (2006). "American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **24**(22): 3693-3704.

Pignataro, L., N. Carboni, et al. (2001). "Clinical relevance of microvessel density in laryngeal squamous cell carcinomas." International journal of cancer. Journal international du cancer **92**(5): 666-670.

Pignon, J. P., J. Bourhis, et al. (2000). "Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer." Lancet **355**(9208): 949-955.

Pignon, J. P., A. le Maitre, et al. (2009). "Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients." Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology **92**(1): 4-14.

Pruneri, G., L. Pignataro, et al. (1996). "p53 protein expression in laryngeal squamous cell carcinomas bearing wild-type and mutated p53 gene." Histopathology **28**(6): 513-519.

Pulkkinen, J. O., P. Klemi, et al. (1999). "Apoptosis in situ, p53, bcl-2 and AgNOR counts as prognostic factors in laryngeal carcinoma." Anticancer research **19**(1B): 703-707.

Puri, S. K., C. Y. Fan, et al. (2003). "Significance of extracapsular lymph node metastases in patients with head and neck squamous cell carcinoma." Curr Opin Otolaryngol Head Neck Surg **11**(2): 119-123.

Rafferty, M., T. R. Helliwell, et al. (2008). "Expression of cell cycle associated proteins influences radiocurability of T2N0 squamous cell carcinoma of the larynx." Oral oncology **44**(10): 975-981.

Ree, A. H., V. A. Florenes, et al. (1997). "High levels of messenger RNAs for tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in primary breast carcinomas are associated with development of distant metastases." Clinical

cancer research : an official journal of the American Association for Cancer Research **3**(9): 1623-1628.

Reinmuth, N., A. A. Parikh, et al. (2003). "Biology of angiogenesis in tumors of the gastrointestinal tract." Microsc Res Tech **60**(2): 199-207.

Resnick, J. M., D. Uhlman, et al. (1995). "Cervical lymph node status and survival in laryngeal carcinoma: prognostic factors." The Annals of otology, rhinology, and laryngology **104**(9 Pt 1): 685-694.

Richard, J. M., H. Sancho-Garnier, et al. (1998). "Randomized trial of induction chemotherapy in larynx carcinoma." Oral oncology **34**(3): 224-228.

Riedel, F., K. Gotte, et al. (2000). "Serum levels of vascular endothelial growth factor in patients with head and neck cancer." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies **257**(6): 332-336.

Rinaldi, S., R. Cleveland, et al. (2010). "Serum levels of IGF-I, IGFBP-3 and colorectal cancer risk: results from the EPIC cohort, plus a meta-analysis of prospective studies." International journal of cancer. Journal international du cancer **126**(7): 1702-1715.

Risau, W., H. Drexler, et al. (1992). "Platelet-derived growth factor is angiogenic in vivo." Growth Factors **7**(4): 261-266.

Robin, P. E., A. Reid, et al. (1991). "The incidence of cancer of the larynx." Clin Otolaryngol Allied Sci **16**(2): 198-201.

Roeckl, W., D. Hecht, et al. (1998). "Differential binding characteristics and cellular inhibition by soluble VEGF receptors 1 and 2." Exp Cell Res **241**(1): 161-170.

Roesch-Ely, M., M. Nees, et al. (2007). "Proteomic analysis reveals successive aberrations in protein expression from healthy mucosa to invasive head and neck cancer." Oncogene **26**(1): 54-64.

Roh, H. J., D. M. Shin, et al. (2000). "Visualization of the timing of gene amplification during multistep head and neck tumorigenesis." Cancer Res **60**(22): 6496-6502.

Rollins, B. J. (1997). "Chemokines." Blood **90**(3): 909-928.

Rosano, L., F. Spinella, et al. (2005). "Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer cells." Cancer research **65**(24): 11649-11657.

Rosin, M. P., X. Cheng, et al. (2000). "Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia." Clin Cancer Res **6**(2): 357-362.

Ruokolainen, H., P. Paakko, et al. (2005). "Serum matrix metalloproteinase-9 in head and neck squamous cell carcinoma is a prognostic marker." International journal of cancer. Journal international du cancer **116**(3): 422-427.

Ruokolainen, H., P. Paakko, et al. (2005). "Tissue inhibitor of matrix metalloproteinase-1 is prognostic in head and neck squamous cell carcinoma: comparison of the circulating and tissue immunoreactive protein." Clinical cancer research : an official journal of the American Association for Cancer Research **11**(9): 3257-3264.

Sanz-Ortega, J., F. Roig, et al. (2007). "17p13 (p53 locus), 5q21 (APC locus) and 9p21 (p16 locus) allelic deletions are frequently found in oral exfoliative cytology cells from smoker patients with non-small-cell lung cancer." Histology and histopathology **22**(5): 541-545.

Sasieni, P. D., J. Shelton, et al. (2011). "What is the lifetime risk of developing cancer?: the effect of adjusting for multiple primaries." British journal of cancer **105**(3): 460-465.

Sauter, E. R., M. Nesbit, et al. (1999). "Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck." Clinical cancer research : an official journal of the American Association for Cancer Research **5**(4): 775-782.

Sawano, A., T. Takahashi, et al. (1996). "Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor." Cell Growth Differ **7**(2): 213-221.

Seetharam, L., N. Gotoh, et al. (1995). "A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF." Oncogene **10**(1): 135-147.

Segal, N. H. and L. B. Saltz (2009). "Evolving treatment of advanced colon cancer." Annual review of medicine **60**: 207-219.

Seitz, H. K., S. Matsuzaki, et al. (2001). "Alcohol and cancer." Alcoholism, clinical and experimental research **25**(5 Suppl ISBRA): 137S-143S.

Seiwert, T. Y. and E. E. Cohen (2008). "Targeting angiogenesis in head and neck cancer." Seminars in oncology **35**(3): 274-285.

Shah, J. P., L. H. Karnell, et al. (1997). "Patterns of care for cancer of the larynx in the United States." Arch Otolaryngol Head Neck Surg **123**(5): 475-483.

Shah, J. P., J. E. Medina, et al. (1993). "Cervical lymph node metastasis." Current problems in surgery **30**(3): 1-335.

Shang, Z. J., Z. B. Li, et al. (2006). "VEGF is up-regulated by hypoxic stimulation and related to tumour angiogenesis and severity of disease in oral squamous cell carcinoma: in vitro and in vivo studies." International journal of oral and maxillofacial surgery **35**(6): 533-538.

Shigyo, H., S. Nonaka, et al. (2007). "Inducible nitric oxide synthase expression in various laryngeal lesions in relation to carcinogenesis, angiogenesis, and patients' prognosis." Acta oto-laryngologica **127**(9): 970-979.

Shin, D. M., J. Y. Ro, et al. (1994). "Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis." Cancer Res **54**(12): 3153-3159.

Shin, D. M., N. Voravud, et al. (1993). "Sequential increases in proliferating cell nuclear antigen expression in head and neck tumorigenesis: a potential biomarker." J Natl Cancer Inst **85**(12): 971-978.

Shirakawa, K., S. Furuhata, et al. (2002). "Induction of vasculogenesis in breast cancer models." Br J Cancer **87**(12): 1454-1461.

Siu, L. L., D. Soulieres, et al. (2007). "Phase I/II trial of erlotinib and cisplatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck: a Princess Margaret Hospital phase II consortium and National Cancer Institute of Canada Clinical Trials Group Study." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **25**(16): 2178-2183.

Slaughter, D. P., H. W. Southwick, et al. (1953). "Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin." Cancer **6**(5): 963-968.

Smith, A. H., M. A. Handley, et al. (1990). "Epidemiological evidence indicates asbestos causes laryngeal cancer." J Occup Med **32**(6): 499-507.

Smith, D. R., P. J. Polverini, et al. (1994). "Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma." J Exp Med **179**(5): 1409-1415.

Sobin, L. H., C. Wittekind, et al. (1997). TNM classification of malignant tumours. New York, J. Wiley.

Soulieres, D., N. N. Senzer, et al. (2004). "Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **22**(1): 77-85.

Spector, J. G., D. G. Sessions, et al. (2001). "Delayed regional metastases, distant metastases, and second primary malignancies in squamous cell carcinomas of the larynx and hypopharynx." The Laryngoscope **111**(6): 1079-1087.

Stell, P. M. (1990). "Prognosis in laryngeal carcinoma: host factors." Clinical otolaryngology and allied sciences **15**(2): 111-119.

Stell, P. M. (1990). "Prognosis in laryngeal carcinoma: tumour factors." Clin Otolaryngol Allied Sci **15**(1): 69-81.

Stellmach, V., O. V. Volpert, et al. (1996). "Tumour suppressor genes and angiogenesis: the role of TP53 in fibroblasts." Eur J Cancer **32A**(14): 2394-2400.

Stephenson, W. T., D. E. Barnes, et al. (1991). "Gender influences subsite of origin of laryngeal carcinoma." Arch Otolaryngol Head Neck Surg **117**(7): 774-778.

Stewart, C. E. and P. Rotwein (1996). "Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors." Physiological reviews **76**(4): 1005-1026.

Stoeltzing, O., S. A. Ahmad, et al. (2003). "Angiopoietin-1 inhibits vascular permeability, angiogenesis, and growth of hepatic colon cancer tumors." Cancer Res **63**(12): 3370-3377.

Strieter, R. M., S. L. Kunkel, et al. (1992). "Interleukin-8. A corneal factor that induces neovascularization." The American journal of pathology **141**(6): 1279-1284.

Sullu, Y., S. Gun, et al. (2010). "Poor prognostic clinicopathologic features correlate with VEGF expression but not with PTEN expression in squamous cell carcinoma of the larynx." Diagnostic pathology **5**: 35.

Sun, J. M., H. J. Jun, et al. (2011). "Insulin-like growth factor binding protein-3, in association with IGF-1 receptor, can predict prognosis in squamous cell carcinoma of the head and neck." Oral oncology **47**(8): 714-719.

Syrjanen, K., S. Syrjanen, et al. (1983). "Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis." Int J Oral Surg **12**(6): 418-424.

Syrjanen, S. (2005). "Human papillomavirus (HPV) in head and neck cancer." Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology **32 Suppl 1**: S59-66.

Syrjanen, S., G. Lodi, et al. (2011). "Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review." Oral diseases **17 Suppl 1**: 58-72.

Tae, K., A. K. El-Naggar, et al. (2000). "Expression of vascular endothelial growth factor and microvessel density in head and neck tumorigenesis." Clinical cancer research : an official journal of the American Association for Cancer Research **6**(7): 2821-2828.

Tae, K., B. J. Jin, et al. (2011). "The role of laryngopharyngeal reflux as a risk factor in laryngeal cancer: a preliminary report." Clinical and experimental otorhinolaryngology **4**(2): 101-104.

Tang, K., Y. Li, et al. (2010). "The PstI/RsaI and DraI polymorphisms of CYP2E1 and head and neck cancer risk: a meta-analysis based on 21 case-control studies." BMC cancer **10**: 575.

Tanigawa, N., M. Matsumura, et al. (1997). "Tumor vascularity correlates with the prognosis of patients with esophageal squamous cell carcinoma." Cancer **79**(2): 220-225.

Tantawy, A., L. Youins, et al. (1999). "Expression of c-erb B-2 oncoprotein in cancer of the larynx in relation to invasion of the cartilagenous framework and prognosis." Eur Arch Otorhinolaryngol **256**(2): 72-77.

Tartour, E., L. Deneux, et al. (1997). "Soluble interleukin-2 receptor serum level as a predictor of locoregional control and survival for patients with head and neck carcinoma: results of a multivariate prospective study." Cancer **79**(7): 1401-1408.

Taylor, D., W. M. Koch, et al. (1999). "Immunohistochemical detection of p53 protein accumulation in head and neck cancer: correlation with p53 gene alterations." Hum Pathol **30**(10): 1221-1225.

Teknos, T. N., C. Cox, et al. (2002). "Elevated serum vascular endothelial growth factor and decreased survival in advanced laryngeal carcinoma." Head & neck **24**(11): 1004-1011.

Terhaard, C. H., G. J. Hordijk, et al. (1992). "T3 laryngeal cancer: a retrospective study of the Dutch Head and Neck Oncology Cooperative Group: study design and general results." Clinical otolaryngology and allied sciences **17**(5): 393-402.

Thorpe, P. E. (2004). "Vascular targeting agents as cancer therapeutics." Clinical cancer research : an official journal of the American Association for Cancer Research **10**(2): 415-427.

Torrente, M. C., S. Ampuero, et al. (2005). "Molecular detection and typing of human papillomavirus in laryngeal carcinoma specimens." Acta oto-laryngologica **125**(8): 888-893.

Torrente, M. C. and J. M. Ojeda (2007). "Exploring the relation between human papilloma virus and larynx cancer." Acta oto-laryngologica **127**(9): 900-906.

Torry, R. J. and B. J. Rongish (1992). "Angiogenesis in the uterus: potential regulation and relation to tumor angiogenesis." Am J Reprod Immunol **27**(3-4): 171-179.

Tse, G. M., A. W. Chan, et al. (2007). "Strong immunohistochemical expression of vascular endothelial growth factor predicts overall survival in head and neck squamous cell carcinoma." Annals of surgical oncology **14**(12): 3558-3565.

Tsuji, T., Y. Sun, et al. (2005). "Angiogenin is translocated to the nucleus of HeLa cells and is involved in ribosomal RNA transcription and cell proliferation." Cancer research **65**(4): 1352-1360.

Tuyns, A. J. (1994). "Laryngeal cancer." Cancer Surv **19-20**: 159-173.

Tuyns, A. J., J. Esteve, et al. (1988). "Cancer of the larynx/hypopharynx, tobacco and alcohol: IARC international case-control study in Turin and Varese (Italy), Zaragoza and Navarra (Spain), Geneva (Switzerland) and Calvados (France)." Int J Cancer **41**(4): 483-491.

Uno, M., T. Otsuki, et al. (2001). "Anti-HER2-antibody enhances irradiation-induced growth inhibition in head and neck carcinoma." Int J Cancer **94**(4): 474-479.

Vaezi, M. F., M. A. Qadeer, et al. (2006). "Laryngeal cancer and gastroesophageal reflux disease: a case-control study." The American journal of medicine **119**(9): 768-776.

Vermorken, J. B., R. Mesia, et al. (2008). "Platinum-based chemotherapy plus cetuximab in head and neck cancer." The New England journal of medicine **359**(11): 1116-1127.

Vlachtsis, K., A. Nikolaou, et al. (2005). "Clinical and molecular prognostic factors in operable laryngeal cancer." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies **262**(11): 890-898.

Voravud, N., D. M. Shin, et al. (1993). "Increased polysomies of chromosomes 7 and 17 during head and neck multistage tumorigenesis." Cancer Res **53**(12): 2874-2883.

Ward, P. H. and D. G. Hanson (1988). "Reflux as an etiological factor of carcinoma of the laryngopharynx." Laryngoscope **98**(11): 1195-1199.

Wei, Q., L. Sheng, et al. (2008). "EGFR, HER2, and HER3 expression in laryngeal primary tumors and corresponding metastases." Annals of surgical oncology **15**(4): 1193-1201.

Weidner, N., J. Folkman, et al. (1992). "Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma." Journal of the National Cancer Institute **84**(24): 1875-1887.

Weidner, N., J. P. Semple, et al. (1991). "Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma." N Engl J Med **324**(1): 1-8.

Weinstat-Saslow, D. and P. S. Steeg (1994). "Angiogenesis and colonization in the tumor metastatic process: basic and applied advances." The FASEB journal : official publication of the Federation of American Societies for Experimental Biology **8**(6): 401-407.

Weinstein, G. S., I. F. Nuamah, et al. (1996). "Evaluation of HER-2/neu (c-erbB-2) oncogene expression in whole organ sections of supraglottic squamous cell carcinoma." Ann Otol Rhinol Laryngol **105**(4): 275-279.

Weiss, M. H., L. B. Harrison, et al. (1994). "Use of decision analysis in planning a management strategy for the stage N0 neck." Archives of otolaryngology--head & neck surgery **120**(7): 699-702.

Weller, M. D., P. C. Nankivell, et al. (2010). "The risk and interval to malignancy of patients with laryngeal dysplasia; a systematic review of case series and meta-analysis." Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery **35**(5): 364-372.

Wolk, A., C. S. Mantzoros, et al. (1998). "Insulin-like growth factor 1 and prostate cancer risk: a population-based, case-control study." Journal of the National Cancer Institute **90**(12): 911-915.

Woutersen, R. A., L. M. Appelman, et al. (1986). "Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study." Toxicology **41**(2): 213-231.

Wu, D. Q., M. K. Kan, et al. (1991). "Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors." The Journal of biological chemistry **266**(25): 16778-16785.

Wu, X., H. Zhao, et al. (2004). "Serum levels of insulin growth factor (IGF-I) and IGF-binding protein predict risk of second primary tumors in patients with head and neck cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **10**(12 Pt 1): 3988-3995.

Wynder, E. L., I. J. Bross, et al. (1956). "A study of environmental factors in cancer of the larynx." Cancer **9**(1): 86-110.

Wynder, E. L., L. S. Covey, et al. (1976). "Environmental factors in cancer of the larynx: a second look." Cancer **38**(4): 1591-1601.

Xie, K. (2001). "Interleukin-8 and human cancer biology." Cytokine Growth Factor Rev **12**(4): 375-391.

Xu, Z. P., T. Tsuji, et al. (2002). "The nuclear function of angiogenin in endothelial cells is related to rRNA production." Biochemical and biophysical research communications **294**(2): 287-292.

Yang, P. C., D. B. Thomas, et al. (1989). "Differences in the sex ratio of laryngeal cancer incidence rates by anatomic subsite." J Clin Epidemiol **42**(8): 755-758.

Yaylaci, A., S. Cakir, et al. (2006). "[The correlation of vascular endothelial growth factor with angiogenesis and p53 gene in laryngeal squamous cell carcinoma]." Kulak burun bogaz ihtisas dergisi : KBB = Journal of ear, nose, and throat **16**(3): 105-111.

Yilmaz, T., S. Hosal, et al. (1998). "Prognostic significance of depth of invasion in cancer of the larynx." The Laryngoscope **108**(5): 764-768.

Yoo, W. J., S. H. Cho, et al. (2004). "Loss of heterozygosity on chromosomes 3p,8p,9p and 17p in the progression of squamous cell carcinoma of the larynx." Journal of Korean medical science **19**(3): 345-351.

Yoshioka, N., L. Wang, et al. (2006). "A therapeutic target for prostate cancer based on angiogenin-stimulated angiogenesis and cancer cell proliferation." Proceedings of the National Academy of Sciences of the United States of America **103**(39): 14519-14524.

Young, M. R. (2006). "Protective mechanisms of head and neck squamous cell carcinomas from immune assault." Head Neck **28**(5): 462-470.

Yu, H., M. R. Spitz, et al. (1999). "Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis." Journal of the National Cancer Institute **91**(2): 151-156.

Yuan, Y., F. Wang, et al. (2009). "Angiogenin is involved in lung adenocarcinoma cell proliferation and angiogenesis." Lung cancer **66**(1): 28-36.

Zatonski, W., H. Becher, et al. (1991). "Tobacco, alcohol, and diet in the etiology of laryngeal cancer: a population-based case-control study." Cancer Causes Control **2**(1): 3-10.

Zeng, Y., K. Opeskin, et al. (2006). "Tumor-induced activation of lymphatic endothelial cells via vascular endothelial growth factor receptor-2 is critical for prostate cancer lymphatic metastasis." Cancer Res **66**(19): 9566-9575.

Zeng, Z. S., A. M. Cohen, et al. (1995). "Elevated tissue inhibitor of metalloproteinase 1 RNA in colorectal cancer stroma correlates with lymph node and distant metastases." Clinical cancer research : an official journal of the American Association for Cancer Research **1**(8): 899-906.

Zhang, H., L. B. Travis, et al. (2012). "Impact of radiotherapy on laryngeal cancer survival: a population-based study of 13,808 US patients." Cancer **118**(5): 1276-1287.