Expression of

Hypoxia Inducible Factor 1α

as a Prognostic Indicator in

Oesophageal Cancer

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Declaration

This thesis contains no material that has been submitted for the award of another degree or diploma at this or any other university or institution. I declare this is my own original work and, except where reference is made in the text, contains no material previously published by another person.

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Acknowledgements

Abstract

Introduction

Hypoxia Inducible Factor 1α (HIF 1α) plays a major role in the pleitropic response observed secondary to hypoxic conditions in tumours. Its expression in the tumour cells has been correlated to tumour aggressiveness but its prognostic value in squamous cell carcinoma (SCC) of the oesophagus remains unclear. To date, limited information is available on the prognostic role of HIF 1α in SCC of oesophagus in UK population. This information may help in choosing appropriate therapeutic strategies and possibly developing a monoclonal antibody with therapeutic potential targeting the HIF 1α.

Methods

Tumour samples from 36 patients diagnosed with SCC of oesophagus were collected in this prospective observational study. Prepared tissue sections were stained with validated specific monoclonal antibodies for HIF 1α in controlled experiment and the expression of HIF 1α was scored by blind assessment. This expression score was correlated with the disease pattern and survival over a period of 4 year 8 months.

Results

Out of 36 patients, 17 patients showed low and 19 high expression of HIF 1α. A trend of better median overall survival was observed in the group with low expression of HIF 1α compared to high expression (238 vs. 196 days) but this was not statistically significant ($p > 0.05$, log rank test). Regression analysis showed that HIF 1α was not an independent prognostic factor for survival ($p > 0.05$).

Absence of metastases at diagnosis ($p = 0.05$) and treatment with curative intent ($p = 0.001$) were statistically significant prognostic factors on univariate analysis; on multivariate analysis, treatment with curative intent was the only independent statistically significant prognostic factor ($p < 0.001$).

Conclusion

HIF 1α expression did not show prognostic value in SCC of oesophagus in present study in spite of a trend in improved survival, in agreement with previous studies. Further study is required to support this observation.

Similar knowledge on the role of HIF 1α expression in oeosphageal adenocarcinoma is scarce. As the incidence of adenocarcinoma is increasing, research in this field is recommended. Novel strategies on the therapeutic manipulation of HIF 1α in cancer are to be explored further and may have a role to play in improving treatment outcome in oesophageal cancer.

Key words: Oesophageal squamous cell cancer, Hypoxia inducible factor 1α, prognostic factor

Publications and Presentations

The following papers were published and presented as a result of this work:

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P C Munipalle, Y K S Viswanath, P A Davis, D Scoones

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P C Munipalle, A Ahitan, D Scoones, P A Davis, M Wilson, Z Ali, Y K S Viswanath

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(Appendix 11)

List of Abbreviations

A1B1   Amplified in breast cancer

ACC   Adenocarcinoma

AIS   Amplified in squamous cell carcinoma

AJCC   American Joint Committee on Cancer

ANN   Artificial Neural networks

ARNT   Aryl-hydrocarbon-receptor nuclear translocator

bFGF   Basic fibroblast growth factor

bHLH   basic helix loop helix

CAD/ C-TAD   C terminus/ domain

CDK   Cyclin dependent kinases

CEA   carcinoembryogenic antigen

CRM   Circumferential resection margins

CRP   C-reactive protein

CRT   Chemoradiotherapy

CSA   Catalysed signal amplification

CT   Computerised tomography

CXR   Chest X-ray

DNA   Deoxy ribonucleic acid

EBM   Evidence based medicine

EDTV   Endoscopic ultrasound defined tumour volume

EGFR   Epidermal growth factor receptor

ELISA   Enzyme Linked Immunosorbent Assay test

eLNMC   endoscopic lymph node metastatic count

EMT   epithelial-mesenchymal transition

EORTC	Organisation for Research and Treatment of Cancer
EpCAM	Epithelial cell adhesion molecule
ERCC	Excision repair cross complementing
EUS	Endoscopic ultrasound
FNA	Fine needle aspiration
GIST	Gastrointestinal stromal tumour
GOJ	Gastro-oesophageal junction
GORD	Gastro-oesophageal reflux disease
HER	Human epidermal receptor
HIF 1α	Hypoxia inducible factor 1α
HMER	Heat-mediated epitope retrieval
HRE	Hypoxia response element
Hsp90	Heat shock protein 90
IMS	Industrialised methylated spirit
IPAS protein	Inhibitory PAS protein
LOH	Loss of heterozygosity
MDT	Multidisciplinary team discussions
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MTV	Metabolic tumour volume
MVD	Microvessel density
NAD/ N-TAD	N terminus domain
NECN	North of England Cancer Care Network
NHS	National Health Service
ODDD	Oxygen-dependent degradation domain
OGD	Oesopahgao-gastro-duodenoscopy

PAS   **P**eriodic circadian protein, **A**ryl hydrocarbon receptor nuclear translocator protein, **S**ingle minded protein

PCR   Polymerase chain reaction

PDGF   Platelet derived growth factor

PDT   Photodynamic therapy

PET   Positron emission tomography

pTNM   Pathological Tumour, Node, Metastasis (staging)

pVHL   Von Hippel-Lindau tumour suppressor protein

QOL   Quality of life

RACK   Receptor of activated protein C kinase

RCC   Ranal cell carcinoma

RT-PCR   Reverse Transcriptase – Polymerase Chain Reaction

SCC   Squamous cell carcinoma

STROBE group   **St**rengthening the **R**eporting of **Ob**servational studies in **E**pidemiology group

TACSTD   Tumour-associated calcium signal transducer

TBS   Tris-buffer saline

TGF α   Transforming growth factor α

TNM   Tumour, Node, Metastasis (staging)

TSO   Two-stage oesophagectomy

UICC   International Union Against Cancer

VEGF   Vascular endothelial growth factor

WECC   Worldwide Esophageal Cancer Collaboration

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CHAPTER ONE

INTRODUCTION

1.1 Prologue

Oesophageal cancer has a poor prognosis in spite of advances in diagnosis and treatment. There is a constant need for more prognostic information to improve the outcome of this disease. The outcome may be improved by identifying potential sub-populations of patients with better prognosis and targeting them with multi-modality therapy where applicable. This strategy also may avoid the significant morbidity caused by the intensive neoadjuvant, adjuvant and radical surgery treatment strategies in those sub-populations with poorer prognosis, who are unlikely to benefit from such treatments and hence can have alternate treatment options with better quality of life (QOL).

The current study aimed at evaluating the role of Hypoxia inducible factor 1α (HIF 1α) in providing prognostic information about SCC of oesophagus. Information about the role of HIF 1α in such UK patient population has not been previously reported and the results of this study aim to fill the gap. I hope this study can suggest further areas of research for better patient outcome.

1.2 Outline of thesis

I have reported my study in six chapters. In chapter one, I have introduced the epidemiology of oesophageal cancer which shows the relevance of this study to the target population. The role of prognostic indicators including HIF 1α in oesophageal cancer and the ongoing search for better indicators is presented. The applied aspects and management of cancer of oesophagus are mentioned. In chapter two, methodology used in the study is presented. This included the recruitment of patients, collection of samples and their processing towards the identification of HIF 1α expression. Chapter three contained the results of the present study. The clinic-pathological and survival data, in relation to the expression levels of HIF 1α is presented with the aid of figures and statistical tests. In chapter four, I discussed the results and analysed then in comparison with existing literature. Chapter five summarised and concluded the significance of the findings of the present study. Future work is discussed in chapter six, including recommendations for further studies for advancement of the existing knowledge regarding HIF 1α.

1.3 Epidemiology of oesophageal cancer

1.3.1 Incidence

Oesophageal cancer is one of the commonest cancers worldwide with an Age-Standardized Rate of 7.0 cases per 100,000 populations in 2008 (World Cancer Research Fund International, www.wcrf.org). Currently oesophageal cancer is the 8th most common cancer worldwide. Wide geographic and ethnic variation is always noted in the incidence of oesophageal cancer. In developing countries it is ranked as the 5th most common cancer, while it is ranked as 13th most common cancer in developed countries. The Age-Standardized Rate for this cancer in 2008 was 8.7 and 3.6 per 100,000 of these populations respectively (www.wcrf.org).

UK has a higher incidence of oesophageal cancer within Europe compared to many other nations, with the highest incidence rate in women and the second highest incidence rate in men (www.cancerresearchuk.org) among European nations. Over all, oesophageal cancer is the 9th most common cancer in UK (www.cancerresearchuk.org). In 2007, 5226 males and 2740 females were newly diagnosed with oesophageal cancer, with European Age Standardised Rate per 100,000 populations of 14.4 (95% CI 14 − 14.8) and 5.5 (95% CI 5.3 − 5.7) respectively. As per the latest data available from 2009, the life time risk of developing this disease is 1 in 64 for men and 1in 116 for women UK. The incidence rate is negligible before the 4th decade of life and reaches a peak in the 9th decade of life in both sexes. However the highest numbers of cases are seen in the 8th decade in males and towards the end of 9th decade in females (www.cancerresearchuk.org), making this a disease of the aging population. There is a clear north/south divide in UK with the highest incidence in Scotland and some areas of the North of England (Quinn et al, 2005).

There has been a gradual increase in the incidence rate of oesophageal cancer over the last 25 years (www.cancerresearchuk.org). The rise was seen especially amongst men. Their incidence rose from 8.8 per 100,000 populations in 1975 to 14.4 in 2006. Comparatively slighter increase in incidence rates was noticed among women in the same period, rising from 4.8 per 100,000 populations to 5.7. The causes for this difference are probably multifactorial.

There are some differences in the incidence and behaviour of the two main types of oesophageal cancer, namely adenocarcinoma (ACC) and squamous cell carcinoma (SCC). The majority of the oesophageal cancers globally are of SCC type, while ACC is more prevalent in developed nations (Quinn et al, 2001). There is a significantly higher incidence rate of oesophageal cancer in most deprived groups within developed countries. Gilbert et al (2002) in his audit on Scottish population showed clear association between social deprivation and SCC but not ACC, which supports the role of different aetiological factors in causation of SCC and ACC as discussed later. Gender also seems to play a role in the incidence of these two different types of cancer. ACC in males was nearly 5 fold to that of females in 1998 (Newnham et al, 2003) while this gross variation was not seen in SCC.

1.3.2 Mortality

Oesophageal cancer is the 6th most common cause of cancer related mortality globally and in the UK (Parkin et al, 2005). In 2008, 5026 men and 2580 women died due to oesophageal cancer (www.cancerresearchuk.org). The mortality figures are highest in the 8th decade for men and 9th decade for women. The current overall survival rates after diagnosis of oesophageal cancer are 30% at the end of 1 year, 8% at 5 years and 6-7% at 10 years. (www.cancerresearchuk.org). The trend of mortality over the years was in parallel to the rate of incidence. The mortality figures per 100,000 population rose from 8.1 in 1975 to 13.3 in 2008 for men; in women, these figures showed a slight rise from 4.8 in 1975 to 5.4 in 2008. These mortality figures mirror the incidence of oesophageal cancer in both sexes.

1.3.3 Oesophageal cancer in local population

The catchment area for the recruitment of patients for this study was the Cancer Care Alliance network region (www.cancercarealliance.nhs.uk). This network was one of the 34 cancer networks in England at the time of patient recruitment and covered the healthcare organisations and healthcare professionals from Teesside, South Durham and North Yorkshire. The James Cook University Hospital was the tertiary referral centre for oesophageal cancer for this network region.

The incidence and mortality rates of oesophageal cancer in this region during 2003 are shown in Table 1 below:

Table 1. Oesophageal cancer in local catchment area (www.ukacr.org)

	Incidence			Mortality		
	Number	*EASR (95% CI)	Rank in England	Number	*EASR (95% CI)	Rank in England
Men	79	13.5 (10.5 – 16.5)	20	83	14 (11 – 17.1)	12
Women	60	7.4 (5.4 – 9.4)	2	46	5.4 (3.7 – 7.1)	11

*European Age-Standardised Rate for 100,000 populations at risk

The incidence rate in the local catchment area is the second highest in UK amongst women. The mortality rate for both sexes is in the upper 1/3 of the comparative table among network regions of England.

After our study started, the Cancer Care Alliance network merged with the North of England Cancer Care Network (NECN) and hence separate figures for the original network are unavailable for updating. For NECN in 2008, 403 new cancers were reported (266 men and 137 women), with mortality figures of 375 (244 men and 131 women, www.nycric.nhs.uk).

1.4 Role of prognostic indicators

Oesophageal cancer is a disease on the rise with no great improvement in survival over the last few decades. The increase in incidence is probably multi-factorial. Life style factors like alcohol consumption and smoking are major aetiological contributors of oesophageal cancer, especially SCC (Anantharaman et al, 2011) The increased consumption of these in recent decades is reflected in the increased incidence of this type of cancer. Other aetiological factors such as obesity and gastro-oesophageal reflux disease (GORD) with resultant Barrett's metaplasia of the oesophagus are increasing in incidence in the Western world, increasing the incidence of ACC (Anantharaman et al, 2011). Education of the population about cancer symptoms has been making potential patients aware of their symptoms and subsequent identification. General Practitioners are identifying possible cancer symptoms based on the 'alarm symptoms' as advocated by National Guidelines

(www.nice.org.uk), leading to the early identification of the cancer through the 'two week referral rule'. In addition, surveillance programmes of patients at risk of developing cancer based on the genetic history and pre-malignant conditions like Barrett's metaplasia are contributing for increasing incidence rates. This trend of a rise in oesophageal cancer makes it a priority to identify the disease at an early stage, and to provide appropriate and timely treatment.

The minimal improvement in survival rates in spite of major advances in the management represents the poor prognosis from this disease. The 5 year survival rates for both men and women have increased from 3% in 1971 to only 8% in 2001 (www.cancerresearch.org.uk). The 10 year survival rates showed a slower trend of improvement: for men, 3% (1971) to 6% (2001); for women, 3% (1971) to 7% (2001).

One of the ways of reducing the morbidity and mortality is to improve the prognostic efficacy of current investigations in order to predict the individual course of the cancer in a particular patient. This prognostic information helps in planning the treatment with the aim of providing the best possible care. In the subgroup of patients with early resectable disease but with aggressive nature, neo-adjuvant therapy can be considered before offering them two-stage oesophagectomy (TSO). If an early cancer is found to have high chances of recurrence post-operatively based on histology of the tumour, adjuvant therapy can be offered following TSO (Vallbohmer & Lenz, 2006). In patients with advanced stage of the disease but possibly good biological behaviour and prognosis, aggressive multi-modality therapy can be considered in place of palliative therapy with the aim of prolonging survival with reasonable QOL.

Over estimating the spread and malignant potential of the cancer in an individual could deprive him or her of the appropriate treatment modalities available. This could have a detrimental effect on the disease progression and subsequent mortality of the patient. On the other hand, under-estimating the biological staging of the cancer may subject the patient to treatment regimens with potential complications and side effects (Avery et al, 2007).

Surgical options for treating oesophageal cancer have significant morbidity and risk of mortality. The in-hospital mortality, 30-day mortality and 90-day mortality were 4.5%, 3.8% and 5.7% consecutively as per the National Oesophago-gastric Cancer

Audit 2010 (www.ic.nhs.uk). The post-operative complication rate causing morbidity was 29.8%. Oesophageal cancer is a disease of the elderly and subjecting them to a major procedure from which they may not benefit is best avoided.

The potential side effects of chemotherapy for oesophageal cancer include immunosuppression, anaemia, increased bleeding tendency, sickness & diarrhoea, hair loss and soreness of hands & feet. Radiotherapy to the oesophagus can cause sore throat resulting in dysphagia, reduced appetite and malnutrition. These effects are worse in older patients. For example, data available from the National Oesophago-gastric Cancer Audit 2010 (www.ic.nhs.uk) shows that the proportion of patients who completed palliative chemotherapy falls from 60% under 60 years of age to 43% in aged 80 years and above. These side effects usually interfere with the QOL of the patients and can be minimised by outweighing the benefits of the particular treatment over the potential side effects and risks, and offering them only to the patients deemed to be benefitting from them.

The aim of treatment for each patient influences his or her quality of life too. A study of QOL using European Organisation for Research and Treatment of Cancer (EORTC) validated questionnaires showed that patients of oesophago-gastric cancer treated with palliative intent reported worse global QOL (mean score 55) than those treated with curative aim (mean score 67; National oesophago-gastric cancer Audit 2010). They also complained about symptoms like fatigue, nausea and vomiting, dyspnoea and appetite loss worse than in those treated with curative intent. While the severity of some of these symptoms might be due to the advanced disease itself, the modality of therapy would undoubtedly have a part to play. Hence the clinicians shall have access to more information about the prognosis of each patient before approaching a particular aim of treatment.

So, it is essential that the best available evidence based prognostic information is provided to the clinicians to enable that SCC patients are treated in their best interests. Prognostic indicators are the main stay of providing such evidence. The pros and cons of the various prognostic indicators of SCC and their current usage are discussed below.

1.4.1 Current prognostic indicators

A wealth of information is currently available about prognostic indicators of SCC. Evidence based medicine (EBM) provides guidance on which of these indicators could be accepted into clinical practice to obtain useful information about disease behaviour. This information forms a basis for multidisciplinary team (MDT) discussions, clinical audits and as a tool to measure outcomes of the disease. These are discussed under clinical prognostic indicators.

Many *in vivo* and *in vitro* studies provided useful information on the prognostic value of various molecular compounds in relation to human cancers, and in particular to SCC. These compounds do have potential value to provide prognostic information that can be utilised for clinical application if stronger evidence is provided. I discussed them further as molecular prognostic indicators.

1.4.1.1 Clinical prognostic indicators

1.4.1.1.1 TNM (Tumour, Node, Metastasis) Staging

Currently TNM staging is the best available evidence based prognostic indicator in universally accepted practice. The TNM staging system for malignant tumours was originally compiled by Pierre Denoix in 1946. The first comprehensive classification for various tumours was published in 1968 by UICC (International Union Against Cancer). The TNM classification and staging for oesophageal cancer is described in detail in 1.5.3.2 and can be briefly mentioned as two separate classifications, clinical and pathological.

The clinical TNM classification is based on the evidence pertaining to the anatomical tumour spread. The various staging investigations as mentioned later provide this evidence. The clinical TNM stage at the time of diagnosis is used as a practical basis to plan the treatment of the particular individual and provides some prognostic information as to the likely course of the disease. The pathological classification is a better provider of information than the clinical TNM stage alone, and is based on the supplementary histological information available from a resected tumour specimen. These histopathology based indicators are the grading of the tumour, pathological T N M staging (pT, pN and pM), and the number of lymph nodes resected. Therefore the pTNM staging has obvious superior prognostic value compared to the clinical stage, and helps plan the postoperative management. TNM staging guides to the

appropriate intent of treatment for a particular individual, modality of primary treatment, extent of resection of involved organ where surgery is chosen, response to the primary treatment, and decision making regarding neoadjuvant treatment (Mackillop et al, 1998). In addition, staging guides research on tumour management and helps manage cancer control programmes.

Current TNM staging for oesophageal cancer is not foolproof though. Significant difference between the assessments of spread of upper oesophageal cancer by clinical and pathological TNM stagings was shown by Peracchia et al (1990). Their study showed that the clinical TNM staging of cervical oesophageal cancer was not in agreement with the pathological TNM stage in nearly 50% of the cases. They felt that the clinical TNM staging alone is hence inaccurate and unreliable for therapeutic decision making and estimating prognosis in patients with cancer in the cervical oesophagus. There is a dearth of information about the accuracy of TNM staging in oesophageal cancer affecting other regions of oesophagus.

Another limitation of TNM is that complete pTNM staging information is only available in patients undergoing surgical resections of their oesophageal tumours; a significant number of patients do not fit into this category. Large numbers of patients present at a later stage of the disease due to minimal symptoms from the tumour and hence are not suitable for resection. Patients with cancer beyond T3N1 stage are considered advanced and curative resection is not attempted in this group. The majority of the patients are also elderly with significant co-morbidities, which have major implications for the management of their disease. Poon et al (1998) have shown that resection rate for oeosphageal cancer in patients aged above 70 years is 48%, in contrast to younger patients with a resection rate of 65% (p<0.001). Their study also showed that the post-operative complications arising from associated medical conditions are also much higher in the older age group. Zehetner et al (2010) studied 560 patients of oesophageal cancer who underwent curative surgery over a period of 15 years and showed that only 8% of this group are aged 80 years and above. While the results of the operation including survival rates in those aged above 80 years were similar to younger patients in their study, there was a careful patient selection based on their cardiopulmonary status that made these results possible. Contrasting results were shown by Braiteh et al (2009) who showed that the complications and survival following surgery is worse in patients aged above 70

years compared to younger patients and this still seems to be the general opinion among surgeons. Recent studies showing similar survival rates with either surgery or chemo-radiation in patients aged above 70 years (Davies et al, 2010) influence the current management opinions regarding this group of patients.

This stresses on the need for very careful selection criteria for the surgical management in the elderly. Many centres including the one where the current study was carried out do not generally consider elderly patients for elective curative surgery and hence pTNM information which is more accurate is unavailable in a significant number of patients. This limits the usefulness of TNM staging system and supports search for a better prognostic marker, which can provide useful information across the patient groups with no undue dependence on the mode of treatment.

The number of involved lymph nodes alone has independent prognostic value, which was not considered as a criterion in the current TNM staging. Prognostic systems based on the number of involved lymph nodes (0, 1 - 3, or >3) or ratio of involved to normal looking lymph nodes (0, <0.15, or >0.15) were shown to be of help in assessing the prognosis of patients who underwent TSO (Van Sandick et al, 2002; Wijnhoven et al, 2007). The number of metastatic lymph nodes counted during endoscopic ultrasound (EUS), known as endoscopic lymph node metastatic count (eLNMC) has shown significant independent prognostic value in a recent study from Cardiff (Twine et al, 2010). Kato et al (2009) showed that the number of metastatic lymph nodes identified by Positron Emission Tomography (PET) scan has independent prognostic value regarding disease free and overall survival. The current TNM staging does not take these into account for staging purposes. Griffiths et al (2006a) criticised TNM staging system for not including the number of metastatic lymph nodes in the staging for cancer of the lower oesophagus and gastro-oesophageal junction (GOJ). He cites the TNM staging of gastric cancer as an example. In gastric cancers, the number of involved nodes determines the N stage as pN1 (1-6 nodes), pN2 (7-15 nodes) and pN3 (>15 nodes). His study involved only the GOJ tumours, but similar argument can be made for all oesophageal cancers to make the current TNM staging a more robust prognostic indicator.

The other criticism of TNM staging is that the information forming the basis of the system is provided mostly by studies on SCC of the upper and middle oesophagus (Lagarde et al, 2006a). As the incidence of lower oesophageal ACC is the currently

predominant form of this cancer, this information is inadequate and hence a new staging system for ACC of gastro-oesophageal junction is proposed to be considered by UICC.

Studies are going on to improve the efficacy of the staging by the addition of various parameters through the evidence base. Rice et al (2010) have incorporated histopathological type of the cancer, histological grade and cancer location to the existing TNM classification to propose a joint AJCC/ UICC staging classification. This was shown to provide better prognostic information that TNM alone (Wijnhoven et al, 2007) but has not yet been tested in practice. More recently Gaur et al (2010) published the staging system recommended by the Worldwide Esophageal Cancer Collaboration (WECC) wherein incorporating the number of lymph nodes involved with cancer to the AJCC system has shown better prognostic potential for ACC of gastro-oesophageal cancers.

1.4.1.1.2 Other clinical prognostic indicators

While TNM staging is a main source of prognostic information, there are other dependable sources that assist in assessing prognosis. Some of them are currently in routine usage for this purpose.

The extent of tumour removal by TSO plays a major role in future local recurrence and has prognostic significance. Involvement of proximal (Mariette et al, 2003a) and distal (Casson et al, 2000) margins of the resected specimen by tumour has shown poor survival. The involvement of circumferential resection margins (CRM) is debatable. Various studies have shown contrasting results of positive CRM on survival rates (Khan et al, 2003; Sheepers et al, 2009). This is a possible reflection on the extent of mediastinal dissection and hence is surgeon dependent. Khan et al (2003) summarised in their review article based on the available literature that in those patients who received neoadjuvant chemotherapy, CRM showed independent prognostic value, while its role in the surgery alone group was unclear.

The extent of residual disease left behind (denoted as: R0 = no residual disease, R1 = microscopic residual disease and R2 = macroscopic residual disease) has important independent prognostic value (Hofstetter et al, 2002; Ott et al, 2009) and is a frequently reported parameter following TSO. The patients who had R0 status are deemed to have complete removal of the primary tumour and have better prognosis

in terms of loco-regional control than R1 status patients. R1, and sometimes R2 status patients are generally considered for adjuvant therapy to improve the loco-regional control of the disease.

The length of the tumour at the time of diagnosis has shown to be of independent prognostic value. Griffiths et al (2006b) reported that patients with tumours longer than 3.5 cm at histology have a poorer prognosis than those with shorter tumours. This may represent a larger tumour volume at the time diagnosis, with possible inherent aggressiveness.

Vascular and peri-neural invasion by cancer cells has shown poor prognosis (Watanabe et al, 2000; Brücher et al, 2001) and has independent prognostic value. It is considered as part of the histological grading of tumour and again this information is available only on resected specimens. Extra-capsular spread of tumour cells in the involved lymph nodes is also reported to be of good prognostic value (Yoon et al, 2010).

Association with Barrett's oesophagus either as a precursor or as concomitant feature along with ACC has been found to be an indicator of better prognosis. The ACC not arising from Barrett's mucosa is probably of a different histological behaviour; also the presence of Barrett's mucosa and the associated symptoms allow early endoscopic identification of the ACC and early treatment, with subsequent better results (Menke-Pluymers, 1992).

The severity of the patients' symptoms like dysphagia and weight loss was traditionally perceived to be a predictor of poor prognosis; there is now evidence to support this. Plaisant et al (2005) reported that weight loss is an independent predictor of survival in oesophageal cancer patients. Dysphagia was a prognostic factor in a univariate analysis (Mariette, 2003b) but did not show independent prognostic value. Low serum albumin levels in patients undergoing chemo-radiation has shown poorer prognosis in a recent study by Cincibuch et al (2010). Deans et al (2006) reported that the tumour inflammatory cell infiltrate and systemic inflammatory response as measured by the circulating levels of C-reactive protein (CRP) were associated with predicting patient survival.

With the advent of recent techniques which from an integral part of work-up of oesophageal cancers, evidence is emerging about additional prognostic markers.

During PET scan, the amount of 18-Fluorodeoxyglucose absorbed by the tumour cells can be calculated and this is known as 'metabolic tumour volume (MTV)'. This parameter was shown to be of independent prognostic value in oesophageal cancer tumours (Hyun, 2010) with tumours having higher MTV showing worse prognosis. On similar lines, during EUS, length of the diseased segment of oesophagus and tumour thickness could be measured and the endoscopic ultrasound defined tumour volume (EDTV) calculated. This information has independent prognostic value as shown by Twine et al (2010a). They studied 174 consecutive patients and showed that if the EDTV is less than 25 cm^3 there was significant survival advantage.

It is evident that there is potential for identifying more prognostic indicators for precise recognition of the behaviour of the cancer. But none of these indicators described so far could show strong enough association with survival of the patients which was significant enough to employ them on their own in predicting the prognosis. Lagarde et al (2006b) suggested Artificial Neural networks (ANN) and nomograms to incorporate various other prognostic indicators discussed above, in view of the increasing knowledge about the biological behaviour of these tumours. This information will arm the healthcare team with the best available knowledge of the prognosis of a particular patient and as a consequence assists in decision making.

Prognostic information available from different markers could also be pooled together to determine a score of severity of oesophageal cancer. The Royal Marsden Hospital prognostic index is such system based on the pooled data from 1,080 patients with locally advanced and metastatic gastro-oesophageal cancer enrolled in multicentre randomized controlled trials. Based on the presence of four risk factors, namely performance status, liver metastases, peritoneal metastases and alkaline phosphatase levels, patients are categorized into good (no risk factor), moderate (1 or 2 factors) and poor (3 or 4 factors) risk groups. Of these, the first three factors have independent prognostic value as well. The one year survival rates in these three groups were 52.4%, 33.1% and 13.7% respectively. However this index is of not much use in early stages of the cancer and in clinical practice may have minimal impact on patient management.

1.4.1.2 Molecular prognostic indicators

Several molecular prognostic indicators are currently being studied in oesophageal cancer. Some examples are epidermal growth factor receptor (EGFR), angiogenic factors (e.g. vascular endothelial growth factor (VEGF), Cox-2 protein, basic fibroblast growth factor (bFGF), Transforming growth factor (TGF) α, tumour suppressor gene p53, cell cycle regulators (Cyclin D1 protein, p21 protein, p27 protein), DNA repair system (Excision repair cross complementing (ERCC) 3 protein), apoptotic factors (ERCC1 gene, Bax protein, Bcl-2 protein and Bcl-X protein), matrix metalloproteinases (MMP-7, MMP-9, MMP-2 and MMP-11) and Human epidermal receptor – 2/ HER – 2 (Vallbohmer & Lenz, 2006).

Ogata et al (2003) in their long term follow up study showed that the expression of EGFR was positively related to the survival in node positive SCC. EGFR gene amplification was also discovered to be a predictor of poor survival in SCC (Kitagawa et al, 1996). Recent studies have shown significance of mutations in the EGFR gene. The patients with mutation of the EGFR gene caused by single nuclear polymorphism at codon 787 of exon 20 showed significantly poorer overall survival rates (Kaneko et al, 2010).

VEGF is a signal protein that induces formation of *de novo* capillaries (vasculogenesis) and proliferation of the existing capillaries (angiogenesis). It plays a major role in the survival of tissues in presence of ischaemia, including tumour tissues and in subsequent adaptability of the tumour to hypoxia. This reflected in the finding of higher T and N stages in oesophageal SCC patients expressing higher expression of VEGF (Shih et al, 2000). The tumour microvessel density (MVD) which in turn is influenced by the action of VEGF has also showed worse prognosis in those with high expression. These two hence have good prognostic potential.

Cyclins and cyclin dependent kinases (CDK) form a protein-kinase complex which participates in the mitotic cycle of the cell during G1 phase. Alterations and mutations of the genes controlling these proteins were known to be causative factors for tumours. Shinozaki et al (1996) studied the role of amplification of cyclin D1 gene in oesophageal SCC. They showed the amplification of cyclin D1 gene in 11q13 region cause significantly lower survival in their study group, stage to stage in comparison with those showing normal gene. The occurrence of distant metastasis

was also found to be higher in those with gene amplification. P16 is a tumour suppressor protein that acts by inhibiting CDK. Loss or low expression of the p16 gene was found to result in worse prognosis in SCC, with an inverse relation to cyclin D1 expression (Tekeuchi et al, 1997). They believed these genetic expressions can be utilized as independent prognostic indicators. Similar results were published recently by Kawakubo et al (2005).

p53 is a tumour suppressor gene whose vital role in preventing human cancers was discovered in 1989 by Baker et al. Mutations of this gene were shown to be common in many visceral cancers. Up to 50% of human tumours show mutation or alteration of this important gene (Hollstein et al, 1991). Similar changes were observed in the p53 expression of oesophageal tumour cell lines and specimens (Hollstein et al, 1990). Patients with tumours with allelic loss of both 17p and 18q of the p53 gene had worse survival than those who did not (Galipeau et al, 1996). P53 mutations as identified by Polymerase chain reaction (PCR) showed significant predictive value in the response to chemotherapy of SCC patients. Yamasaki's study observed objective response to chemotherapy in 65.9% of patients with original genotype, but in only 16.7% of patients with the mutant gene (2010).

Epithelial cell adhesion molecule (EpCAM), also known as tumor-associated calcium signal transducer 1 (TACSTD1) is a component of cadherin-catenin pathway. It has shown correlation with tumour depth, stage of the disease, blood vessel invasion and survival in oesophageal cancer (Kimura et al, 2007). Cytokeratin 19 in combination with EpCAM has improved the accuracy of this identification. Estimating the expression of various molecular markers in combination may improve their overall prognostic accuracy in a similar fashion.

ΔNp63 (Amplified in SCC or AIS) protein detection in the blood of patients with primary and recurrent SCC of oesophagus has shown promising results in identifying the presence of SCC cells (Koike et al, 2002). This protein is produced by the ΔNp63 gene which is a homologue of p53 gene. Regular measurement of this protein may help in the monitoring of the patients with known oesophageal cancer. Squamous cell carcinoma associated antigen (SCC) and carcinoembryogenic antigen (CEA) have limited value in the monitoring of SCC patients, and ΔNp63 expression was found to be more accurately associated with this cancer.

Ross & McKenna (2001) showed that HER-2/*neu* oncogene expression on chromosome 17q encodes tyrosine kinase growth factor receptor and its overexpression is an independent prognostic factor in ACC of oesophagus. But there is lack of evidence to attribute the same role to it in SCC of oesophagus.

Amplified in breast cancer (A1B1) gene over expression (He et al, 2009) was associated with distant metastases, late stages of the tumour and resistance to chemoradiation in oesophageal SCC. Moreover, this gene overexpression was an independent predictor of survival in these patients.

With the advent of genome mapping, studies were done to detect alterations in various genes areas that could help identify the biological behaviour of oesophageal SCC. Carneiro et al in 2008 applied array based comparative genomic hybridization technique to 30 SCC specimens and detected recurrent alterations in 19% of them. They included recurrent gains of up to 60% in chromosome regions 5p, 7p, 7q, 8q, 10q, 11q, 12p, 14q, 16p, 17p, 19p, 19q, and 20q; losses of up to 40% in regions 3p, 5q, 8p, 9p and 11q. The number of alterations was also found correlated with the tumour stage, degree of differentiation and metastatic potential, with fewer alterations in less aggressive tumours. They concluded that there is independent prognostic role for gain of 7p22.3 in nodal metastases and gains of 1p36.32 and 19p13.3 in survival. The genetic profiling may help identify the subset of patients with aggressive tumours at a very early stage, with subsequent implications for targeted therapy. Further studies are needed to develop a practical application tool utilizing this information.

Hu et al (2009) went further and tried to identify the genes identifiable with oesophageal carcinogenesis itself. Their group studied the genomic instability in SCC cells from a high risk region of China using genetic arrays and identified 30 regions with potential carcinogenic genes. High levels of loss of heterozygosity (LOH) were observed on selected chromosome arms, which could be targeted to identify genes responsible for carcinogenesis. They compared the tumour DNA with germ-line DNA obtained from peripheral venous blood and found identical genetic changes within each patient's samples.

Currently none of these molecular tumour markers are incorporated in the protocol for the management of oesophageal cancer and they have no practical application.

The limited choice of available molecular prognostic indicators encouraged our current study in evaluating the possible role of an important molecule, Hypoxia Inducible Factor 1α in oesophageal cancer of the local population.

1.4.1.3 Hypoxia Inducible Factor 1α

1.4.1.3.1 Molecular biology

Hypoxia inducible factor (HIF) was described by Semenza and Wang in 1992 during their studies on the hypoxia response element (HRE) of erythropoietin. They later (1993) described the role of HIF in the hypoxic response related activation of many genes. HIF has basic-helix-loop-helix (bHLH) structure, and belongs to the PAS (Periodic circadian protein, Aryl hydrocarbon receptor nuclear translocator protein, Single minded protein) subfamily of transcription factors (Wang & Semenza, 1995a; 1995b).

HIF is composed of α and β subunits, and is activated when both the subunits join and form a heterodimer. HIF α was known to exist in three paralogues, namely HIF 1α, HIF 2α and HIF 3α. HIF 1β was initially known as aryl-hydrocarbon-receptor nuclear translocator (ARNT) (Semenza & Wang, 1992). This exists as three paralogues, ARNT1, ARNT2 and ARNT3. Either HIF 1α or HIF 2α can heterodimerise with HIF1β to from functional HIF. The domain structures of HIF are shown in Figure 1.
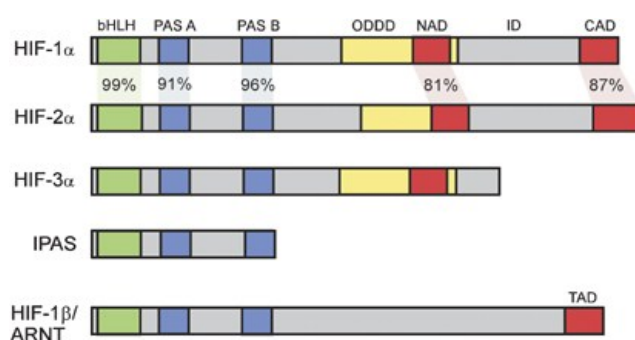
Figure 1. Domain structures of the HIF transcription factors
(Lisy & Peet, 2008. Reproduced by permission)

The three HIF α subunits show partially overlapping expression and functional patterns both *in vitro* and *in vivo* (Lisy & Peet, 2008). HIF 2 and HIF 3 may cross-

react with HIF 1 target genes, which can lead to either up-regulation of the function of HIF 1, or down-regulation by forming inhibitory PAS domain protein or IPAS. But their role is still to be defined (Wenger, 2002).

1.4.1.3.2 Functional structure

The three HIF α paralogues, HIF 1α, HIF 2α and HIF 3α (including the HIF 3α splice variant IPAS, and common binding partner HIF 1β ARNT), all contain N-terminus/ domain (NAD/ N-TAD) which is a bHLH domain for DNA binding; central region which is PAS domain that facilitates heterodimerization and C-terminus/ domain (CAD/ C-TAD) to recruit transcriptional coagulatory proteins (Zhulin 1997; Ponting 1997; Yang 2005). In addition, all the three HIFα isoforms contain Oxygen-dependent degradation domain (ODDDs). HIF 1α and HIF 2α each contain oxygen regulated CAD. The functional domains of HIF 1 were shown in Figure 2.
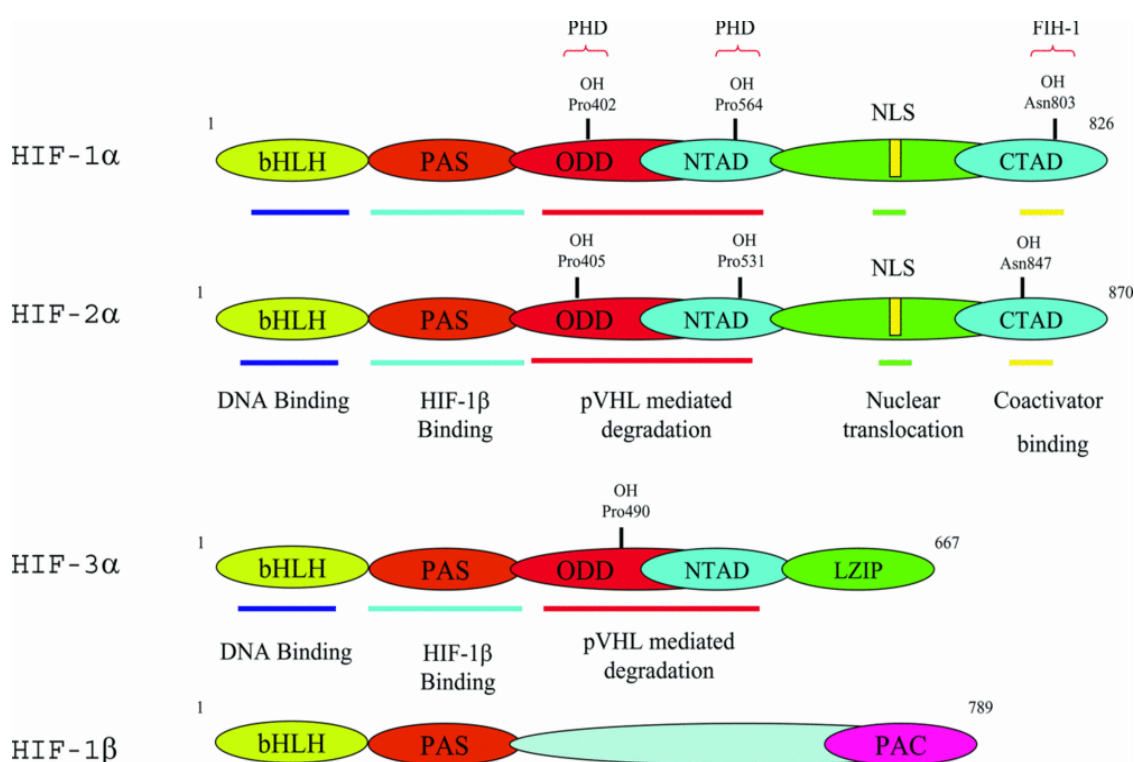
Figure 2. Schematic diagram showing functional domains of HIF family members HIF-1α, HIF-2α, HIF-3α and HIF-1β

(Kenneth & Rocha, 2008. Reproduced by permission)

The modifications of specific residues are highlighted above each protein, and the proteins that perform those modifications are shown. Coloured bars below each

protein delineate particular interaction regions within HIF proteins (CTAD - C-terminal transactivation domain; LZIP - leucine zipper; NLS - nuclear localization signal; NTAD - N-terminal transactivation domain; PAS - Per/ARNT/Sim domain; PAC - PAS-associated C-terminal domain).

1.4.1.3.3 Regulation of HIF 1α protein levels

HIF 1α concentration significantly increases as a response to exposure of the cell to hypoxia and is mostly Oxygen regulated (Matsumuto et al, 2003). In contrast, HIF 1 β is constitutively present in the intracellular content and its expression usually does not change following exposure to hypoxia (Bardos & Ashcroft, 2004).

The intracellular levels of HIF 1α are maintained at low levels with a half life of less than 5 minutes (Bardos & Ashcroft, 2004) by a balance between its continuous production and degradation as shown in Figure 3.
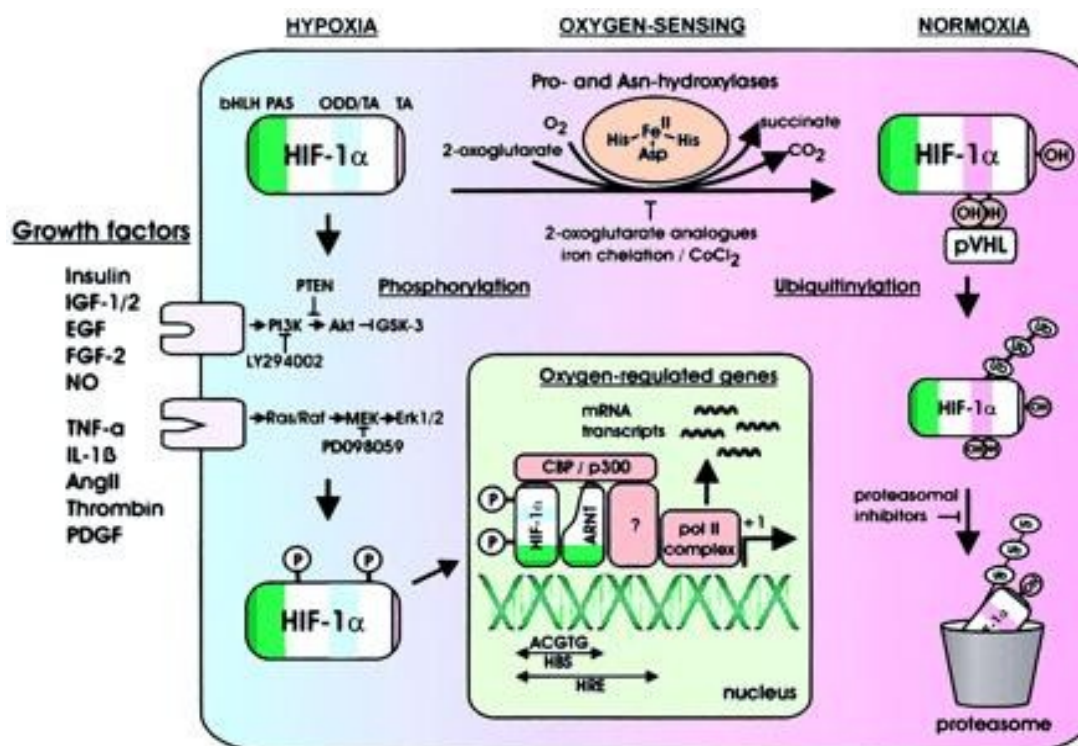
Figure 3. Pathways of HIF 1α regulation (Wenger, 2002. Reproduced by permission)

During normoxia, the two proline residues (Pro 402 and Pro 564) in HIF 1α are hydroxylated, mediated by prolyl- 4- hydroxalase in presence of iron, oxygen and 2 − oxoglutarate (Jakkola et al, 2001). This is shown in Figure 4. These two proline

residues then enable the binding of HIF 1α to the Von Hippel-Lindau tumour suppressor protein (pVHL). The carboxyl-terminal *trans*-activation region of HIF 1α is hydroxylated which modifies the asparagine residue Asn803. This phenomenon regulates activity rather than stability by interfering with the recruitment of the transcriptional coactivator CBP/p300 (Lando et al, 2002).
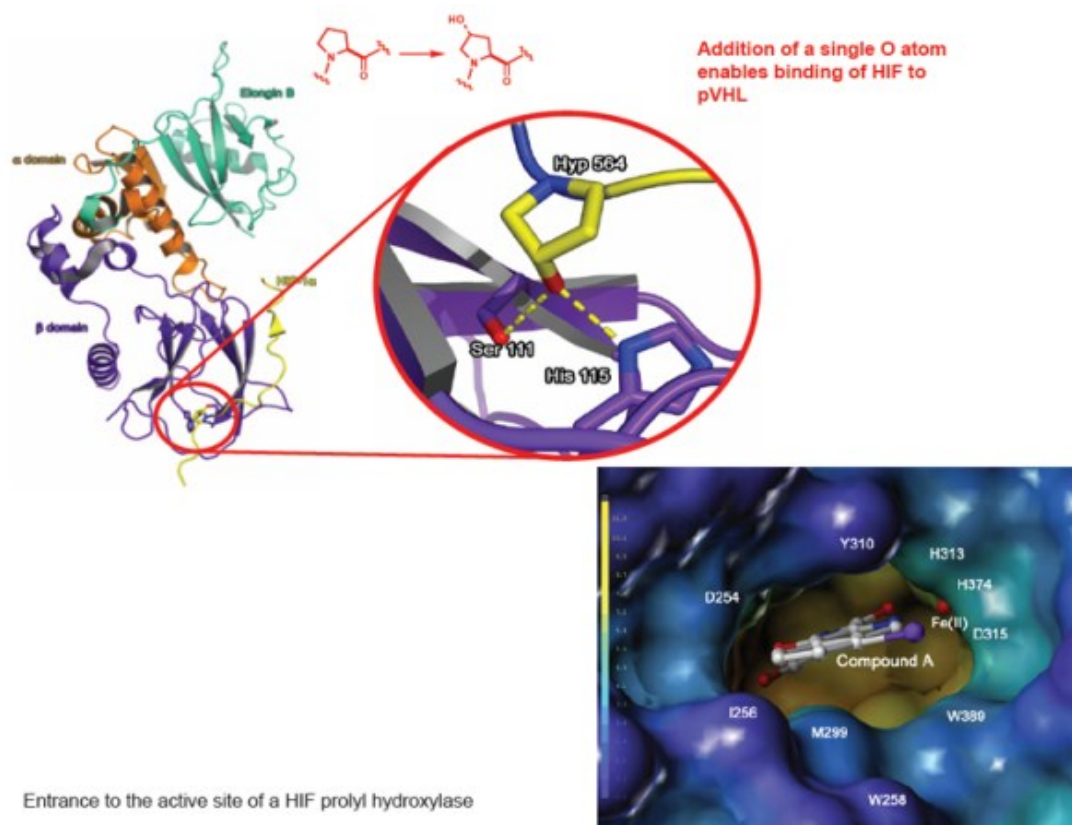
Figure 4. Formation of HIF prolyl hydroxylase (Flashman & Leonarz, Schoffield group, Oxford University; www.chem.ox.ac.uk. Reproduced by permission)

The HIF 1α which is prolyl-hydroxylated is bound to an ubiquitin ligase complex, and is subsequently ubiquitinylated and targeted for degradation (www.chem.ox.ac.uk). This final step is mediated by the ubiquitin–proteasome pathway known as 26S proteasome pathway, resulting in hydrolysis of HIF 1α (Maxwell et al, 2001). This is the main and well known pathway of HIF 1α regulation in normoxia.

During hypoxia, HIF 1α proline residues remain unmodified and hence do not bind to pVHL, thus resulting in increased levels of HIF 1α. The HIF 1α then migrates to the cell nucleus and dimerises with HIF 1β to form the active transcription factor HIF 1, which recognises the hypoxia response element (HRE) present in the enhancers of several target genes (Ryan et al, 1998) and recruits coactivators (Wenger, 2002):

Alternate pathways of HIF 1α regulation were described. Regulation of HIF 1α stability is also mediated by an oxygen-independent pathway. In the cytoplasm, HIF 1α is bound by heat-shock protein 90 (Hsp90) and this association leads to enhanced stability of the subunit. However, upon displacement of Hsp90 by Hsp90 inhibitors, the receptor of activated protein C kinase (RACK1) is able to bind and acts to recruit ubiquitin ligase machinery and potentiate the degradation of the α subunit (Lisy & Peet, 2008).

Wenger (2002) reviewed the mechanisms of stabilization of HIF 1α, other than pVHL mediated pathway. These are mediated through various agents including insulin, insulin like growth factors 1 and 2, epidermal growth factor, fibroblast growth factor2, interleukin 1β, tumour necrosis factor, angiotensin 2, thrombin, transforming growth factor ß$_1$, platelet-derived growth factor and hepatocyte growth factor. Genius and Fandrey (2000) described that nitric oxide initially increases and on prolonged exposure inhibits HIF 1α expression.

It is believed this mode of stabilization of HIF 1α is mediated by via common cellular kinase pathways (Wenger, 2002), but the picture is unclear.

1.4.1.3.4 Activation of HIF 1α

Once the HIF 1α is stabilised, various further steps are involved in the activation of HIF 1α. These include post-translational protein phosphorylation, nuclear translocation, ARNT heterodimerization, DNA binding, recruitment of general and tissue specific transcriptional cofactors and target gene transactivation, as reviewed by Wenger (2002).

1.4.1.3.5 Functional mechanism of HIF 1α

To date, the transcription of more than 70 genes are found to be directly regulated by HIF 1α. These genes are involved in cellular processes that act to directly address the cellular hypoxia by decreasing oxygen dependence and consumption by cells, and by increasing the efficiency of oxygen delivery to cells. These processes include vasculogenesis and angiogenesis, metabolism, vasodilatation, cell migration, signalling and cell fate decisions and were reviewed by Semenza (2010). They are tabulated in Table 2.

Table 2: Selected HIF 1α target genes, whose products contribute to cancer progression (Reprinted by permission from Macmillan Publishers Ltd.: *Oncogene*, Semenza (2010))

Gene Product	*Role in cancer progression*
Angiopoietin 2	Angiogenesis, lymphangiogenesis
Angiopoietin-like 4	Metastasis
Breast cancer resistance protein (ABCG2)	Multidrug transport, stem cell maintenance
Carbonic anhydrase 9 and 12	pH regulation
C-MET	Invasion
CXCR4	Metastasis
DEC1	Genomic instability
Endothelin 1	Invasion
Fibronectin 1	Invasion
Glucose phosphate isomerase	Cell motility, glucose metabolism, immortalization
Glucose transporter 1	Glucose uptake
Hexokinase 1 and 2	Glucose phosphorylation; cell survival
Inhibitor of differentiation 2	Angiogenesis, proliferation
Insulin-like growth factor 2	Cell survival, proliferation
JARID1B	Stem cell maintenance
Kit ligand (stem cell factor)	Angiogenesis, stem cell maintenance
Lactate dehydrogenase A	Glucose metabolism
Lysyl oxidase	Metastasis
Matrix metalloproteinase 2 and 14	Invasion
NT5E (ecto-5′-nucleotidase/CD73)	Immune evasion, multidrug resistance
OCT4	Stem cell maintenance

Gene Product	*Role in cancer progression*
Placental growth factor	Angiogenesis
Platelet-derived growth factor B	Cell proliferation/survival, angiogenesis
Pyruvate dehydrogenase kinase 1	Glucose metabolism
Pyruvate kinase M2	Glucose metabolism
Stromal-derived factor 1	Angiogenesis
Survivin	Cell survival
Telomerase	Immortalization
Transforming growth factor α	Cell proliferation/survival
TWIST1	Epithelial-mesenchymal transition
Urokinase plasminogen activator receptor	Invasion
Vascular endothelial growth factor	Angiogenesis
WSB1	Cell survival
ZEB1 (ZFHX1A), ZEB2 (ZFHX1B)	Epithelial-mesenchymal transition

1.4.1.3.6 Actions of HIF 1α

1.4.1.3.6.1 Physiological role

Many animal experiments demonstrated that HIF 1α is required for mesenchymal cell survival during embryonic development. HIF 1α knockout embryos die around mid-gestation, showing cardiovascular malformations and open neural tube defects (Iyer et al, 1998; Ryan et al, 1998; Kotch et al, 1999). Mice containing only one mutant HIF 1α allele have shown impaired physiological responses to chronic hypoxia (Yu et al, 1999; Shimoda et al, 2001). In partially HIF 1α deficient mice, although the ventilatory response to acute hypoxia was normal, ventilatory adaptation to chronic hypoxia was impaired as evident by the diminished response to subsequent exposure to acute hypoxia (Kline et al, 2002). These results clearly demonstrate the central role of HIF 1α in the adaptation to hypoxia not only at the cellular but at the systemic level.

1.4.1.3.6.2 Role in human cancer

The role of HIF 1α in human cancers is not yet fully understood. But the available evidence supports the hypothesis that this factor acts at different levels in the causation and progression of cancer (Semenza, 2002).

1.4.1.3.6.2.1. Carcinogenesis & cancer progression

In 1998, Ryan and Johnson studied the role of HIF 1α in the transcriptional activities induced by hypoxia. They created a null mutation at the HIF 1α locus in embryonic murine stem cells and discovered that these cells fail to up-regulate many HIF 1α target genes. The teratomas induced in syngeneic or immunocompromised mice by the introduction of these cells were noticed to be only ¼ of the size compared to those caused by wild embryonic stem cells. Mice embryos which were homozygous for null HIF 1α showed failed yolk sac vascularization, defective neural tube closure and apoptosis. Hypoxic regulation of VEGF was also severely affected. They were the first authors to report that the embryonal response to hypoxia was regulated by HIF 1α. Later on, Goda et al (2003) experimented on murine embryonic fibroblasts and splenic B lymphocytes showing that HIF 1α was the determinant of hypoxia induced cell arrest. The levels of HIF 1α correlated with p27 expression, p27 and p21 transcription and Retinoblastoma protein phosphorylation. This experimental evidence paved way for similar studies in human cancer cells.

HIF 1α was found to play a role in the process of carcinogenesis itself. Hypoxia mediated by HIF 1α was shown to mediate angiogenesis, fibrosis and subsequent carcinogenesis in hepatic carcinoma (Rosmorduc & Housset, 2010). These actions are believed to be mediated through the alteration of platelet derived growth factor (PDGF) and VEGF signalling. In 2007, Horree et al studied the role of HIF 1α in gynaecological cancers and found it to be an important factor of carcinogenesis. They noted the expression of HIF 1α and the proteins influenced by it (Glucose transporter-1 protein, Carbonic anhydrase IX and VEGF) in normal endometrium, pre-malignant endometrium and endometrial cancer. Their study revealed higher levels of HIF 1α and the other proteins as the morphology of the tissues progressed from normal to malignancy, with an associated increase in microvessel density (MVD). Similar evidence of involvement of HIF 1α pathway in gastric carcinogenesis (Griffiths et al, 2005) and breast carcinogenesis (Chen et al, 2010) was also discovered. Currently there is no evidence to suggest a similar role for HIF 1α in oesophageal carcinogenesis.

In 1999, Zhong et al provided the first clinical data showing the role of HIF 1α in human cancer progression. Once the tumour is formed, oncogenes and tumour

suppressor genes can modify the HIF 1α regulation and activation, which can be modified by the tumour hypoxic levels. HIF 1α expression and function can be enhanced by the mutations of tumour suppressor genes, which include p53 and pVHL (Maxwell et al, 1999). Birner et al (2001) showed that in patients with epithelial ovarian tumours, HIF 1α over expression alone has no impact on the prognosis but indicates poor dismal prognosis with non-functional p53. Similarly, experimental activation of the P13K or ERK MAPK pathways has shown increased recruitment and activation of HIF 1α pathway (Semenza, 2003). This knowledge may help in the identification of genetic therapy towards modulating HIF 1α role in human cancers. Shi and Fang (2004) also showed HIF 1α as a key factor in regulation of VEGF and VEGFR and other angiogenic factors, which are essential for tumour survival and spread. It was shown that HER-2 immunoactivity and gene amplification, VEGF expression, and Ki-67 expression were correlated strongly with HIF 1α expression in lymph node negative invasive breast carcinoma (Bos et al, 2003).

There is increasing evidence of the role of HIF 1α in tumour formation, as demonstrated by many other experimental studies. Many authors have shown the relation between cancer progression and expression of HIF 1α in tumour cells implanted into experimental animals. These include hepatocellular carcinoma, colorectal tumours, pancreatic, renal cell tumours, gliomas, gastric tumours, prostate, breast and melanomas as summarized by Semenza in 2010.

Telomerase was known to be influencing the cell survival by its action on prolonging the telomeres as well as lengthening them. It is essential for tumour proliferation and progression and this action of it was targeted in identifying new anti-cancer strategies (Newbold, 2002). It was shown very recently that the activation of telomerase and subsequent carcinogenic activity was induced through P13 dependent transcriptional activation involving HIF 1α as a key transcriptional factor (Heeg et al, 2011). This study was done on cell lines of oral-oesophageal cancer and paved way for further studies on agents modifying HIF 1α function for anti-cancer effects.

New evidence is emerging to suggest an important role for HIF 1α in cancer spread. Shirato et al (2011) hypothesised that glycosyl transferases and nucleotide sugar transporters which modulate glycosylation patterns in the hypoxic environment of

solid tumours are induced by activated HIF 1α. The glycosylation is an important part of tumour spread through metastasis and hence could be influenced by the action of HIF 1α. In cancer cells, HIF1α induces overexpression and increased activity of several glycolytic protein isoforms that differ from those found in non-malignant cells, including transporters (GLUT1, GLUT3) and enzymes (HKI, HKII, PFK-L, ALD-A, ALD-C, PGK1, ENO-alpha, PYK-M2, LDH-A, PFKFB-3) (Marin-Hernandez et al, 2009). These HIF 1α induced isoforms make various tumour cells more resistant to physiological inhibitors.

1.4.1.3.6.2.2 Clinical & prognostic role

Tumour hypoxia is a feature of rapidly growing solid tumours as they outgrow their blood supply and the tumour attempts to overcome this by adapting to the hypoxic environment. HIF 1α is a master regulator of this adaptive response and hence is integral in the malignant potential of tumours. Activated HIF 1α has been shown to target genes required for angiogenesis and survival of tumours in hypoxic environments, through of regulation of factors like VEGF and VEGFR (Shi & Fang, 2004). In addition, HIF 1α overexpression in association with deficiency or mutation of tumour suppressor genes such as VHL, p53 and PTEN, and amplification of oncogenes (Akt, Ras, ERK1/2) has been frequently seen in human cancer and these genetic alterations have been associated with tumour growth, invasion and metastasis (Shi & fang, 2004). It has been shown that HER-2 immunoactivity and gene amplification, VEGF expression, and Ki-67 expression are correlated strongly with HIF 1α positivity in lymph node negative breast carcinoma (Bos et al, 2003). Most cancers over-expressing HIF 1α are associated with increased mortality. These include breast, stomach, brain, ovarian, endometrial, cervical, prostate and colon cancers (Semenza, 2002). In head and neck and non-small cell lung cancers, studies have shown conflicting results, probably due to the interaction of HIF 1α with apoptotic genes.

Evidence is accumulating regarding the significance of HIF 1α expression in various solid organ cancers. HIF 1α has shown prognostic value in predicting the response of oropharyngeal tumours to radiotherapy (Aebersold et al, 2001). Over expression of HIF 1α was shown to be marginally associated with poor outcome in non-small cell lung cancer (Giatromanolaki et al, 2001). Hung et al (2009) showed that in resectable

non-small cell lung cancers, the expression of HIF 1α is related to shorter recurrence free and overall survival. They concluded that HIF 1α in association with either 'Snail' or 'TWIST1', which are epithelial-mesenchymal transition (EMT) regulators, was an independent prognostic marker for recurrence free and overall survival. Studies were reported on HIF 1α expression in gastrointestinal tract. HIF 1α expression had positive association with tumour size, TNM stage, cellular proliferation and micro vessel density/ neo-angiogenesis in pancreatic carcinoma (Kitada et al, 2003). Zhang et al showed significantly poorer survival in HIF 1α positive pancreatic ductal adenocarcimas, but did not report on its role as independent prognostic factor. Over expression of HIF 1α is correlated with poor prognosis in gastrointestinal tumour (GIST) of stomach (Takahashi et al, 2003). Griffiths et al (2007) studied HIF 1α expression in gastric and gastro-oesophageal adenocarcinomas and reported that that expression at invasive edge of the tumour was associated with cancer specific survival, lymph node metastases and advanced TNM stage. However, HIF 1α did not show any independent prognostic value in their study. HIF 1α expression had positive association with tumour size, TNM stage, cellular proliferation, micro vessel density/ neo-angiogenesis and prognosis in colorectal carcinoma (Kuwai et al, 2003).

HIF 1α can serve as a prognostic marker for an unfavourable outcome in breast cancer patients with T1/T2 tumours and positive axillary lymph nodes (Gruber et al, 2004). Bos et al's study (2003) could not show similar results in patients with positive axillary lymph nodes: survival only in node negative invasive breast cancer patients was independently related to the expression of HIF 1α in their study. Generali et al (2006) showed that overall response to therapy and disease free survival progressively decreased with increasing tumour HIF 1α and HIF 1α was an independent predictor of response. Local recurrence, metastastic recurrence and disease free survival after radiation therapy for patients with Stage IIIB squamous cell carcinoma of the cervix is predictable with the degree of expression of HIF 1α, with high degree of expression demonstrating aggressive tumour (Birner et al, 2001; Burri et al, 2003; Ishikawa et al, 2004). HIF 1α protein expression correlated with grade, growth pattern, p53 oncoprotein expression, and PCNA index in transitional cell carcinoma of the upper renal tract (Nakanishi et al, 2005). Furthermore, a

significant correlation was found between HIF 1α protein expression and both overall and disease-free survival rates in this study.

The results in certain cancers are equivocal. Ma et al (2007) showed that HIF 1α correlated with MVD and VEGF expression in gastric cancers, which can be used as prognostic indicators; Griffiths et al (2005) study has shown improved survival in distal gastric cancers with high HIF 1α expression. Two studies do not appear to confirm the significant association between HIF 1α over expression and tumour aggressiveness or unfavourable prognosis in colorectal cancers (Furlan et al, 2008; Kuwai et al, 2003). These results may be due to the ability of HIF 1α to induce apoptosis and decrease cellular proliferation in certain morphological types of cancers (Griffiths et al, 2005).

1.4.1.3.6.2.3 HIF 1α in oesophageal cancer

The role of HIF 1α expression in oesophageal cancer was reported first by Koukourakis et al (2001). They studied the role of HIF 1α on the response of early oesophageal tumours in 37 patients treated with photodynamic therapy (PDT). PDT needs a pre-requisite of cellular normoxia for effective results and their study showed that the tumours with higher expression of HIF 1α can be predicted to show less response to PDT. But this expression did not translate into effect on survival. Their group brought the relation of HIF 1α and oesophageal cancer into limelight, but did not report any bench mark for marking the expression of this factor and this makes future attempts to repeat their results difficult. They significantly did not mention the type of oesophageal cancer (SCC or ACC) in their patient group and also on the relation between HIF 1α and other clinicopathological characteristics.

The potential role of this new molecular factor in the management of oesophageal cancer caused interest in the Far East, where the incidence of oesophageal cancer is much higher compared to Western population. Kurokawa et al (2003) showed that high HIF 1α expression in 130 oesophageal cancer specimens was associated with tumour invasion, lymph node metastasis, distant metastasis, pTNM stage, lymphatic invasion and worse survival. HIF 1α however did not show independent prognostic value in predicting survival. Their group reported that in their patients where in the specimens' margins tested positive for tumour the HIF 1α expression in the tissues

was higher. But this is debatable, as positive margins reflect surgical technique rather than molecular expressions.

Sohda et al (2004) first reported a study to identify the role of HIF 1α in predicting response to chemoradiotherapy (CRT). They examined 65 biopsy specimens endoscopically obtained before administering chemoradiation. Their results failed to show any significant relation between HIF 1α expression and the depth of tumour invasion, degree of differentiation, TNM staging and 5 year survival. But there was complete response to chemoradiotherapy in tumours with low HIF 1α expression (13/27) vs. high expression (3/38) and this effect was significantly predictable. They concluded that as CRT requires tissue normoxia for optimum results, the HIF 1α can act as a surrogate marker to measure levels of tissue oxygenation in oeosphageal tumours. They failed to report whether the cancers were SCC or ACC.

Kimura et al (2004) was the first to explore the relation between HIF 1α and VEGF expressions both *in vitro* and *in vivo* of SCC of oesophagus. They studied the levels of these two factors in oesophageal cancer cell lines *in vitro* and discovered that their levels rise in proportion with degree of hypoxia created. In 82 resected specimens, HIF 1α expression correlated with venous invasion, VEGF expression and microvessel density. The survival of the patients with high HIF 1α was significantly lower than those with low expression, but not as an independent indicator of survival. They further published their results on the relation between HIF 1α and p53 (2005). They reported that p53 showed over-expression in early cancers implicating its role in carcinogenesis, but did not show any association with HIF 1α, VEGF expression, angiogenesis or apoptosis. They concluded that factors other than p53 could influence HIF 1α activity. Matsuyama et al (2005) detected similar positive relation between expression of HIF 1α and VEGF in their series of 215 SCC patients who underwent resection, but they too could not show any significant relation between HIF 1α and p53 levels in their specimens. They reported poorer disease free survival on univariate analysis in patients with high expression of HIF 1α, but no independent prognostic potential for HIF 1α and no effect at all on disease free survival. Katsuta et al (2005) showed that high HIF 1α expression was associated with lymphatic invasion, lymph node metastasis and VEGF expression in their study on 5 oesophageal SCC cell lines and 48 operated specimens of oesophageal SCC. They

believed this was the result of induction of the lymphangiogenic effect of VEGF by HIF 1α but they did not study the relationship of HIF 1α on patient survival. Tzao et al (2008) studied HIF 1α and VEGF expression in 85 resected SCC specimens and reported a concordance of 69.5% between these factors, suggesting at their combined role in SCC progression. More significantly this was one of the very few studies where independent prognostic value in predicting survival with both HIF 1α and VEGF was reported. The only other study which confirmed the role of HIF 1α in survival of oesophageal cancer was published recently by Ogane et al (2010). They showed that HIF 1α could be employed as an independent prognostic marker, with HIF 1α expression reflecting the lymph node involvement, disease free and overall survival rates in 96 patients who underwent surgical curative resections in pT1 tumours.

Attention has also focussed on the genetic control of HIF 1α and relationship of genes to tumour biological behaviour. Matsuyama et al (2005) showed that the level of expression of HIF 1α mRNA correlated with HIF 1α protein expression, but with no further relation to any clinicopathological factors or survival of the patients. Yu et al (2008) showed HIF 1α mRNA expression was higher in SCC cells than normal oesophageal cells, and this was significantly associated with lymph node metastasis and lymphatic invasion. Similar results were obtained between HIF 1α expression and lymph node metastasis & histological grading. Again, survival data was lacking in their results. Liu et al (2007) studied the HIF 1α mRNA and HIF 1α levels in normal (23), dysplastic (26) and tumour tissues (70) of SCC. The positive expression and correlation of both mRNA and HIF 1α increased in carcinoma tissues. While they both showed association with tumour depth, metastasis and invasion there was no survival data.

Polymorphisms of the HIF 1α gene were detected in carcinomas of the kidney, breast, head & neck, prostate and colorectum. The transcriptional activity of the polymorphic gene was found to be higher in hypoxia of prostate cancer (Chau et al, 2005). Ling et al (2005) tested the HIF 1α gene polymorphism to identify any effect this may have on various clinicopathological features of SCC of oesophagus. They identified C/T mutations of C1772T gene on a similar level in both test (95) vs. control specimens (104). But, C/T expression group compared to others was

associated with larger tumours and higher incidence of metastatic lymph nodes. They did not identify the relation between the polymorphism and HIF 1α protein expression in their study and information on survival was lacking from their results. It still remains to be seen if any vital information on the genetic make up HIF 1α could be available through further studies, which can have a prognostic potential.

With the emerging significance of HIF 1α in oesophageal cancer, studies were done to identify its role in predicting response to therapy. Wu et al (2007) reported on the strength of expression of HIF 1α and survival in patients undergoing chemotherapy in SCC. Their study on 48 patients showed that the 2 year survival rate in the group with high HIF 1α expression was significantly lower, but they did not further evaluate its role as an independent prognostic factor. Hence their results are inconclusive.

Chen et al (2009) studied the relation between HIF 1α expression and Beclin-1, a known mediator of autophagy in 54 SCC specimens. There were significantly higher survival rates in patients with high Beclin-1 and high Beclin-1/ low HIF 1α expression tumours. While the HIF 1α expression was positively correlated with differentiation, invasion and clinical stage, it per se did not show significant survival advantage.

Most of the above studies were done on far Eastern populations, reflecting the higher occurrence of oesophageal cancer and subsequent interest in this molecule in those regions. Limited information is available on the prognostic role of HIF 1α in oesophageal carcinoma of western population. Ling et al (2006) published results of their study conducted on 53 patients in Germany who underwent neoadjuvant chemoradiation followed by curative resection. They showed that HIF 1α mRNA or HIF 1α protein expression did not correlate with histomorphological regression or survival following treatment, in contrast with many other previously reported studies. Griffiths et al (2007a) published their results on the role of HIF 1α expression on a combined series of gastro-oesophageal and gastric adenocarcinoma patients, which as discussed earlier have different aetiopathological, morphological and biological behaviour from the SCC.

It is evident that there were no uniform results from the studies reported so far regarding the significance of expression of HIF 1α in oesophageal cancer, particularly western population. This lacuna in existing scientific evidence prompted our current study.

1.4.1.3.6.3 Detection of HIF 1α

Immunohistochemistry is an ideal and most commonly employed analytical assay for the detection of prognostic markers. The other methods are Western blot technique and Enzyme Linked Immunosorbent Assay test (ELISA).

Immunohistochemistry technique uses an antigen-antibody reaction as the basis for the identification or quantification of the factor being studied, originally described by Coons et al in 1941. In the initial stages of development of immunohistochemistry, Coons introduced a fluorescent molecule conjugated to an antibody to identify the antigen – called 'direct immunoassay'. In indirect immunoassay which he developed as a more effective method, the antibody which identifies the antigen is called as primary antibody. This antibody is used as an antigen in a different species to produce the secondary antibody. The secondary antibody labels the primary antibody when introduced into the test and aids in enhancing the signal amplification through conjugation. Immunohistochemistry is an established method to identify and quantify many molecules in laboratory medicine.

Monoclonal antibodies were discovered by Köhler & Milstein in 1975. They are produced *in-vivo* as a result of the antibody response induced against a particular 'clone' of antigen producing cells. These reagents are preferably used in various antigen-antibody reactions because of the advantages like greatly reduced background staining, minimal contamination with other non-specific antibodies and consistency between various batches for uniform results. Another feature of the monoclonal antibodies is their ability to be used as secondary antibodies for advanced Immunohistochemical investigations like indirect assays (Ramos-Vara, 2005).

The following are the various methods employed to highlight the antigen-antibody reactions during the immunohistochemistry assay for the identification of HIF 1α:

Affinity labelling methods: The extremely high affinity of certain non-immunological substrates is utilised to provide links between the antigen and detection agents through secondary antibody. Examples include Avidin-Biotin complex and Streptavidin-Biotin complex. Streptavidin-Biotin complex contributes to lower background staining (O'Leary & Timothy, 2003).

Amplification systems: Substrates are added to the assay that generate more targets and hence increase the sensitivity of the assay. These substrates utilise all the binding sites of the substrates to amplify the signal.

Detection systems: Various enzymatic and non-enzymatic systems are in use to detect the presence of the reporter molecule which identifies the antigen being tested (Nakane & Pierce, 1967).

The Catalyzed Signal Amplification (CSA) System is an extremely sensitive immunocytochemical visualisation system based on patented technology (NEN Life Science Products, Inc., U.S.). The procedure uses a peroxidase-catalysed deposition of biotinyl-tyramide, which in turn is reacted with peroxidase-conjugated Streptavidin resulting in a greatly amplified signal (See Figure 5).

Figure 5: Catalysed Signal Amplification (CSA) System

Step 1
Application of
primary antibody.
Incubate 15 min

Step 2
Application of
F(ab)$_2$ biotinylated
secondary antibody.
Incubate 15 min

Step 3
Application of
avidin-biotinylated
peroxidase-complex.
Incubate 15 min

Step 4
Application of
biotinyl tyramide.
Incubate 15 min

Step 5
Application of peroxidase
conjugate streptavidin.
Incubate 15 min

Legend for Schematics

Primary
Antibody

Secondary
Antibody

Tissue
Antigen

HRP
Enzyme

Biotin
Label

Streptavidin

Biotinyl
Tyramide

(Reproduced with permission from www.dako.co.uk)

Western blot method and Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) were also used to detect HIF 1α protein and mRNA presence subsequently (Wu et al, 2010). More recently molecular probes that detect HIF 1α activity were described. One such agent was recombinant protein PTD-ODD-Halo tag (POH) that shares the Oxygen dependent regulation pathway as HIF 1α. The POH could be near-infrared fluorescently labelled and employed to monitor the HIF 1α active regions of solid organ cancers in mice (Kuchimaru et al, 2010). This probe showed specific affinity to HIF 1α active cells on further Immunohistiochemical analysis and so could potentially be used to image hypoxia. Immunohistochemistry was the proven method of detecting the presence and strength of expression of HIF 1α. This technique was used in various previously reported studies (Gruber et al 2004, Ishikawa et al 2004, Kurokawa et al 2003, Schindl et al 2002; Giatromanolaki et al 2001; Koukourakis M et al 2001).

CSA technique was used for detecting the expression of HIF 1α in present study.

1.5 Oesophagus

In this section I discussed the aspects of oeosphageal cancer relevant to the understanding of the present study. The applied aspects of oesophageal anatomy and lymphatic drainage are described, followed by aetiology, clinical features and management of the cancer.

1.5.1 Applied anatomy

The oesophagus is the continuation of the pharynx for the purpose of carrying food into the stomach. It is a muscular tube approximately 26 cm long and 2 cm in diameter (Moore et al, 2006). It begins at the level of the C6 vertebra in neck, descends anterior to the vertebral column through the mediastinum following a generally vertical course, passes through the diaphragm at the level of the T10 vertebra and ends at the cardiac orifice of the stomach (GOJ) at the level of the T11 vertebra.

1.5.1.1 Anatomical divisions

The oesophagus is broadly divided into cervical, thoracic and abdominal parts. The cervical oesophagus measuring nearly 3 – 4 cm extends from the lower border of the cricoid cartilage to the thoracic inlet, and ends at approximately 18cm from upper incisors. The thoracic part extends from the thoracic inlet to the diaphragmatic hiatus, measuring approximately 20cm. The abdominal part extends to only 2 - 3 cm below the diaphragm (Alderson, 2000).

1.5.1.2 Blood supply

The oesophagus has a rich blood supply through various arteries, which anastomose with each other (Hermann & Murugasu, 1966). The cervical oesophagus is supplied by the inferior thyroid artery and the thoracic part by the bronchial & oesophageal arteries. The abdominal oesophagus is supplied by the ascending branches of left phrenic & left gastric arteries.

The venous return from the oesophagus drains into a peri-oesophageal venous plexus. The oesophageal veins from this plexus drain into veins corresponding to the arterial supply.

1.5.1.3 Lymphatic supply

The oesophagus has an extensive submucosal lymphatic system that plays a major role in the spread of oesophageal carcinoma. The anatomy of the various lymph nodes is particularly essential for their removal 'en block' during 'three fields of lymphadenectomy' as advocated by Akiyama (1994). Sobin et al (2002) described oesophageal nodes as cervical, intrathoracic and abdominal groups. These involvement of these groups of lymph nodes with oesophageal cancer alters the staging and subsequent management of the disease.

1.5.1.4 Histology

According to standard teaching texts (Kumar et al, 2005), the histology of the oesophagus reflects the general structure of gastrointestinal tract except for absence of an outer serosa. A cross section of the oesophagus shows mucosa, submucosa, muscularis propria and adventitia.

Mucosa is the innermost layer and is formed by (from inside out) non-keratinising stratified squamous epithelium, a non-epithelial layer known as the lamina propria and a delicate layer of muscularis mucosa. The submucosa consists of loose connective tissue including blood vessels, lymphatics, nerve fibres and submucosal glands. The muscularis propria is formed from inner circular and outer longitudinal coat of smooth muscle with intervening Auerbach's myenteric plexus.

1.5.2 Pathology

The spectrum of oesophageal disorders is very wide and a review of all the diseases is outside this thesis. I discussed only the appropriate conditions and diseases which have a role in the aetiology of oesophageal cancer.

1.5.2.1 Benign conditions

The benign diseases of the oesophagus can be classified as congenital, inflammatory, physiological/ motor and neoplastic. Some of these benign conditions are pre-malignant and hence these conditions are discussed further here.

1.5.2.1.1 Congenital

Congenital cysts - These may be classified into inclusion, retention or developmental cysts. Carcinoma arising in these cysts was reported (Olsen et al, 1991).

Diverticula - Outpouchings of the oesophageal wall, usually as a result of intra-oesophageal pressure. The complications include squamous cell type malignancy (Wychulis et al, 1969).

Mucosal webs and rings - Upper oesophageal (especially post cricoid) web in association with iron deficiency anaemia and cheilosis is known as Paterson-Brown-Kelly or Plummer-Vinson syndrome (Paterson, 1919; Kelly, 1919). This condition is a risk factor for postcricoid oesophageal cancer.

1.5.2.1.2 Inflammatory (Oesophagitis)

Reflux oesophagitis – The exposure of the lower oesophageal mucosa to the acidic contents of the stomach causes inflammation of the mucosa and subsequent chronic changes. The causes for this reflux include decreased efficacy of oesophageal antireflux mechanism, hiatus hernia, decreased gastric emptying, and decreased reparative capacity of oesophageal mucosa or a combination of factors. The clinical manifestations are mainly dysphagia and heartburn. Reflux oesophagitis can potentially develop in to Barrett's oesophagus (discussed below under Cancer: aetiopathogenesis section) which is a risk factor for oesophageal cancer (Shaheen & Ransohoff, 2002).

Infective oesophagitis: Human Papilloma Virus (Strains 16 and 18) induced oesophagitis is believed to play a role in the carcinogenesis (Far et al, 2007) but some studies have disputed this causation (Tornesello et al, 2009).

Chemical oesophagitis – This condition is caused by the ingestion of mucosal irritants like alcohol, corrosive acids & alkalis, hot fluids and smoking. The strictures caused by lye ingestion can lead to carcinoma in later life (Appelqvist et al 1980).

1.5.2.1.3 Physiological/ Motor

Achalasia cardia is a functional disorder of swallowing mechanism due to the incomplete relaxation of the lower oesophageal sphincter. This condition can increase the risk of oesophageal cancer by 33 times compared to general population (Streitz et al, 1995). Zendehdel (2011) recently showed that the incidence of both SCC and ACC is substantially higher in male patients with achalasia cardia, but in women this is not confirmed.

1.5.2.1.4 Neoplastic

Benign neoplasms of the oesophagus can be classified into gastrointestinal tumours (GISTs), true smooth muscle tumours and true Schwann cell tumours (Joensuu & Kindblom, 2004). These tumours have varying malignant potential and rarely can cause aggressive tumours in oesophagus.

The common malignant neoplasms (cancer) of the oesophagus are discussed in detail below.

1.5.2.2 Cancer

The cancers of oesophagus are predominantly epithelial in origin (Bennett, 2009). The uncommon types of cancer accounting for <0.1% cases include granular cell tumours, basaloid carcinoma, mucoepidermoid carcinoma and endocrine tumours.

The epithelial cancers are squamous cell carcinoma and adenocarcinoma with different spectra of aetiology and morphology but somewhat similar clinical features.

1.5.2.2.1 Aetiopathogenesis

The aeatiopathogeneis of the SCC and ACC shows distinct differences.

1.5.2.2.1.1 Squamous Cell Carcinoma

Dietary and environmental factors play a major role with a contribution from genetic predisposition in the aetiology of SCC.

Dietary factors are implicated for this cancer in high-incidence regions like China and South Africa. They include deficiency of vitamins and trace elements, fungal contamination of food and pickled food with high levels of nitrites and nitrosamines (Wang et al, 2006). These findings were confirmed on a recent study on Western population also. Anantharaman et al (2011) performed Principle Component Analysis of dietary and life style factors on incidence of oesophageal cancer. They showed meat and nitrate intake was associated with increased risk of both SCC and ACC, while alcohol and smoking were associated with SCC. The life style related factors like alcohol and tobacco usage are implicated in oesophageal cancer aetiology across the world (Morita et al, 2010). These two factors in association can cause a 20 times increased incidence of SCC (Lee et al, 2005).

Genetic factors playing a significant role in the aetiology of SCC were recently reported. Mutation of the p53 gene which is a tumour suppressor, disruption of cell-cycle control in G1phase, activation of oncogenes like EGFR and inactivation of tumour suppressor genes have been shown as contributing to development of SCC of oesophagus (Mandard et al, 2000). ADH2*1*2, CYP1A1 Val allele and the p53 codon 72 Pro/Pro genotypes are shown to increase the risk of SCC of oesophagus (Hiyama et al, 2007). Common alleles of genes including MTHFR C677T and A1298C polymorphisms, the XRCC1 Arg194Trp polymorphisms, the hOGG1 Ser326Cys polymorphism, and the p53 Arg72Pro polymorphism were shown to be moderate risk factors of SCC among Chinese population (Xing et al, 2003).

1.5.2.2.1.2 Adenocarcinoma

The majority of cases of ACC arise from Barrett's mucosa. Barrett's mucosa occurs as a result of chronic exposure of the lower part of oesophagus to gastric juices, usually as a result of GORD: up to 5 – 15% patients with GORD show Barrett's mucosa (Shaheen & Richter, 2009). In this condition, the distal oesophageal squamous mucosa is replaced by metaplastic columnar epithelium. These metaplastic cells have high proliferative activity and in nearly 10% of cases turn severely dysplastic or cancerous. The Barrett's metaplasia increases the risk of oesophageal adenocarcinoma by 30 – 125 fold compared to the general population (Wild & Hardie, 2003). Anantharaman et al (2011) confirmed this association on principle component analysis of dietary and environmental factors in ACC. Recent epidemiological review by Kubo et al (2010) showed that that dietary lack of vitamins A & C, fibre, fruits and raw vegetables was inversely associated with the incidence of Barrett's oesophagus and ACC.

Apart from GORD and Barrett's oesophagus, some isolated genes are known to cause increased risk of adenocarcinoma. DNA repair genes like xeroderma pigmentosum group D in the nucleotide excision repair pathway and X-ray repair cross-complementing gene 1 in the base excision repair pathway can in association with each other enhance the risk (Liu G et al, 2007). ALDH2*1*2 and the CYP1A1 Val allele (Hiyama et al, 2007) are shown to increase the risk of adenocarcinoma of oesophagus. Cyclooxygenase-2 gene polymorphisms contribute to the carcinogenesis through inflammation and Barrett's oesophagus (Ferguson et al, 2008). Matrix metalloproteinase (MMP) polymorphisms (Bradbury et al, 2009) and MicroRNA

related oncogene mutations (Ye et al, 2008) play a role in the process of cancer aetiology. Genetic variants of Caspase-7 and Caspase-9 in the apoptosis pathway may also be associated with adenocarcinoma (Liu et al, 2010).

1.5.2.2.2 Morphology

1.5.2.2.2.1 Squamous cell carcinoma

This carcinoma occurs generally in the upper two thirds of the oesophagus. It starts as *in situ* lesion which is confined to the mucosa without evidence of spread, and is also known as intraepithelial neoplasm. Teaching texts (Kumar et al, 2005) mention four common morphological patterns as the tumour grows in the order of frequency: Polyploid, exophytic, ulcerated and diffuse infiltrative types.

1.5.2.2.2.2 Adenocarcinoma

This type occurs most frequently at lower end of oesophagus (GOJ). The following classification of these tumours is generally followed:

Type I   Arises from Barrett's oesophagus and infiltrates GOJ from above

Type II   True carcinoma of the cardia

Type III   Subcardial gastric carcinoma, which infiltrates GOJ from below

(Siewert & Stein, 1998)

Multiple foci may appear in an area of 'field change' and hence multisite biopsies are essential during Barrett's surveillance programmes (www.bsg.org.uk).

1.5.2.2.3 Clinical Presentation

The symptoms of oesophageal cancer are predominantly related to the narrowing of the oesophagus by the tumour mass (Devitt et al, 1988). These symptoms are dysphagia (difficulty in swallowing, initially for solids followed by liquids), odynophagia (painful swallowing), weight loss & debilitation (due to dysphagia & general manifestation of cancer) and food bolus obstruction. Ulceration of the tumour may cause haemorrhage, aspiration of food via trachea-oesophageal fistula and sepsis.

Atypical symptoms of oesophageal cancer (Levernment & Milne, 1974) include hoarseness due to recurrent laryngeal nerve infiltration by the tumour and palpable lymphadenopathy in the neck due to metastatic spread. Concurrent symptoms of

GORD and sliding hernia e.g. heartburn, regurgitation and epigastralgia may mask the cancer symptoms to some extent, causing delay in diagnosis.

1.5.3 Management of oesophageal cancer

The management of oesophageal cancer is evidence based and multidisciplinary. Some of the investigations and treatment pathways are debated and I discussed our local protocol in this chapter.

1.5.3.1 Clinical investigations

Various modes of investigation are available for the diagnosis of oesophageal cancer. Each mode has an important role in the diagnostic pathway and many of them also significantly contribute to the staging of the disease, as shown below:

Haematology, Biochemistry: Provides information on the general health and nutrition of the patient

Endoscopy +/- biopsy (Oesophago-gastro-duodenoscopy, Bronchoscopy): Generally the first investigation to identify and confirmation of disease, to assess the extent of extent of disease

Peritoneal cytology: Helps in identifying the peritoneal spread of disease (Bryan et al, 2001)

Chest radiograph (CXR): Checks for pulmonary metastases and malignant pleural effusion

Contrast radiology: Provides information on the extent and passability of strictures and trachea-oesophageal fistula (Deans & Patterson Brown, 2009)

Ultrasonography +/- Fine needle aspiration (FNA): Provides imaging of the cervical nodal (Bressani et al, 1998) and abdominal visceral metastases; Staging

Endoscopic Ultrasonography (EUS) +/- FNA: Helps in identifying the extent of involvement of the oesophageal wall and regional lymph nodes; staging. Kelly et al (2001) have shown that EUS +/- FNA is currently the most accurate modality for the 'T' staging of oesophageal tumours.

Laparoscopy +/- Ultrasonography: Shows involvement of abdominal viscera and peritoneum; Staging (Rau and Hunerbein, 2005)

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI): Provide imaging for local, regional and distant spread of disease; Staging (Onbas et al, 2006)

Positron Emission Tomography (PET) +/- CT: Helps in identification of distant metastases: Staging (Van Vliet et al, 2008)

Bone scanning: Helps in identification of skeletal metastases; Staging

1.5.3.2 Staging of the disease

Staging of oesophageal cancer prior to treatment is essential for various reasons. As all the major modalities of treatment have significant morbidity and risk of mortality, staging of the disease helps in choosing the most appropriate form of therapy for each individual case. The results of various treatment methods (including clinical trials) and results between treatment centres can also be compared using the staging system. Final histopathological staging aids in planning adjuvant treatment. Staging is one of the most accurate prognostic indicators available, as discussed earlier.

The various investigative modalities described earlier contribute to the staging of the oesophageal cancer as per our local protocol shown below (Figure 6).

Figure 6. Preoperative staging protocol at our tertiary oesophago-gastric centre (James Cook University Hospital, Middlesbrough) for oesophageal cancer

History, clinical examination

↓

Endoscopy & Biopsy

↓

Diagnosis

↓

CT Thorax & Abdomen   Palliation

↓

No metastases   Metastases

↓

EUS (+/- FNA) & PET-CT

↓

Resectable   Unresectable

↓

Curative resection (+/- neo-adjuvant treatment) offered if fit for surgery
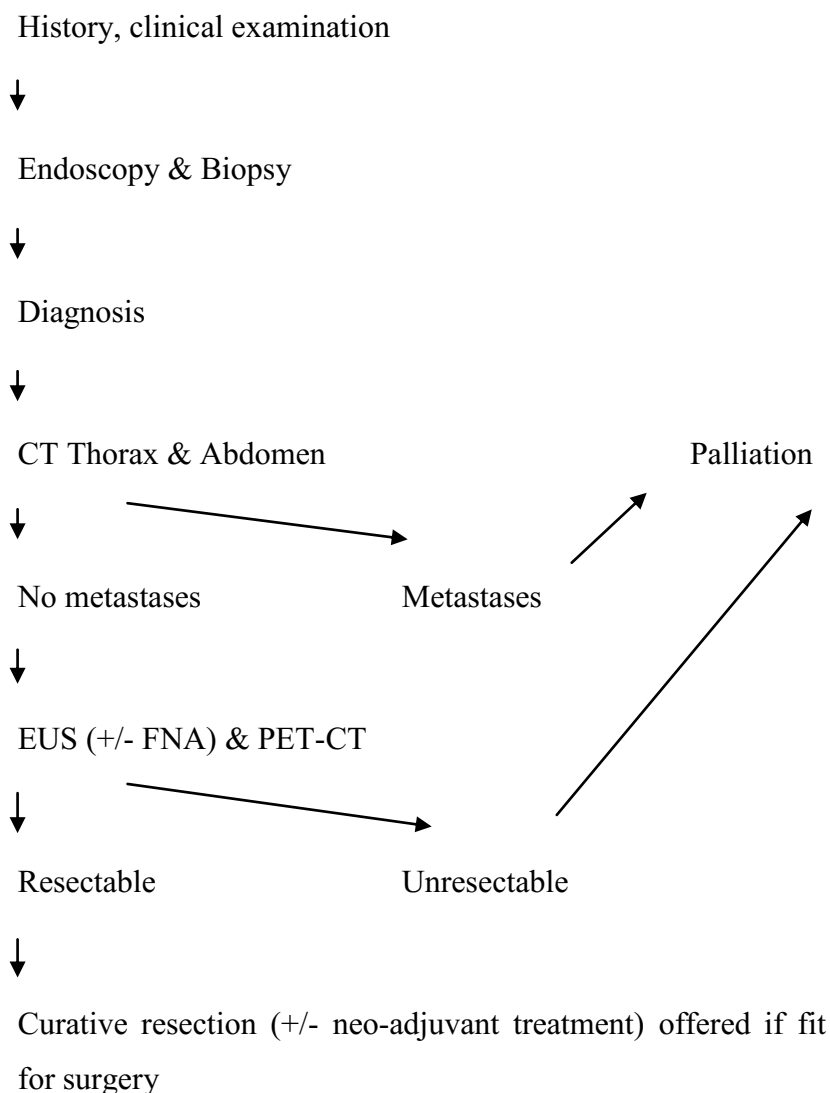
The clinico-pathological staging is the final summative assessment of pre-operative investigations, intra-operative macroscopic findings and postoperative microscopic analysis of the resected tissue specimen (Ferguson and Paterson-Brown, 2001). It forms the base on which treatment decisions are made. The 'T, N, M' staging (UICC) is currently the universally accepted standard system for staging of many visceral cancers, including oesophageal cancer.

Tumour **(T)** staging is assessed by endoscopy (Oesophago-gastro-duodenoscopy/ Bronchoscopy), imaging (CT, EUS) and final histology results from the resected specimen. Node **(N)** staging is assessed by various investigative modalities like imaging (CT, EUS and laparoscopic ultrasound) and/ or the histology from fine needle aspiration (or biopsy) samples. The histology results of the resected lymph

nodes following diagnostic laparoscopy or TSO provide a wealth of information, which could change the treatment decisions regarding adjuvant treatment. Metastases **(M)** status is also assessed based on imaging (CT, EUS and PET-CT) and in some instances histology obtained through EUS-FNA.

Knowledge about the TNM staging is essential to understand the present study. Hence the UICC classification currently used is reproduced here:

TNM classification

T – Primary tumour

TX   Primary tumour cannot be assessed

T0   No evidence of primary tumour

Tis   Carcinoma in situ

T1   Tumour invades lamina propria or submucosa

T2   Tumour invades muscularis propria

T3   Tumour invades adventitia

T4   Tumour invades adjacent structures

N – Regional Lymph Nodes

NX   Regional lymphnodes cannot be assessed

N0   No Regional lymph node metastasis

N1   Regional lymph node metastasis

M – Distant metastasis

MX   Distant metastasis cannot be assessed

M0   No distant metastasis

M1   Distant metastasis

For tumours of the upper thoracic oesophagus

M1a   Metastasis in cervical lymph nodes

M1b   Other distant metastasis

For tumours of the mid-thoracic oesophagus

M1a   Not applicable

M1b   Non-regional lymph node or other distant metastasis

For tumours of the lower thoracic oesophagus

M1a   Metastasis in coeliac lymph nodes

M1b   Other distant metastasis

pTNM pathological classification

The pT, pN and pM categories correspond to the T, N, and M categories.

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T2, T3	N0	M0
Stage IIB	T1, T2	N1	M0
Stage III	T3	N1	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

Table: Internationally unified TNM staging grouping of oesophageal cancer

(*UICC: TNM Classification of Malignant tumours, 2002*)

1.5.3.3 Treatment Pathways

The treatment for oesophageal cancer is increasingly being multi-disciplinary and involves the expertise offered by Surgeon, Anaesthetist, Oncologist, Radiologist, Pathologist, Dietician, Physiotherapist and specialist nurse team. This is an evidence based practice (Stephens et al, 2006) which is followed in all the cancer treatment units of UK. In the MDT meeting, the treatment for each patient is considered on an individual basis taking into account the patient's disease stage, fitness, comorbidity, nutritional status and response to treatment. MDT has been shown to significantly improve the accuracy of staging for oesophageal cancer and enhance the correctness of management decisions (Davies et al, 2006).

Major advances have been seen in the management of oesophageal cancer in the last few decades. The early diagnosis of the disease through education of the general population and surveillance of the Barrett's mucosa patients enabled better results though early institution of treatment. Urgent referral of suspected cancer patient to specialist centres as per the National Guidelines (www.nice.org.uk) reduced the delay before the diagnosis is made. Incorporation of new imaging methods like EUS, EUS FNA and PET-CT scan has increased the accuracy of the staging process. Improved peri-operative care with optimisation of patient's pre-operative general and nutritional status, thrombo-embolic prophylaxis and newer methods of analgesia have resulted in improved outcome from major surgical procedures like TSO. Minimally invasive operative techniques like thoracoscopic and laparoscopic assisted resections have again improved the patient recovery times and analgesic requirements. Availability of less toxic chemotherapy agents and more accurate radiotherapy regimens has reduced the side-effects from these modalities.

The current treatment pathways for oesophageal cancer are summarised as below (Figure 7):

Figure 7. Current treatment pathways at our tertiary oesophago-gastric centre for oesophageal cancer

Diagnosis

Staging

Early/ locally advanced disease   Metastatic disease

Fitness assessment for surgery   Palliation

Fit   Unfit

Surgery   Non-surgical modality
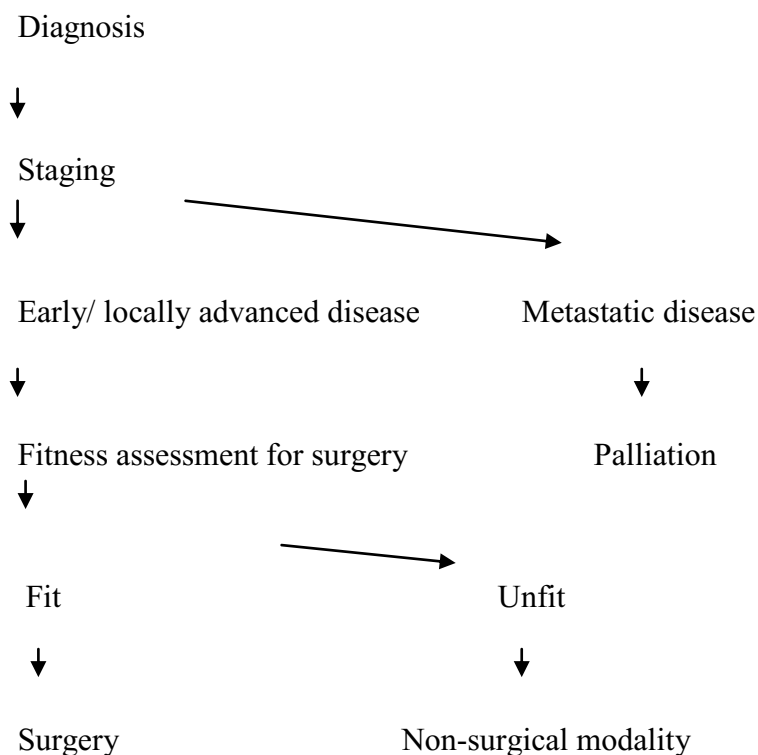
1.5.3.3.1 Curative care

Consistent with the principles of cancer treatment elsewhere, potentially curative care is offered to patients whose disease staging displays absence of metastatic disease. Latest figures show that 36 per cent of patients with SCC were treated with curative plan (National Oesophago-gastric cancer Audit, 2010). The potentially curative care is discussed as surgical and non-surgical modalities which are offered depending on individual characteristics of the disease (Ferguson & Paterson-Brown, 2001).

1.5.3.3.1.1. Surgical modality

As a result of the recent improvements in the multi-disciplinary management of oesophageal cancer, the hospital mortality (or death within 30 days post op) has improved from 28% in 1950-70's (Earlam et al, 1980) to 4.5% in this decade (Portale et al, 2005). These two reviews have also shown that the overall 5 year survival in operated patients has improved from 20% in 1950-70's to nearly 50% in this decade, and supported the surgical modality as the one of the curative treatments for oesophageal cancer.

The principles underlying the oesophageal resection are to remove major part of the oesophagus with enbloc lymphadenectomy, so as to achieve tumour free resection margins of 10 cm if at all possible (Ferguson and Paterson-Brown, 2001). The gap between the two resection margins is channelled using stomach (most common), colon or jejunum. Both hand sewn and stapled anastomoses have been described and used in various centres. Various techniques are described for resection of oesophagus in literature. They include transhiatal oesophagectomy (Turner, 1933), two stage oesophegectomy (TSO) (Lewis, 1946 and Tanner, 1949), thoraco-abdominal approach (Moore, 1955) and three staged resection (McKeown, 1969) . Thoroscopic assisted oesophagectomy was initially described by Gossot et al in 1993. This is a relatively advanced minimally invasive procedure which is currently practiced in many centres.

There are various important factors influencing the surgical technique chosen. These include location and stage of the tumour, patient's anatomy and build and surgeon's expertise. The most commonly performed procedure in our institute for tumours in

the middle and lower one thirds of oesophagus is two stage sub-total oesophagectomy with reconstruction by gastric conduit and a stapled anastomosis.

1.5.3.3.1.2 Non-surgical modalities

Radiotherapy and chemotherapy are the primary non-surgical modes of therapy which are being offered more frequently with potentially curative aim (Crellin, 2009). Radiotherapy and chemotherapy have different modes of action and purpose. Radiotherapy affects the actively dividing cancer cells at the level of nucleus and is more efficient at the periphery of tumour due to direct penetration. This feature of radiotherapy aims at improving resectability of the tumour while reducing the impact of microscopic residual disease and subsequent loco-regional recurrence. Chemotherapy aims at treating the systemic disease in contrast to surgery and radiotherapy, while down-staging potentially resectable tumours (Campbell and Villaflor, 2010).

Some patients with resectable disease may not be fit for a major surgery like TSO, for whom chemotherapy, radiotherapy or both in combination (CRT) may be considered as definitive therapy with the aim of long term disease control (Bartels et al, 1998). Definitive CRT was shown as being significantly effective than radiation alone (Al-Sarraf et al, 1997) and is being increasingly accepted as standard of care in 'inoperable' but potentially curable oesophageal cancer. Chemotherapy decreases the ability of tumour cells to repair radiation mediated DNA damage while acting as radiation sensitizer (Lamont & Vokes, 2001). Even among whole patient population including 'fit' patients, results from definitive CRT rival those of surgery (Chan & Wong, 1999), but this modality is still under 'clinical evaluation' (National Cancer Institute, U.S). In squamous cell carcinoma, a policy of CRT as primary therapy with surgery for salvage is suggested (Wilson & Lim, 2000; Tobias & Ball, 2001). This strategy provided 46% 5- year survival in squamous cell carcinoma and 68% organ preservation in both histological types (Wilson & Lim, 2000).

The ability of tumour markers to predict which patients will respond to non-surgical modality would allow greater certainty in advocating this approach (Crellin, 2009), as discussed earlier.

There is still some role for radical radiotherapy alone in squamous cell carcinoma patients with localised disease, who cannot tolerate CRT (Sykes et al, 1998).

1.5.3.3.1.3 Combined modalities

Treatment of oesophageal cancer is more multi-disciplinary now than ever. This stems from the disappointment of the surgeons at loco-regional recurrence as well as systemic metastasis in spite of good surgical clearance and even the necessity of surgery itself has been questioned in the past (Coia, 1989). The addition of chemotherapy, radiotherapy or CRT to the standard surgical practice as adjuvant or neoadjuvant therapy is advocated in a significant proportion of cases taking into account the individual characteristics of the disease. National Oesophago-gastric cancer Audit (2010) showed that 58 per cent of patients who underwent curative therapy had surgery with associated chemotherapy as the most common modality of treatment.

Neoadjuvant therapy is offered before the main treatment, to achieve better disease control (Crellin, 2009). Adjuvant therapy is the additional treatment given after potentially curative therapy, aimed at reducing the risk of recurrence from occult disease and hence improving disease free and overall survival. It is offered in cases where R0 resection (No macroscopic residual disease left behind) is achieved.

Evidence for benefit from neoadjuvant radiotherapy is not strong, as Arnott et al (1998) showed in their meta-analysis of absolute survival benefit of 4% at 5 years. But neoadjuvant chemotherapy in addition to treating occult metastatic disease may improve the nutritional status of the patient by tumour shrinkage (Crellin, 2009). Many significant trials have confirmed the benefit of neoadjuvant therapies in treating oesophageal cancer. Medical Research Council OEO2 trial started in 2000 as a RCT with surgery alone arm (400 patients) and neoadjuvant chemotherapy/ surgery arm (402 patients) to identify the survival differences if any. The results showed significant survival advantage in the latter arm, with 5 year survival figures of 17.1% vs. 23% respectively (HR 0.84%, p = 0.03). Similar conclusions were drawn from previous trials (Kok et al, 1997; Kelsen et al, 1998) and neoadjuvant chemotherapy is currently an accepted clinical practice. The Medical research council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial studied the role of Peri-operative regimen with ECF (Epirubicin, Cisplatin and Fluorouracil) in lower oesophageal and GOJ adenocarcinomas. This trial concluded in 2006 that this method of treatment decreased tumour size and stage, and significantly improved disease free and overall survival.

Neoadjuvant CRT is currently accepted as a standard of care in many centres (RTOG 85-01 trial: Cooper et al, 1999). CRT can be given either as sequential or synchronous treatment. Both these strategies have shown significant improvement in survival in clinical trials: EORTC trial (Bossett et al, 1997) showed significantly longer disease free survival in the sequential CRT arm at 3 years but no difference in overall survival. Walsh et al (1996) gave synchronous CRT in adenocarcinoma and showed overall survival benefit at 3 years. A RCT (Urba et al, 1997) with both adenocarcinoma and squamous cell cancers showed an overall survival advantage at 3 years in CRT arm. CRT also has given better survival than chemotherapy alone (Gebski et al, 2007).

Adjuvant Radiotherapy has a limited role in node-negative patients with residual mediastinal disease and did not show statistically significant survival advantage (Arnott et al, 2005). Potential drawbacks include morbidity and mortality caused by the exposure of the transposed stomach to the radiation, and are to be considered in weighing the benefits. Adjuvant Chemotherapy may not be feasible in the immediate postoperative period in a good number of cases due to prolonged postoperative phase and resultant poor performance status, hence cannot be relied upon. Currently its role is limited to patients who had resection for early oesophageal cancer without neoadjuvant chemotherapy, but were found to have unexpectedly advanced final staging (Crellin, 2009).

1.5.3.3.2 Palliative care

The aim of palliative care is to minimise the morbidity associated with the advanced disease while maximising the quality of life in the brief period of survival. Sometimes this care may prolong the survival by improving the nutrition in patients with dysphagia.

Various factors influence the selection of patients for palliative care. Clear evidence of metastatic spread to the lung, liver or bone implies the wide spread nature of the cancer, with little hope of cure. Evidence of invasion of adjacent structures like the aorta, bronchus or pericardium by cancer determines the inoperability of the primary tumour and suggests palliative care. Those patients with potentially resectable disease but not fit for either curative surgery or radical radiotherapy due to general debilitation and co-morbidities are considered for palliative care.

However patient preferences form an integral part of the decision making process. MDTs which are a mandatory part of cancer care in UK play an important role in making a decision in this aspect, taking into consideration the wishes of the patient (Blazeby and Alderson, 2009).

The methods for palliative treatment fall into three groups. Oncological therapy constitutes chemotherapy, radiotherapy or a combination of these two, and is currently the most common palliative anti-cancer modality (National Oesophago-gastric cancer Audit, 2010). The endoscopic/ radiological therapy includes placement of stents across the tumour, dilatation of tumour area, thermal ablation and brachytherapy. In some frail patients with advanced disease supportive care is appropriate. Surgical methods for the palliation of symptoms usually show unsatisfactory results and generally non-operative methods are preferred. The symptoms that are targeted for palliative relief are: Dysphagia, aero-digestive fistula, recurrent laryngeal nerve palsy and chronic bleeding.

1.6 Aim of the study

The aim of this research study was to identify the level of expression of HIF 1α in squamous cell carcinoma (SCC) tumour cells of our local population (Teesside, Durham, North Yorkshire) and to assess its possible role as a prognostic indicator in these patients. The null hypothesis (H_0) that there is no relationship between the expression level of HIF 1α and survival rates in the recruited patients with SCC of oesophagus was to be tested.

Tumour samples from newly diagnosed patients of SCC of oesophagus were prospectively collected and the expression of HIF 1α was assessed in our laboratory. The level of expression of HIF 1α was used to stratify the study population into separate groups. This expression pattern was correlated to various clinic-pathological parameters of the disease (Gender, age, T status, N status, M status, UICC stage, tumour differentiation and aim of treatment) to identify any relation. The disease related and overall survival of the patients was correlated with the above parameters and the HIF 1α expression to identify the factors of prognostic significance in SCC of oesophagus. This information could help define the use of HIF 1α in evaluating the expected outcome of SCC of oesophagus in our local population.

This study may possibly influence the management plan and hence the outcome in patients with SCC of oesophagus by providing further knowledge on molecular prognostic markers. Recommendations on further research into the role of HIF 1α in oesophageal cancer could be made based on the findings arising from this study.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study design

The current study was designed as a prospective observational cohort study with clinical and laboratory components.

The clinical component included the identification and recruitment of patients, sample collection from them; follow up of these patients and collection of clinical data. The laboratory based component comprised the processing of the specimens towards the identification of HIF 1α. These were described in the following sections.

2.1.1 Ethics & Approval

All the participants in the study were NHS patients. Their safety, dignity and wellbeing were assured through the approval within the framework of regulations of the NHS and University bodies responsible for Research Governance (www.nres.npsa.nhs.uk; www.tees.ac.uk). This study was carried out conforming to the ethical principles outlined in the Declaration of Helsinki, 1996.

The Research Approval Board and the Local Research Ethics Committee of South Tees Hospitals NHS Trust granted Research and Ethical approval respectively for this project (14 Dec 2004, Appendix 1; 4 Jan 2005, Appendix 2). Subsequently, the Ethics Committee of School of Health and Social Care, Teesside University granted approval on 8 June 2005 (Appendix 3).

The Patient Information Sheet (Appendix 4) and the Consent Form (Appendix 5) which were prepared as per existing trust research guidelines and submitted to the above Boards were used in the project.

2.1.2 Sample size

The aim of the current observational study was to identify if the expression of HIF 1α correlates to the survival of the patients with SCC of oesophagus. It was felt that on an individual patient basis, any change in his or her survival in comparison with a similar patient would have significant impact, if determined by HIF 1α expression. Hence the effect size could not be defined for this study and a formal power study was deemed not possible to determine the sample size. However, available literature

was reviewed to identify the sample size in their studies. Five previous studies were identified with the sample size in the range of 37 – 54 patients (Koukourakis et al (2001), katsuta et al (2005), Wu et al (2007), Ling et al (2006) and Chen et al (2010)).

In consideration of the average number of patients that could possibly be identified in the study period, a sample size of 35 - 40 consecutive patients was considered to be sufficient to identify with good accuracy the difference in tumour's biological behaviour as reflected in the patient's survival, with respect to the expression of HIF 1α.

2.1.3 Participant selection & Recruitment

Patients with confirmed diagnosis of oesophageal cancer and/ or under treatment for oesophageal cancer at the James Cook University Hospital, Middlesbrough (JCUH) during the study period of June 2005 – August 2006 were identified. This information was obtained through a prospectively and regularly updated local cancer database known as '*Infoflex*' system (www.infoflex-cims.co.uk). Forty three patients were identified satisfying this selection criterion.

It was felt that it would be unethical to approach patients with very poor general condition in addition to the presence of advanced disease for recruitment for present study. Hence this was used as an exclusion criterion and accordingly six patients were excluded from the study to safeguard their interests.

The remaining thirty seven patients were contacted and informed about this research project in person by the author (P C Munipalle). The approved Patient Information Sheet was given to these potential participants and explained to them. Those agreeing to participate in the study were recruited by obtaining written consent on the approved Consent Form.

Recruitment of participants was done at the Department of General Surgery, JCUH. Areas where contact with the participants in a confidential environment was assured were chosen for the purpose of recruitment. The upper gastrointestinal outpatient department was a good initial contact point with majority of the participants as this was located away from the clinical areas and busy wards, and hence most

participants were contacted and recruited here. Some participants were recruited at the Endoscopy centre where the patients present for endoscopic procedures. The Upper gastrointestinal ward (Ward 23) was allotted for admissions of oesophageal cancer patients during operative procedures and part of the recruitment was done here.

2.2 Tissue Collection

Tissue samples were obtained from the recruited participants either directly during endoscopy, or from the operative specimen in those patients who underwent TSO.

i) During Upper gastrointestinal endoscopy -

This was a routine investigation in oesophageal cancer patients as discussed in section 1.5.3.1 (Clinical investigations). During this procedure, the endoscope was introduced through pharynx into the oesophagus and stomach either under local anaesthetic spray or conscious sedation (Nagengast 1993, www.bsg.org.uk/guidelines). Multiple biopsies in the form of 4 to 6 samples of tissue were collected from the macroscopic tumour areas using standard tissue biopsy forceps. Tissue biopsies were done during diagnostic as well as therapeutic endoscopy procedures like dilatation, stenting and Argon Plasma Coagulation of tumour tissue.

ii) After the operation – The resected specimen containing the distal part of the oesophagus harbouring the cancer was sent for histopathological examination as a standard practice. Due to the Ivor-Lewis TSO technique, the proximal part of stomach was also resected and hence included in the specimen.

Samples from operative specimens were preferred over endoscopic specimens where available. In participants who had both endoscopy and TSO, large amount of sample could be obtained from the operative specimens with the possibility of preparing multiple slides if need be, in comparison with the specimens obtained through endoscopy. Operative specimens could also provide additional source of sample from the peri-oesophageal lymph nodes, which could not be sampled through endoscopy.

All the endoscopic and operated specimens obtained were labelled with the date of collection, details of the patient and site/ or nature of specimen and sent to the histopathological laboratory in buffered formaldehyde 10% (v/v) (Genta Medical, York, UK).

The tissues were fixed in the formaldehyde for 24 hours in preparation for further processing.

2.3 Tissue processing & Staining

The laboratory at JCUH has optimised the protocol for the preparation, processing and staining of the tissue samples for assessing the expression of HIF 1α in oesophageal SCC tissue. This protocol was validated previously in this laboratory conditions (A Ahitan, personal communication) and is described in this section.

2.3.1 Reagents & Accessories

The Dako CSA kit (Dako, UK) contained all the following reagents except Reagent 3 (Table 3). The reagents were prepared as per the suppliers' instructions:

Table 3. Reagents in Dako CSA kit

Reagent 1   Peroxidase Block

        3% hydrogen peroxide in methanol (v/v) made up of:

                H_2O_2 (BDH) 6%, 300 ml

                Methanol (BDH) 100%, 300 ml

Reagent 2   Protein Block

        Serum-free protein in Phosphate Buffered Saline (PBS) with 0.015 mol/L sodium azide

Reagent 3   Primary Antibody (Novus Biologicals, UK)

Mouse monoclonal (HIF 1α 67-sup clone) antibody to HIF 1α (ab463)

1:1000 concentration (2 μl in 2000 μl Ventana antibody diluent), 2.90 mg/ml

Reagent 4   Link Antibody

Biotinylated rabbit anti-mouse immunoglobulins in Tris-HCl buffer containing stabilising protein and 0.015 mol/L sodium azide

Reagent 5   Streptavidin-Biotin Complex

Reagent A – Streptavidin in PBS buffer containing anti-microbial agent Bronidox (5 – bromo – 5 – nitro - 1, 3 - dioxane, at 0.1% concentration)

Reagent B – Biotin conjugated to horseradish peroxidase in PBS buffer containing anti-microbial agent Bronidox (5 – bromo – 5 – nitro - 1, 3 - dioxane, at 0.1% concentration)

Reagent C – PBS buffer containing stabilising protein and anti-microbial agent Bronidox (5 – bromo – 5 – nitro - 1, 3 - dioxane, at 0.1% concentration)

Reagent 6   Amplification reagent

Biotinyl tyramide and hydrogen peroxide in PBS containing stabilising protein and anti-microbial agent Bronidox (5 – bromo – 5 – nitro - 1, 3 - dioxane, at 0.1% concentration)

Reagent 7   Streptavidin-Peroxidase

Streptavidin conjugated to horseradish peroxidase in PBS containing stabilising protein and anti-microbial agent Bronidox (5 – bromo – 5 – nitro - 1, 3 - dioxane, at 0.1% concentration)

Reagent 8   Substrate Tablets

3, 3'-diaminobenzidine tetrahydrochloride (DAB Chromogen) packaged with desiccant – 10 mg per tablet

Reagent 9   Tris-HCl buffer concentrate

(0.05 mol/L Tris-HCl pH 7.6 containing 0.3mol/L NaCl and 0.1% Tween 20 (v/v)

Reagent 10   0.8% (v/v) Hydrogen Peroxide in water

Reagent 11   Haematoxylin counter-stain

The other reagents used in the immunohistochemistry assay for identification of

HIF 1α were described below in Table 4 (from Genta Medical, York, UK):

Table 4. Other reagents used for HIF 1α identification

Citrate buffer pH 6.0   8.82g sodium citrate (BDH)

3 litres of distilled water

Adjust to pH 6.0 using N Hydrochloric acid

N Hydrochloric acid   HCl (BDH) 34ml

Distilled water 366ml

EDTA buffer pH 8.0   EDTA (Sigma) 370 mg

Distilled water 900 ml

Adjust to pH 8.0 using NaOH pellets

3% (v/v) H_2O_2 in methanol

H_2O_2 (BDH) 30 ml

Methanol (BDH) 300 ml

Tris buffered saline (TBS; NewEngland Biolabs, Hitchin, Herts, UK)

Sodium chloride 8g

Tris 0.605g

Distilled water 1000ml

Adjust to pH 7.6 with N HCl and 0.1 M Tris

Accessories   Target retrieval solution (S1699/ S1700/ K1499)

5ml calibrated test tubes with cap for use in preparation of Streptavidin

Substrate container

10 ml calibrated test tubes with cap for use in preparation of substrate

Forceps

2.3.2 Technique

Tissue samples were selected from the endoscopic/ operative specimens by the reporting pathologist and the tissue was processed for preservation on a Leica tissue processor model TP 1050 (Appendix 6).

The tissue samples were then embedded in paraffin wax with a melting temperature of 56-57°C using a Leica embedding centre model EG 1160.

Sections were cut at 4μm on a Leica microtome model RM2235 and mounted on separate electrostatic slides to increase the adhesion of the sections to the slide. The slides were stained with Haematoxylin & Eosin on Shandon varistain gemini automated stainer as below:

Haematoxylin & Eosin Staining protocol

Step	Reagent	Conc. % (v/v)	Time (min)	Agitate
1	Dry Storage		00:00	None
2	Heater Station		30:00	None
3	Xylene		02:00	None
4	Xylene		02:00	None
5	Alcohol	100 %	01:00	None
6	Alcohol	100 %	01:00	None
7	Alcohol	70 %	01:00	None
8	Running Water Wash		01:00	None
9	Harris unacidified		05:00	None
10	Running Water Wash		01:00	None
11	Acid Alcohol	0.1%	00:10	None
12	Running Water Wash		00:30	None
13	Bluing Reagent		01:00	None
14	Running Water Wash		01:00	None
15	Alcohol	90 %	01:00	None
16	Alcoholic Eosin		01:20	None
17	Alcohol	90%	00:30	None
18	Alcohol	100 %	01:00	None
19	Alcohol	100 %	01:00	None
20	Xylene		01:00	None
21	Xylene		01:00	None
22	Xylene		01:00	None

The slides showing the following areas were selected for the study:

- Oesophageal tumour

- Peri-oesophageal/ peri-gastric Lymph nodes (where available)

The formaldehyde fixed paraffin processed (FFPP) blocks corresponding to these selected slides were retrieved for HIF-1α staining.

Control material

To enhance the validity of the laboratory protocol, the experiments were controlled through the use of positive and negative controls. The positive control is an experiment using a substrate which from previous experience was known to produce a positive result in the given uniform experimental conditions. Use of such a positive control ensures that the experiment was carried out in the specified manner; if the positive control does not produce satisfactory result, the experiment is void and repeated. This was useful in eliminating false negative results (Johnson & Besselsen, 2002, p204). Representative tissue from a previously reported grade 4 renal cell carcinoma (RCC), which was known to express very high HIF 1α levels was anonymously selected from archives of Histopathology, JCUH and processed as described earlier. One slide of this control material was used as positive control for each batch of test slides.

The negative control is an experiment done in such a way that a negative result is produced, to compare against the results from the test. This helps in identifying any contamination of the test or presence of any artefacts mimicking the presence of HIF 1α, hence avoiding false positive results (Johnson & Bessesen, 2002). Both external and internal negative controls were employed to ensure the identification of false positive results, if any. A slide prepared from the RCC tissue was used as external negative control for each batch of slides. One slide of 'test' tissue was used as internal negative control for each batch of slides. For both these negative controls, the application of primary antibody at step 3 was replaced with Tris Buffer Saline (TBS). As per the principles of usage of negative control, this control slides shall not show any evidence of identification of HIF 1α or similar artefacts.

2.3.2.1 Preparation of slides for HIF 1α staining

For each block selected for the study, two sections were cut at 4μm and floated out on a water bath containing distilled water at 50°C. Each section was mounted on separate electrostatic slides, which were used to increase adhesion of the section to the slide. Each slide was labelled with an assigned unique identification number, which would ensure complete patient anonymity. A suffix number relating to tissue type was also recorded on the slide. One of these slides was designated as negative control as described earlier.

The slides were dried in an incubator at 37°C or in a wooden box at room temperature until staining started.

When ready to stain, the prepared slides were placed in metal baskets and heated to 60°C for 1 hour prior to immunohistochemical staining. The control slides were also treated the same way. It took 5 hours to complete one cycle of staining process from these prepared slides. Using the equipment available for the project, up to 40 slides including controls could be stained in one cycle.

2.3.2.2 Processing

De-paraffinization and rehydration

The slides were de-paraffinized in 3 changes of xylene (Genta Medical, York, UK) for 5 minutes in each solution. This was followed by rehydration of the sections with 2 changes of 100% v/v Industrialised Methylated Spirit (IMS) (Genta Medical, York, UK) for 5 minutes each, then into 95% v/v and 70% v/v strength IMS for 5 minutes each. Finally they were immersed in running tap water.

Antigen retrieval

During fixation with formalin, the antigenic sites of the tissues would be masked by the formation of methylene bridges between various proteins. It was vital to unmask these sites for effective binding with antibodies through a process called 'antigen retrieval'. The two common methods employed for this task utilize either heat or enzymes for the retrieval. The suppliers of the antibodies used in this study

(Novus Biologicals, UK) recommended Heat-mediated epitope retrieval (HMER) for this purpose. In previous experiments in the JCUH laboratory, the following method was validated for providing higher expression of antigen staining in the nuclei, with minimal background staining (A Ahitan, personal communication):

Three litres of citrate buffer at pH6.0 was brought to the boil in a pressure cooker (Prestige model 6193, operating pressure 103kPa/15 p.s.i) using a solid sealed hot plate (S & J Juniper & Co., UK). Re-hydrated slides placed in metal slide baskets were rapidly immersed into this boiling liquid. The lid of the pressure cooker was secured and the pressure cooker was continually heated on the hot plate until pressure was reached and maintained for a further 6 minutes.

The pressure cooker was then removed from the hot plate and immersed in a sink of cold running tap water to rapidly decrease the pressure. Once pressure had reduced, the lid was opened and cold running tap water was flushed inside the pressure cooker to replace the citrate buffer. The slides were covered with Shandon coverplates and placed in a Shandon sequenza tray (Shandon Sequenza Immunostaining Center, Thermoscientific, UK) for further staining.

2.3.2.3 Immunohistiochemical staining of slides

The protocol of staining the SCC slides for the identification and quantification of the expression of HIF 1α was optimised at the Histopathology laboratory of JCUH, based on previous experiments (Ms A Ahitan, personal communication). These results have been validated and accepted as appropriate for this laboratory conditions. The protocol is described below:

As a first step, the slides in each Sequenza tray were washed with 3 ml TBS for 5 minutes.

The slides were then incubated with Reagent 1 (Peroxidase Block) for 5 minutes, followed by a rinse in TBS for 5 minutes. This process was repeated with Reagent 2 (Protein Block). Each reagent was added at 100μl per slide.

The slides (except the negative control slides) were then incubated with Reagent 3 (Primary Antibody) for 60 minutes followed by TBS wash in 3 steps of 5 minutes each. Negative control slides were incubated with TBS in place of Primary Antibody.

This was followed by sequential 15 minute incubations with Reagent 4 (Biotinylated Link Antibody), Reagent 5 (Streptavidin-Biotin-Peroxidase Complex), Reagent 6 (Amplification reagent) and Reagent 7 (Streptavidin-Peroxidase). Each 15 minute incubation period was followed by three TBS washes of 5 minutes each.

Finally, slides were incubated with Reagent 8 (DAB chromogen) for 5 minutes followed by a rinse in distilled water. The slides were then counter-stained with Haematoxylin as described earlier and rinsed in distilled water (See appendix 7 for Staining Protocol Worksheet).

The slides were mounted and were ready for analysis of the HIF 1α antibody expression.

2.4 HIF 1α Scoring

HIF 1α antigen was expressed in the cell nucleus as well as cytoplasm as per published literature. The suitability of using the expression levels in these two different parts of a cell was debatable.

Hypoxia in a tumour cell results in enhanced expression of HIF 1α and subsequent activity only in the nucleus. This nuclear activity in turn mediates the various mechanisms of action of HIF 1α throughout the cell. For this reason majority of studies on HIF 1α in SCC determined the strength of expression by the extent of nuclear staining alone (Kurokawa, 2003; Sohda, 2004; Katsuta, 2005; Matsuyama, 2005). Similar approach was taken in present study. The HIF 1α expression was considered positive if the nucleus stains intensely with the antibody, which results in dense brown colouring of the nuclear material.

Under an Olympus microscope (Olympus Optical, Tokyo, Japan) with × 400 magnification, concentrated foci of tumour cells were selected in each slide for the scoring of expression. The percentage of tumour cells in that field which stain

positive for HIF 1α compared to those cells staining negative was scored. Negative control slides were also checked to confirm the absence of nuclear staining.

A scoring system was employed based on previous studies (Schindl et al, 2002; Kurokawa et al, 2003). The samples where ≥10% cells showed positive staining were scored as 'High' expression and the samples with <10% cells with positive staining were scored as 'Low' expression. This was the common form of scoring system in previous studies. Kimura et al (2004) calculated the mean values for the expression of HIF 1α and used these to determine the cut-off points between high and low expressions – the author's impression was that this method has intentionally divided the whole patient population into similar sized groups.

The expression of HIF 1α in all the slides was scored independently by author (P.C.M.) and a histopathologist (D.S.) without knowledge of clinicopathological data on each individual case, conforming to the principles of blind assessment. If there was a significant difference in the scoring for a particular case, a mutual agreement was reached after a re-reading and discussion.

The scores were compared for inter-observer validity and the average of the two scores was used for analysis of results.

2.5 Follow up, Data collection & Data Storage:

Clinical follow-up of the patients was done as per the existing clinical protocol of the responsible healthcare teams. This includes the Surgeon, the Oncologist and the Gastroenterologist, with the support of the Radiologist and other healthcare professionals like Specialist nurses, Physiotherapists, Dieticians and Pain care team. The progress of the patient, subsequent tests done and their results etc. were entered into the dedicated and secure cancer database called 'Info*flex*' by data managers as per nationally accepted current clinical practice (www.cancerimprovement.nhs.uk). In addition, these details were documented in the patients' case notes.

The patients' data were collected from the above resources by the author, which was then entered into the project database. The demographic parameters of the participants were collected from the case notes. The disease related follow-up information was collected from the Info*flex,* radiology and histopathology reports.

All the data was anonymised with a serial number to denote each participant throughout the study and entered in the project database. The security of the data was ensured through password enabled secure storage systems.

2.6 End points of the study

Survival of the study participants in relation to the expression of HIF 1α was considered as primary end point of the study. Survival in relation to various other clinic-pathological factors was considered as secondary end point.

In addition, the relationship between the expression pattern of HIF 1α and other clinic-pathological factors was also analysed.

2.7 Statistics

Inter-observer variability (measurement of the agreement between the HIF 1α expressions scoring of two independent observers) was calculated using Cohen's kappa test. Where there was disagreement, both observers repeated the reading of the expression and agreed upon a single score. A value of 1 was considered as perfect agreement (Cohen J, 1960).

Correlation between HIF 1α expression in the tumour mucosa and other clinicopathological parameters was assessed with χ^2 test or Fisher's exact test. These were gender, age, T stage, N stage, M stage, integrated UICC TNM tumour stage, tumour differentiation, aim of treatment and positivity of resection margins in the TSO group. A difference of $p<0.05$ was considered statistically significant.

Kaplan Meier curves were used to plot the survival characteristics, which were the most commonly employed statistical method for this purpose (Altman, 1991). The curves separately represent the survival data of patient groups with high and low HIF 1α. For each patient died during the study, his or her death was represented as an 'event' on the curve and the 'time to event' of each patient was plotted. This was considered as 'censored' data. To identify the difference between two curves with censored data, log rank test (also known as Mantel-Cox test) was the appropriate method and hence was employed. Disease related and overall survival curves in relation to the expression of HIF 1α were constructed using the Kaplan-Meier

method. Overall survival curves were plotted in relation to gender, age, T stage, N stage, M stage, integrated UICC TNM tumour stage, tumour differentiation and aim of treatment. The difference between survival curves was compared with Log Rank test. A difference of $p<0.05$ was considered statistically significant.

The prognostic value of individual variables was assessed by Cox proportional hazards model. A difference of $p<0.05$ was considered statistically significant.

SPSS for Windows version 12.0 (SPSS, Chicago, Illinois, USA) was used to process the data.

2.8 Reporting the study

In order to ensure that the results of observational studies are presented in a scientific manner, **St**rengthening the **R**eporting of **Ob**servational studies in **E**pidemiology group issued guidelines (www. strobe-statement.org, 2007). The results from the current study were reported adhering to the principles of the above statement with the aid of the check list provided for this purpose.

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CHAPTER THREE

RESULTS

3.1 Patient recruitment pathway

43 patients with SCC were identified during the study period. Six of them were frail with advanced disease stage, and hence they were excluded from the study as per the defined exclusion criteria stated earlier (Section 2.1.3). The remaining 37 patients were approached for informed consenting, and all of them consented to take part in the research. One patient had rethought and retracted from the study citing personal reasons, and hence was excluded from the study. The remaining 36 patients were recruited for the study.

The pathway and the number of patients at each stage of the study are presented below as a flow chart (Figure 8), as per the STROBE guidelines (www. strobe-statement.org, 2007). The HIF 1α score sheet is presented as Appendix 8.
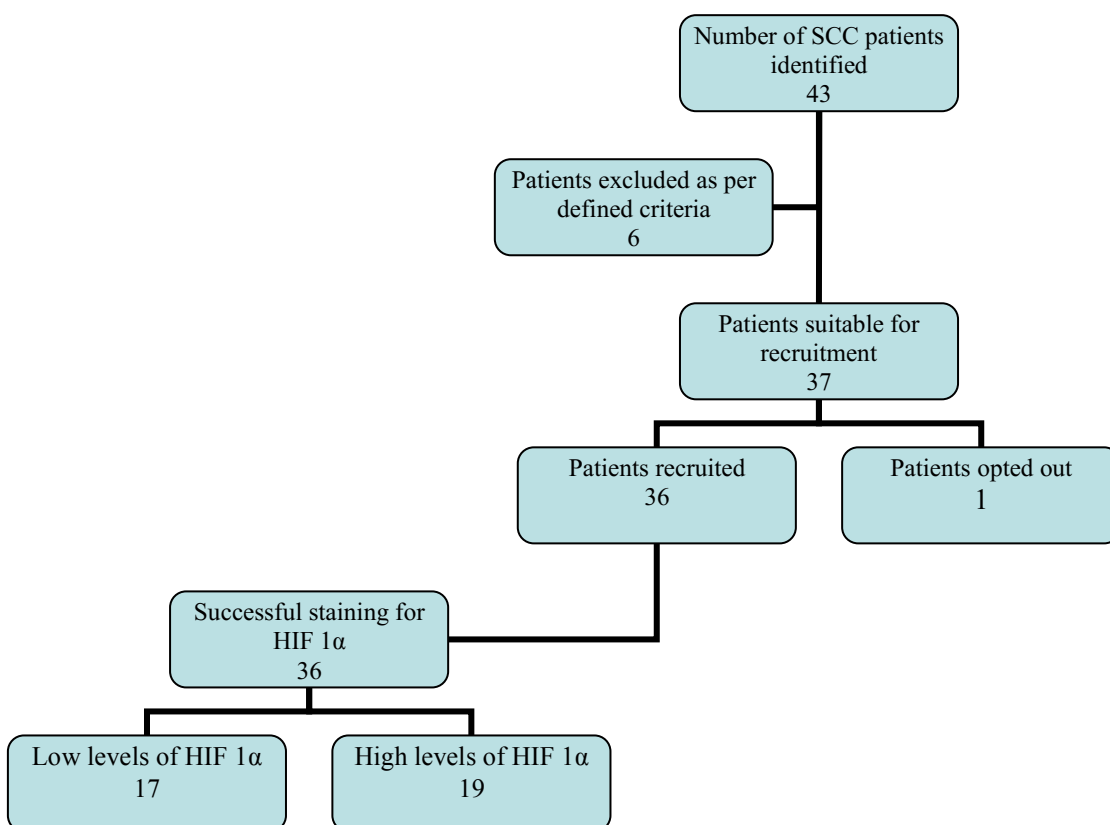
Figure 8. Pathway of patient recruitment

3.2 Expression of HIF 1α in SCC mucosa

The expression of HIF 1α was found to be predominantly in the cancer tissue rather than adjoining normal oesophageal epithelium. Within the cancer cells, the expression was found to be concentrated within the nuclei in comparison with the cytoplasm as shown below in Figures 9 and 10:
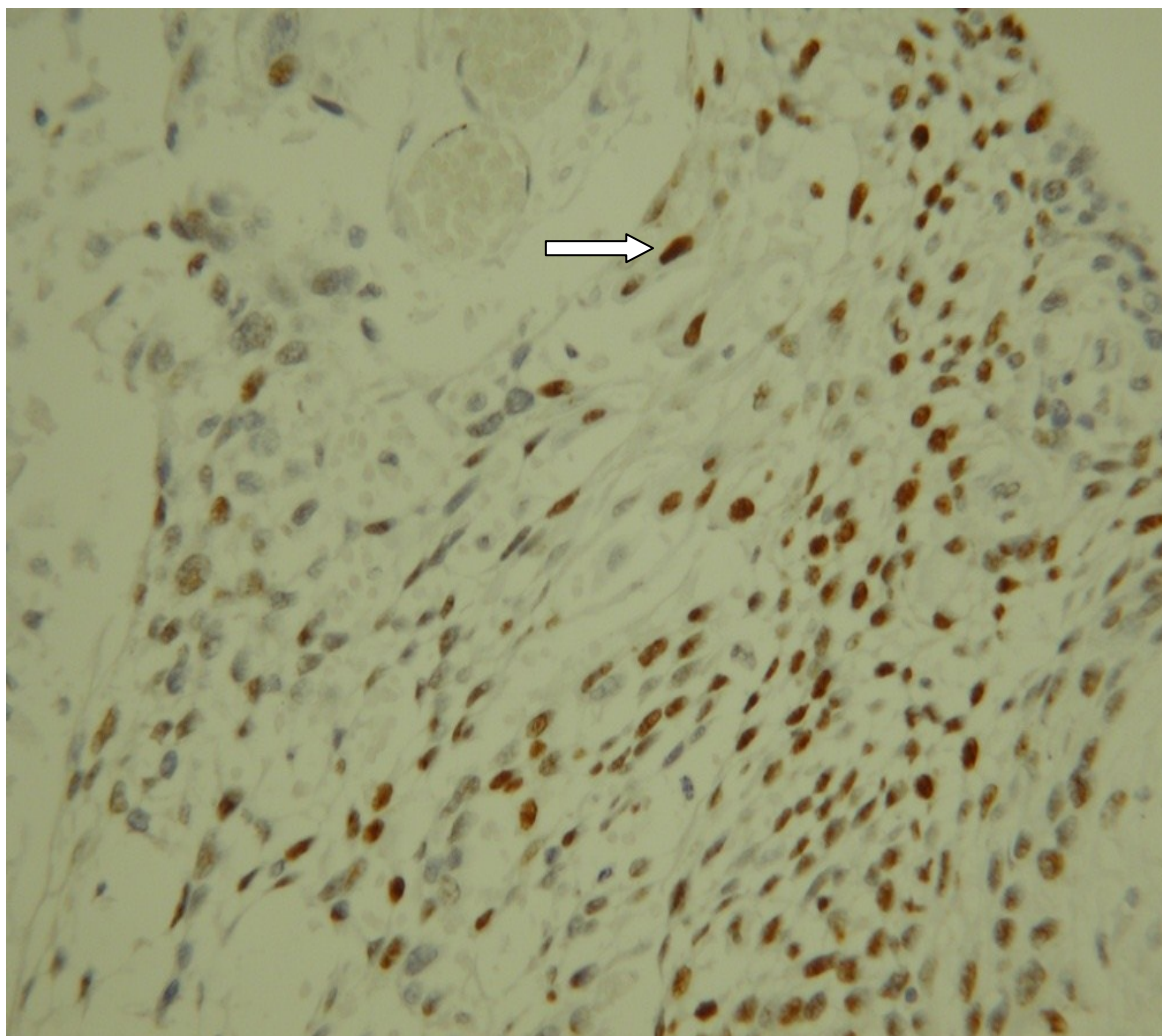
Figure 9. Expression of HIF 1α in SCC cells of oesophageal epithelium × 200 magnification

*Arrow points to a positively stained nucleus.
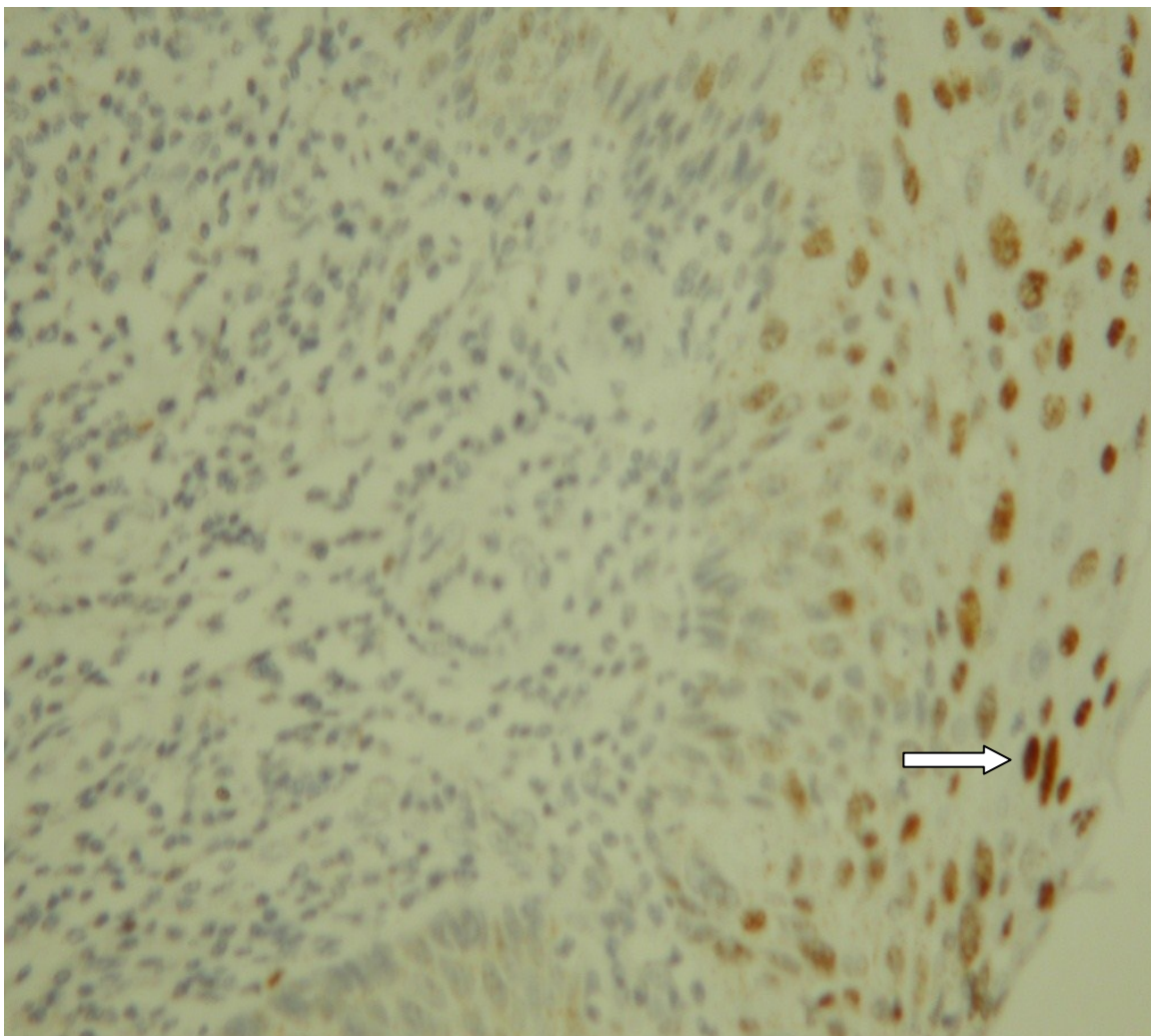
Figure 10. Expression of HIF 1α in SCC cells of oesophageal epithelium × 400 magnification

*Arrow points to a positively stained nucleus.

3.3 Expression of HIF 1α in lymph nodes

Lymph node tissue was available in all the patients who underwent TSO, eight in total. The results showing the expression levels of HIF 1α in these lymph nodes (LN) are presented below as a flow chart (Figure 11) as per STROBE guidelines.
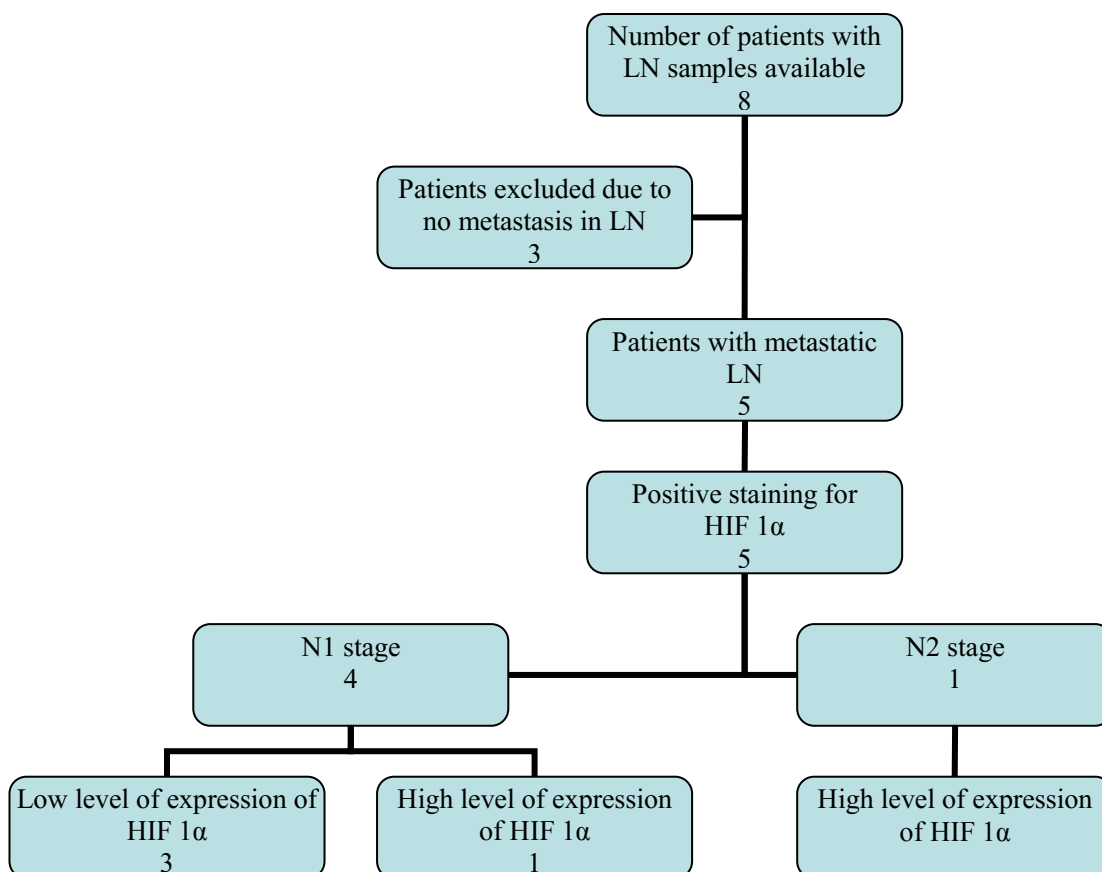
Figure 11. Pathway of HIF 1α expression identification in lymph nodes of SCC

Four operative specimens yielded regional lymph nodes with metastases, which were N1 stage. One specimen contained non-regional metastatic lymph node, which was N2 stage. This information is represented in the histogram below (Figure 12):
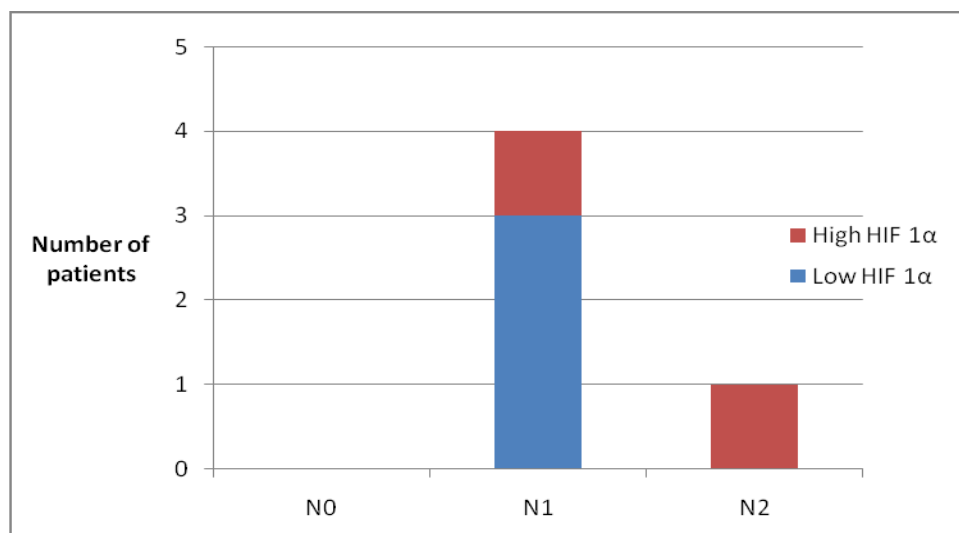
Figure 12. HIF 1α expression pattern in SCC metastatic lymph nodes

Study of the expression of HIF 1α in lymph nodes infiltrated with cancer cells also showed good nuclear expression as shown in Figure 13. Normal lymph nodes with no evidence of cancer cell presence did not show any positive staining for HIF 1α expression, similar to normal oesophageal tissues.
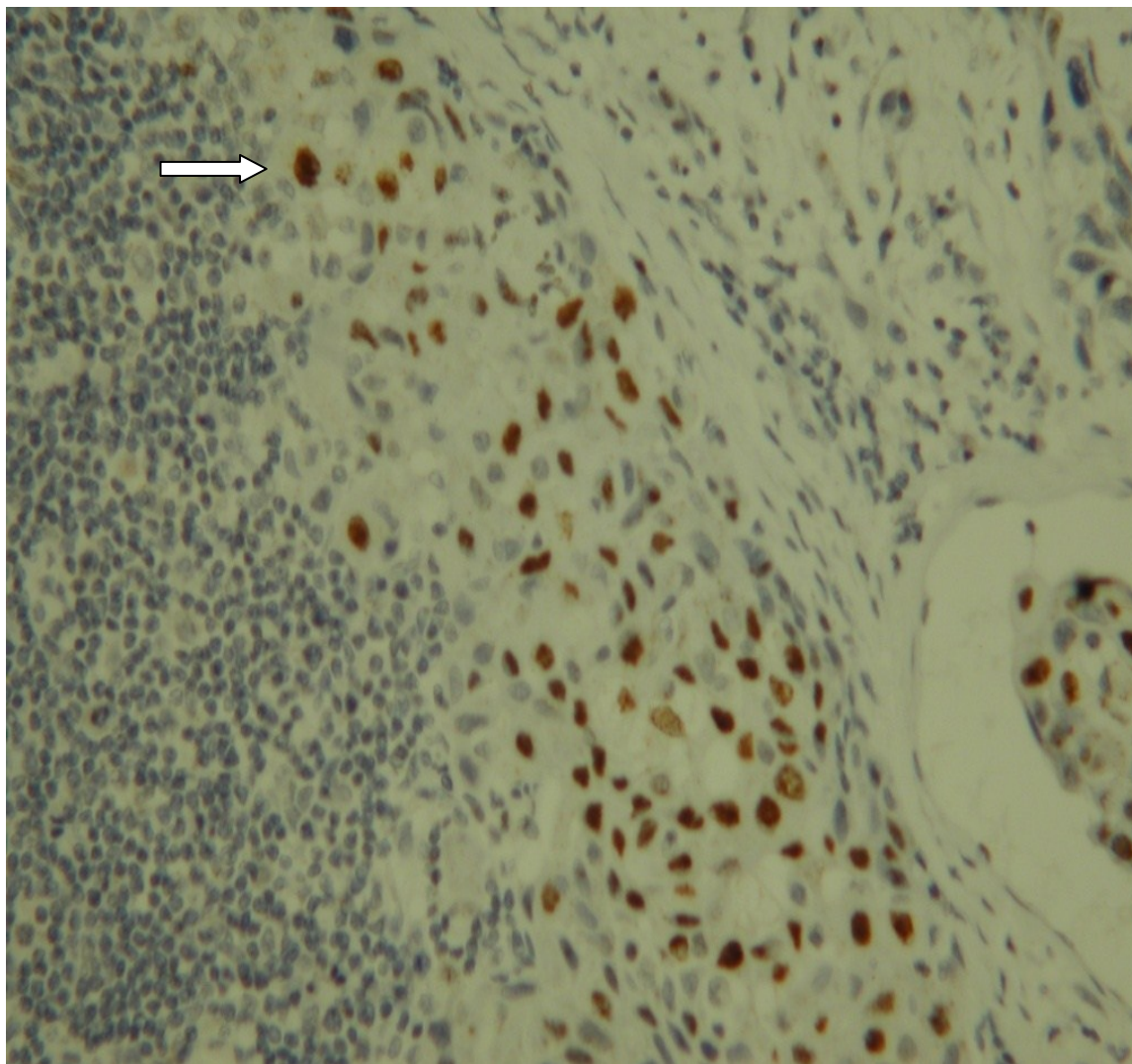
Figure 13. Expression of HIF 1α in lymph node with SCC metastasis × 200 magnification

*Arrow points to a positively stained nucleus.

3.4 Inter-observer variability

The inter-observer variability test showed good agreement between the two independent observers regarding the reading of the expression of HIF 1α (Cohen's Kappa 0.82, p<0.01). The score sheet is attached as Appendix 8.

3.5 Follow up period

The recruited patients (n = 36) were studied for a period of 1 – 56 months, with a median of 42 months.

3.6 Patient characteristics

The study population comprised 15 males and 21 females, with a median age of 76 years (range 52 – 95).

Out of the 36 patients, 15 were offered treatment with curative intention (TSO for 9, one of them after neoadjuvant chemotherapy; Chemoradiotherapy 5; Radical radiotherapy 1). 16 patients were offered Palliative treatment (radiotherapy 8, chemotherapy 8). 5 patients were offered Supportive care (Figure 14).
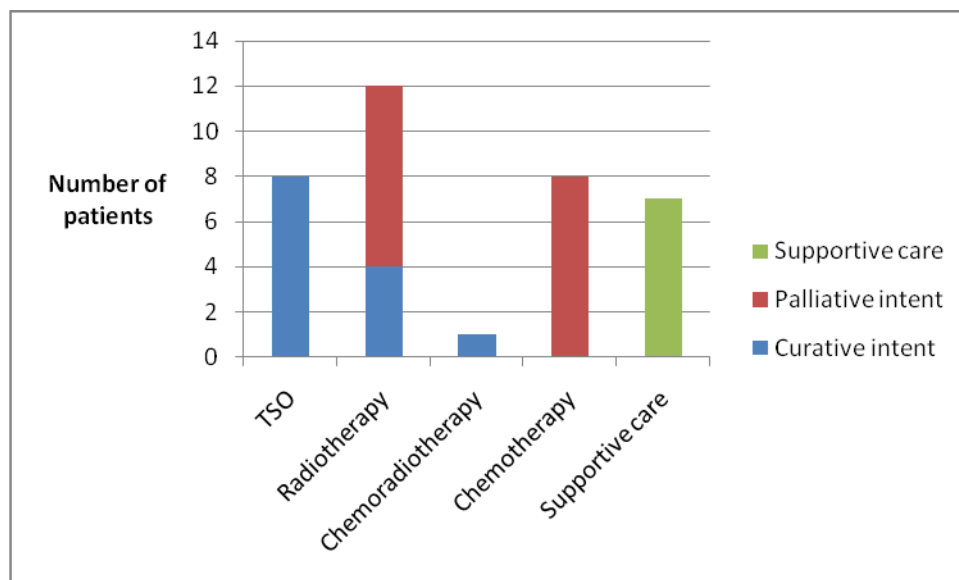
Figure 14. Aim and mode of treatment pattern

3.7 HIF 1α expression and clinico-pathological variables

The correlation between the HIF 1α expression and various clinico-pathological variables is presented below.

3.7.1 Gender

Among male patients HIF 1α expression was high in 8 and low in 7 patients; among female patients HIF 1α expression was high in 11 and low in 10 patients (Figure 15).
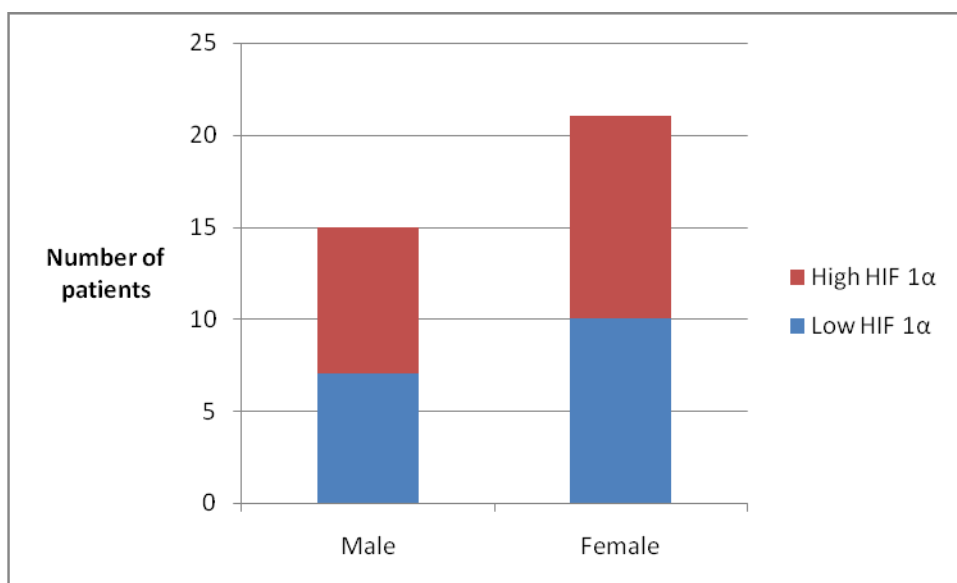
Figure 15. HIF 1α and gender

There was no correlation between the gender of the patient and HIF 1α expression (p=0.709).

3.7.2 Age

HIF 1α expression was significantly high (p= 0.022) in patients aged above 70 years (13 high: 5 low) and low in patients aged below 70 years (12 low: 6 high) (Figure 16).
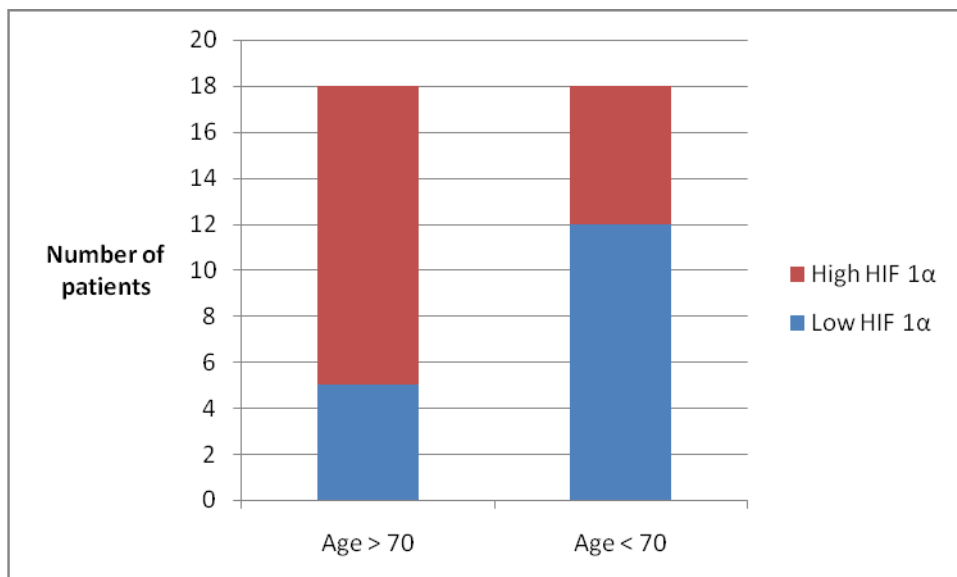
Figure 16. HIF 1α and age

3.7.3   Tumour (T) stage

There was no correlation between T stage of the disease and HIF 1α expression (p=0.408) (Figure 17).
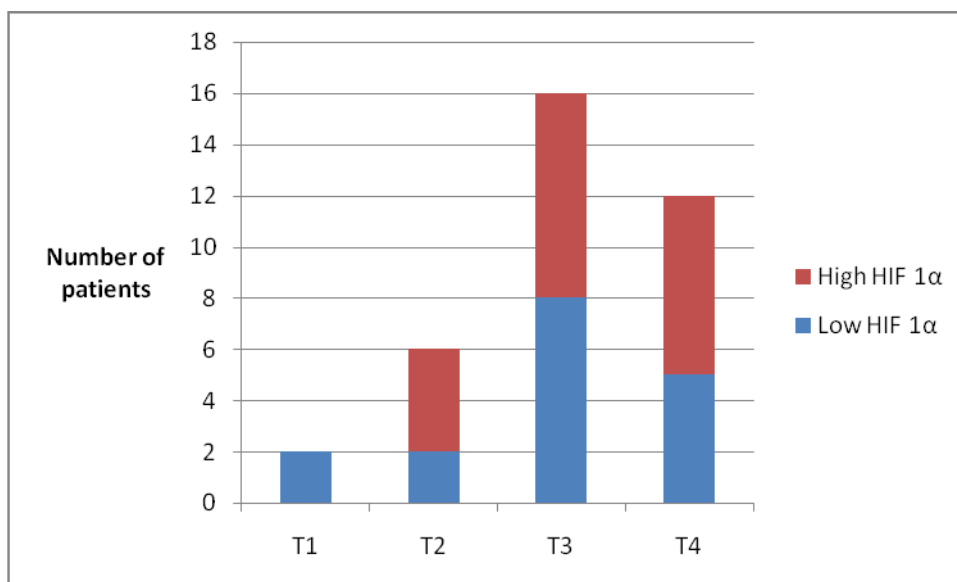
Figure 17. HIF 1α and T stage

This trend continued whether the T stage was compared to HIF 1α expression in 4 stages (T1, T2, T3 and T4) or the T stage was categorised into 'early stage' (T1 and T2) and 'late stage' (T3 and T4).

3.7.4 Lymph node (N) stage

There was no correlation between N stage of the disease and HIF 1α expression
(p=0.846) (Figure 18).
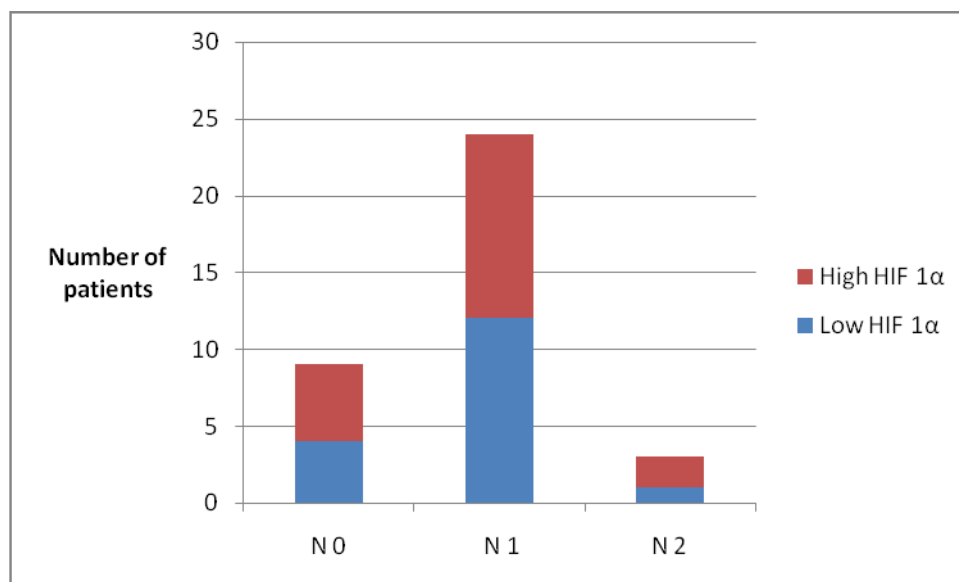
Figure 18. HIF 1α and N stage

3.7.5 Metastases (M) stage

In patients with metastatic disease, the expression of HIF 1α was high in 8 patients and low in 4 patients. While this is an interesting trend, the difference in rates of expression is not statistically significant (p=0.238) (Figure 19).
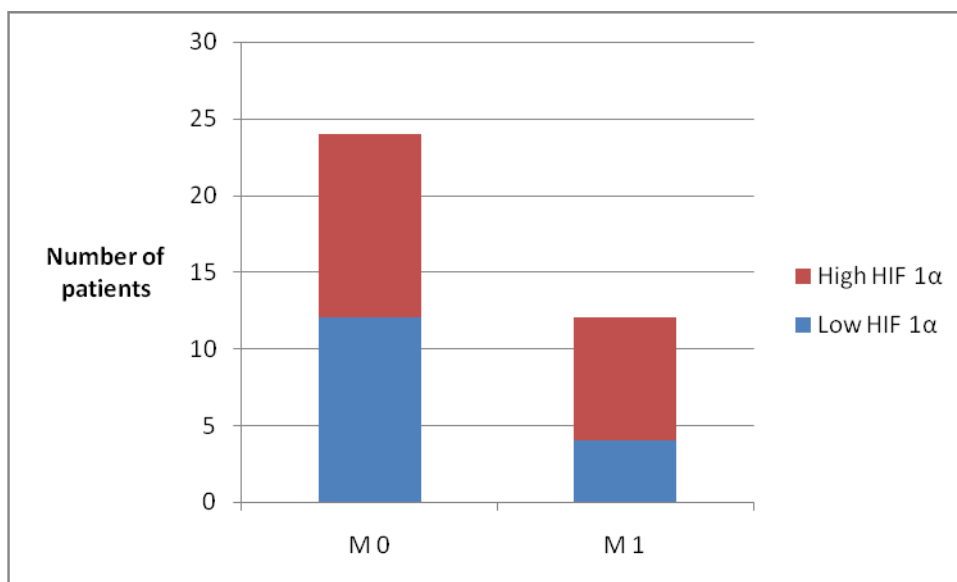
Figure 19. HIF 1α and M stage

3.7.6 Integrated UICC TNM stage

The expression of HIF 1α in the various stages of the disease correlates with the stages (p= 0.067) which was nearing statistical significance (Figure 20).
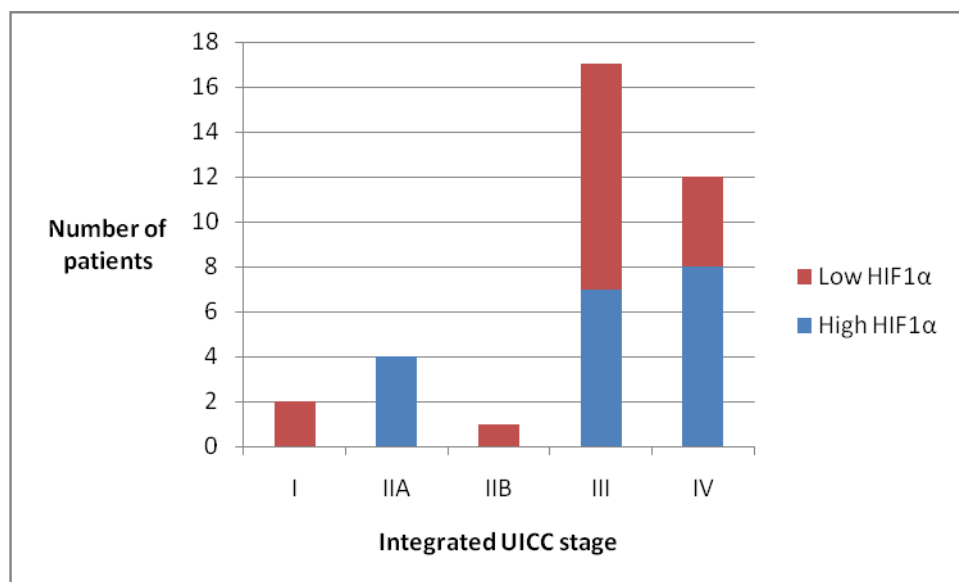
Figure 20. HIF 1α and Stage of the disease

3.7.7 Margins of the resected specimens

Eight patients had TSO with curative intent. Of these, 5 specimens had tumour free margins which were categorised as proximal, distal and circumferential margins. One specimen had positive proximal margin (HIF 1α expression score of 9) and 2 had positive circumferential margins (score of 0 and 14).

3.7.8 Tumour differentiation

No significant trend was observed with the expression of HIF 1α and the differentiation of the tumour (p=0.221) (Figure 21).
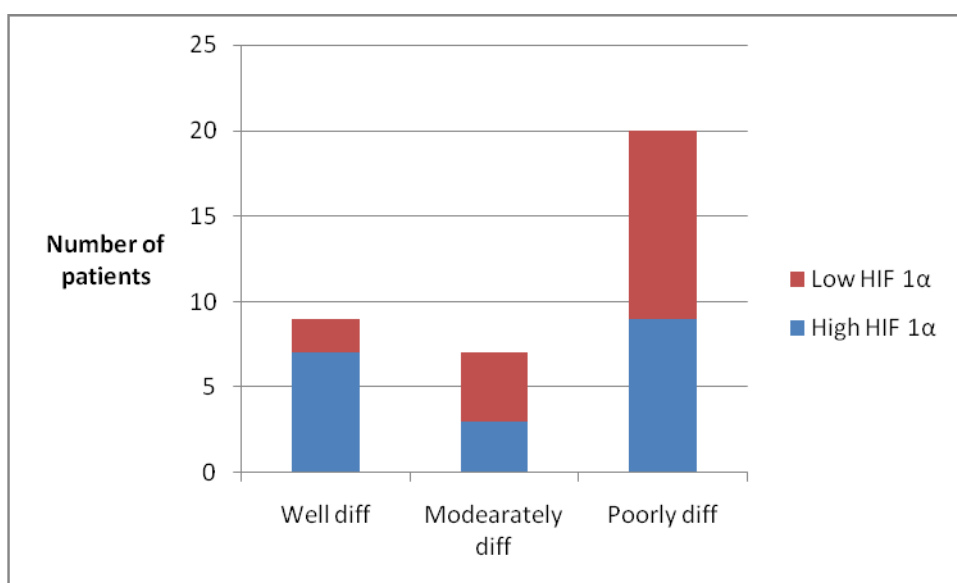
Figure 21. HIF 1α and degree of tumour differentiation

3.7.9   Aim of treatment

Aim of treatment in a way indicates the predicted prognosis of the disease and is mentioned as either treatment with a 'curative' intent or 'palliative' intent. Fifteen patients received treatment with curative aim, while the remaining 21 were treated either by palliative or supportive care.

There was no correlation between the expression of HIF 1α and the intended aim of treatment (p=0.169) (Figure 22).
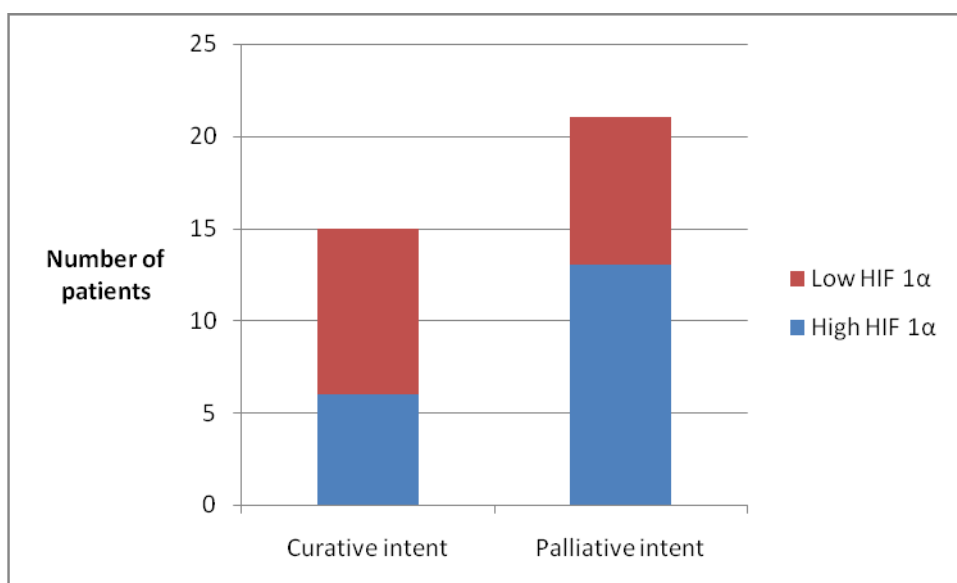
Figure 22. HIF 1α and aim of treatment

3.7.10 Summary

Summary of the correlation between the above mentioned clinico-pathological variables and the expression of HIF 1α in the tumour is summarised below in Table 5:

Table 5. HIF 1α and clinico-pathological variables

Variable	P value	Total (36)	Low expression of HIF 1α (17)	High expression of HIF 1α (19)
Gender	0.709			
Male		15	7	8
Female		21	10	11
Age	0.022*			
< 70 years		18	12	6
> 70 years		18	5	13
T stage	0.408			
T1		2	2	0
T2		6	2	4
T3		16	8	8
T4		12	5	7
N stage	0.846			
N0		9	4	5
N1		24	12	12
N2		3	1	2
M stage	0.238			
M0		24	13	11
M1		12	4	8

Variable	P value	Total (36)	Low expression of HIF 1α (17)	High expression of HIF 1α (19)
Integrated UICC TNM stage	0.067			
Stage I		2	2	0
Stage IIA		4	0	4
Stage IIB		1	1	0
Stage III		17	10	7
Stage IV		12	4	8

Tumour differentiation	0.221			
Well differentiated		9	2	7
Moderately differentiated		7	4	3
Poorly differentiated		20	11	9

Aim of treatment	0.169*			
Curative intent		15	9	6
Palliative intent		21	8	13

* Fisher's exact test

3.8 HIF 1α expression and survival analysis

The survival duration of the patients from the time of diagnosis of oesophageal cancer to the time of end of study period (or death) is presented below. The relation between survival pattern of specific patient groups and HIF 1α expression is presented, followed by overall survival pattern and HIF 1α expression. The correlation between various other demographical & clinic-pathological parameters and overall survival is then presented along with statistical analysis of prognostic role of HIF 1α in the study population.

Persistent disease is the presence of oesophageal cancer in a particular patient from the time of diagnosis till the end of follow up period, with no evidence of cure at any stage: twenty three patients had persistent disease. Of the remaining 13 patients, who were initially cured of the disease as evident on clinical, radiological and endoscopic evidence, 8 were diagnosed with recurrence of cancer during follow up and 5 patients showed no evidence of recurrence till the end of the study period. This information is represented in Table 6 below:

Table 6. Status of disease and survival of patients

Status of the disease at the end of study period	No. of patients alive	No. of patients dead	Total no. of patients
Persistent disease	3	20	23
Recurrence	3	5	8
Recurrence free	2	3	5

Further analysis of the survival in the above groups is presented below.

3.8.1 Patients with persistent disease (Table 7 and Figure 23)

Table 7. HIF 1α and survival in patients with persistent disease

HIF 1α expression	No. of patients alive (median survival in days)	No. of patients dead (median survival in days)	Total no. of patients (median survival in days)
High	2 (15%, 813)	11 (85%, 139)	13 (147)
Low	1 (10%, 1169)	9 (90%, 182)	10 (188)
Combined	3 (13%, 1011)	20 (87%, 155)	23 (171)
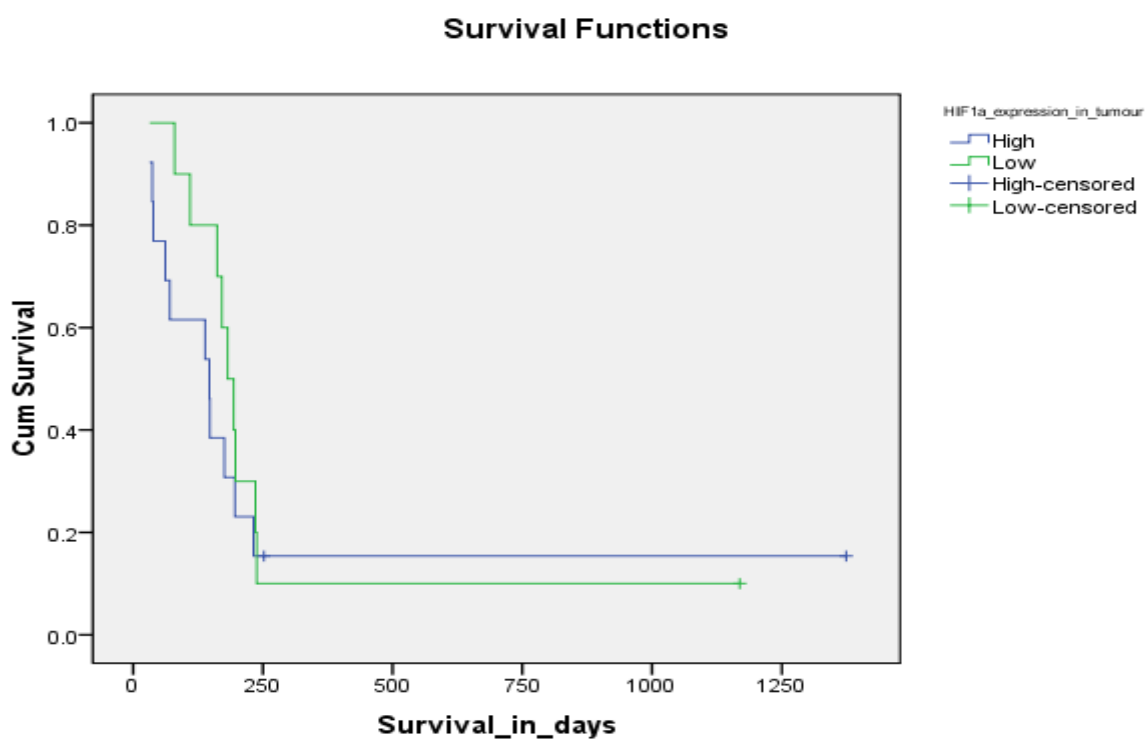
Figure 23. HIF 1α and survival curve in patients with persistent disease

The censored data corresponds to the patients who died during the study period.

There was no statistically significant correlation between the survival rate in patients with persistent disease and the strength of expression of HIF 1α (p = 0.457, log rank test).

3.8.2 Patients with recurrent disease (Table 8 and Figure 24)

Table 8. HIF 1α and survival in patients with recurrent disease

HIF 1α expression	No. of patients alive (median survival in days)	No. of patients dead (median survival in days)	Total no. of patients (median survival in days)
High	2 (40%, 508)	3 (60%, 267)	5 (428)
Low	1 (33.3%, 239)	2 (66.6%, 298)	3 (239)
Combined	3 (37.5 %, 428)	5 (62.5 %, 267)	8 (341)
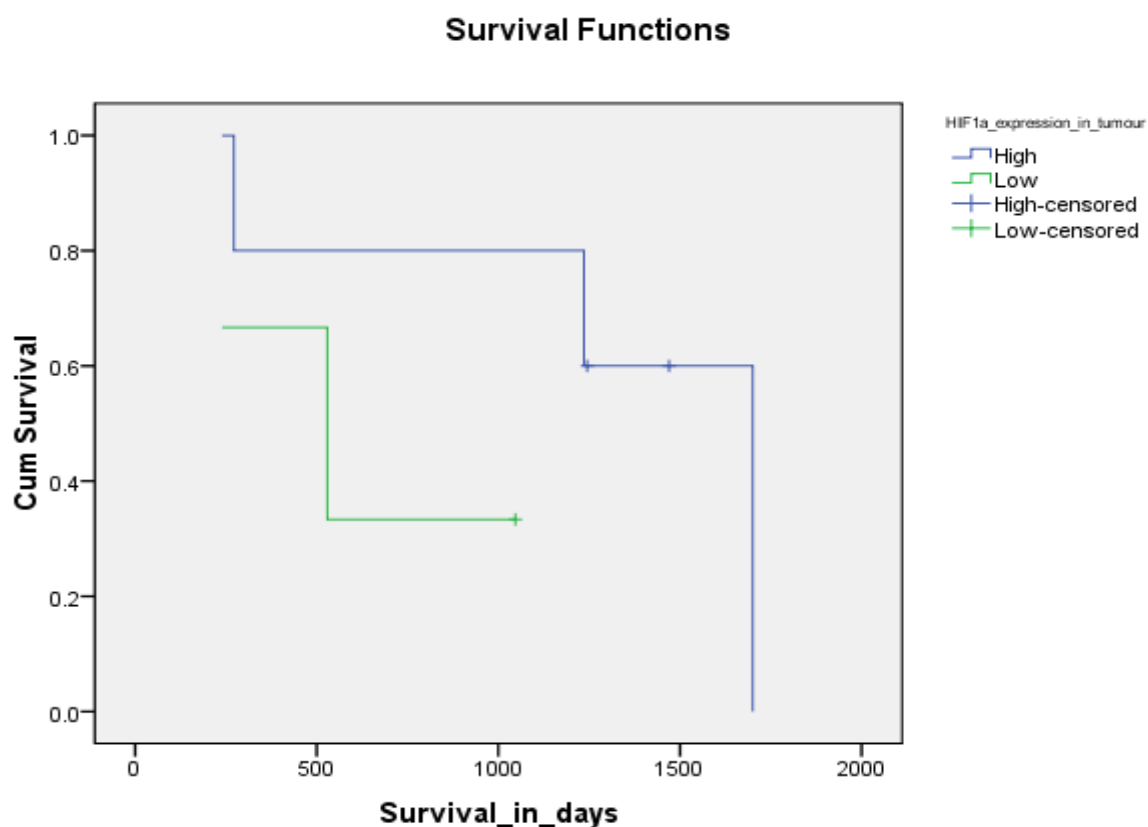
Figure 24. HIF 1α and survival in patients with recurrent disease

There was no statistically significant correlation between the survival rate in patients with recurrent disease and the strength of expression of HIF 1α ($p = 0.216$, log rank test).

3.8.3 Patients with recurrence free disease (Table 9 and Figure 25)

Table 9. HIF 1α and survival in patients with recurrence free disease

HIF 1α expression	No. of patients alive (median survival in days)	No. of patients dead (median survival in days)	Total no. of patients (median survival in days)
High	1 (100%, 1314)	0	1 (1314)
Low	1 (25%, 1495)	3 (75%, 473)	4 (559)
Combined	2 (40%, 1404)	3 (60%, 473)	5 (646)
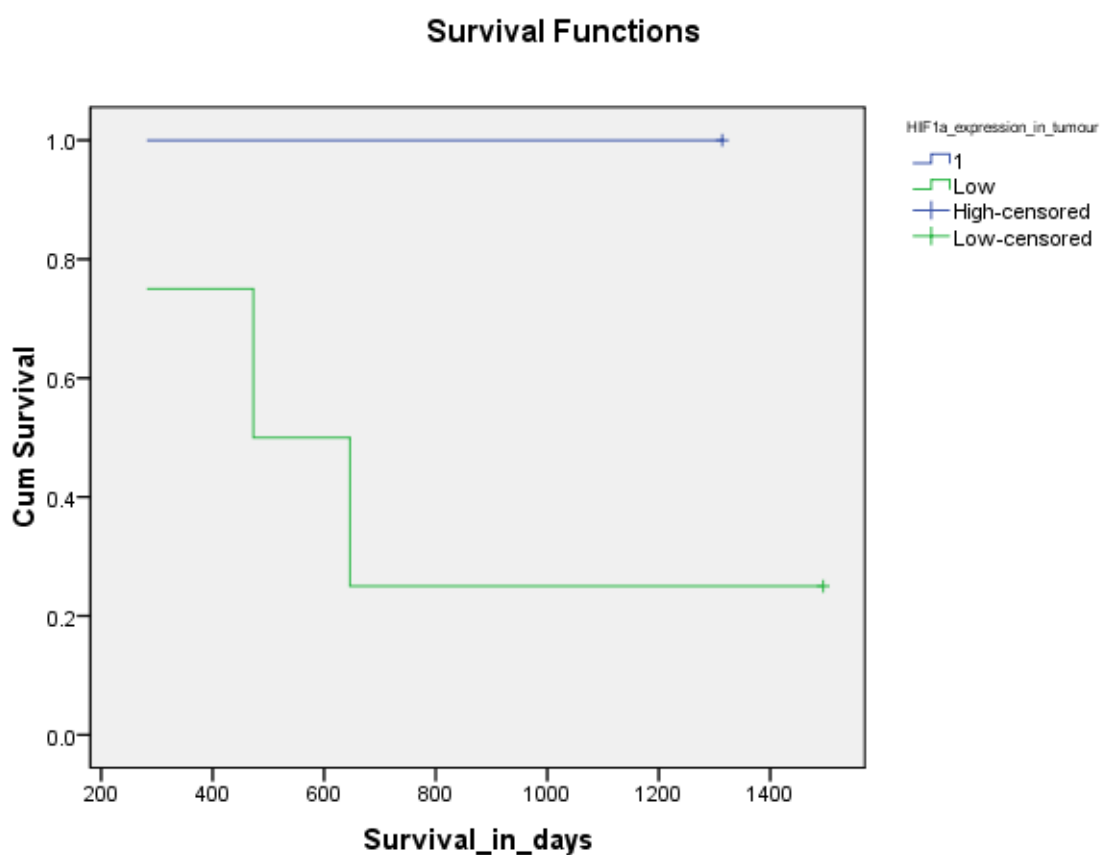
Figure 25. HIF 1α and survival curve in patients with recurrence free disease

There was no statistically significant correlation between the recurrence free survival rate and the strength of expression of HIF 1α ($p = 0.299$, log rank test).

3.8.4 Overall survival

Eight of the 36 patients were alive at the end of the study period spanning 4 years, with an overall survival rate of 22%. The overall survival of these 8 patients ranged from 252 – 1495 days. The survival of the remaining 28 patients who died in this period ranged from 32 – 1700 days.

The median overall survival for the 36 patients was 232 days after diagnosis. The overall survival rate at the end of 1 year (365 days) was 33.3%, 2 years (730 days) was 25%, 3 years (1095 days) was 25% and 4 years (1460 days) was 22%. The cumulative overall survival curve is presented below (Figure 26):

Survival Function
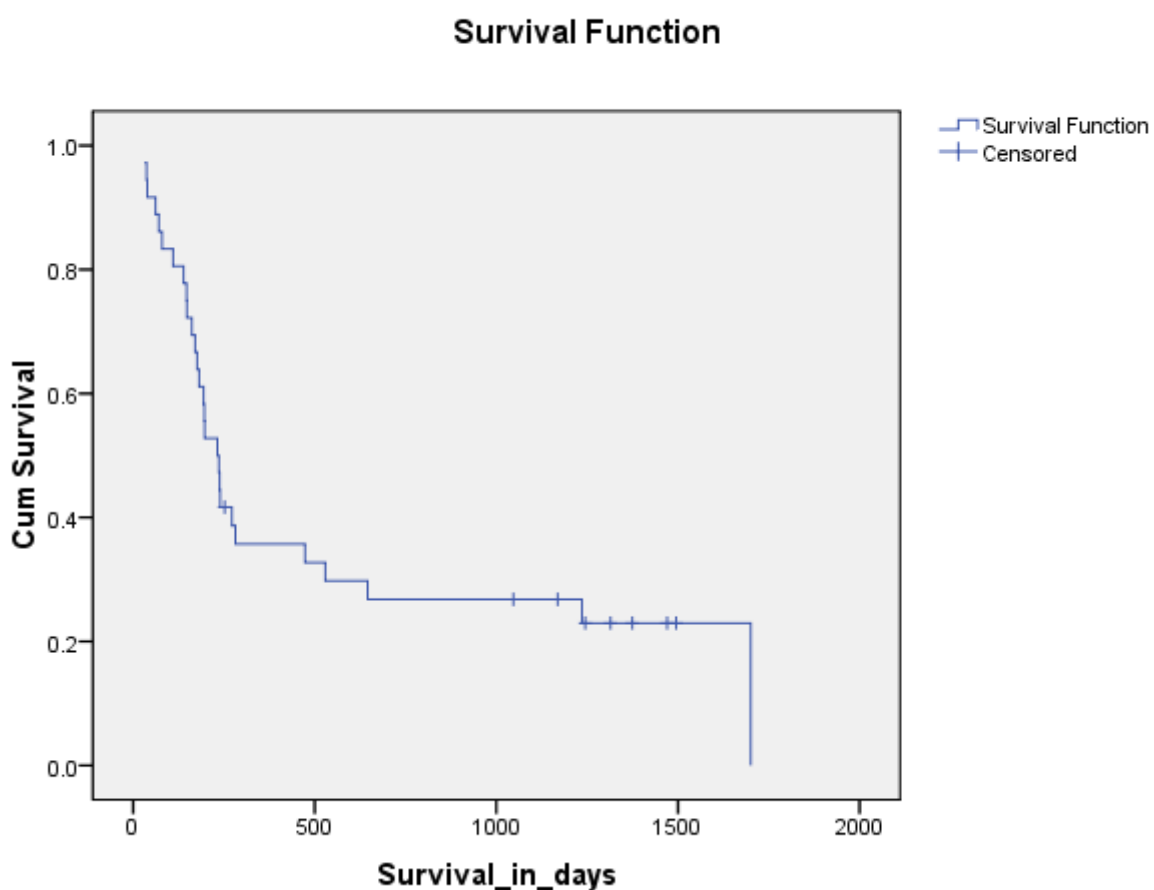
Figure 26. Overall survival curve in patients with SCC

The censored data corresponds to the patients who died during the study period.

Correlation of overall survival and HIF-1α expression:

Out of 17 patients showing a low expression of HIF 1α, 14 (82.36 %) died and 3 (17.64 %) were still alive at the end of study period, with a mean survival of 438 days and median survival of 238 days.

Out of 19 patients showing high expression of HIF 1α, 14 patients died (73.68 %) and 5 (26.32) were still alive, with a mean survival of 533 days and median survival of 196 days (Figure 27).
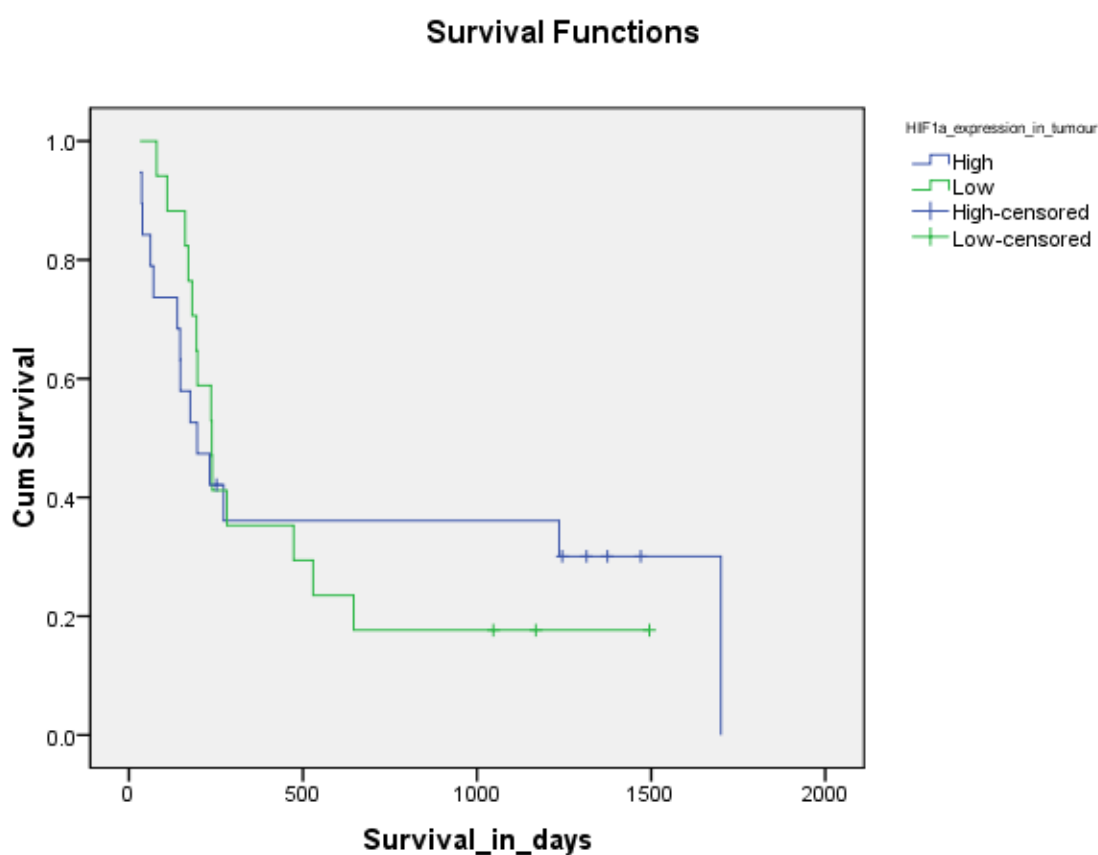
Figure 27. HIF 1α and overall survival curve

The censored data corresponds to the patients who died during the study period. There was no statistically significant correlation between the overall survival rate and the level of expression of HIF 1α ($p = 0.908$, log rank test).

3.8.5 Demographical & clinico-pathological variables and survival analysis

The correlation between various other demographical & clinic-pathological parameters and the overall survival is shown below:

3.8.5.1 Gender (Figure 28)
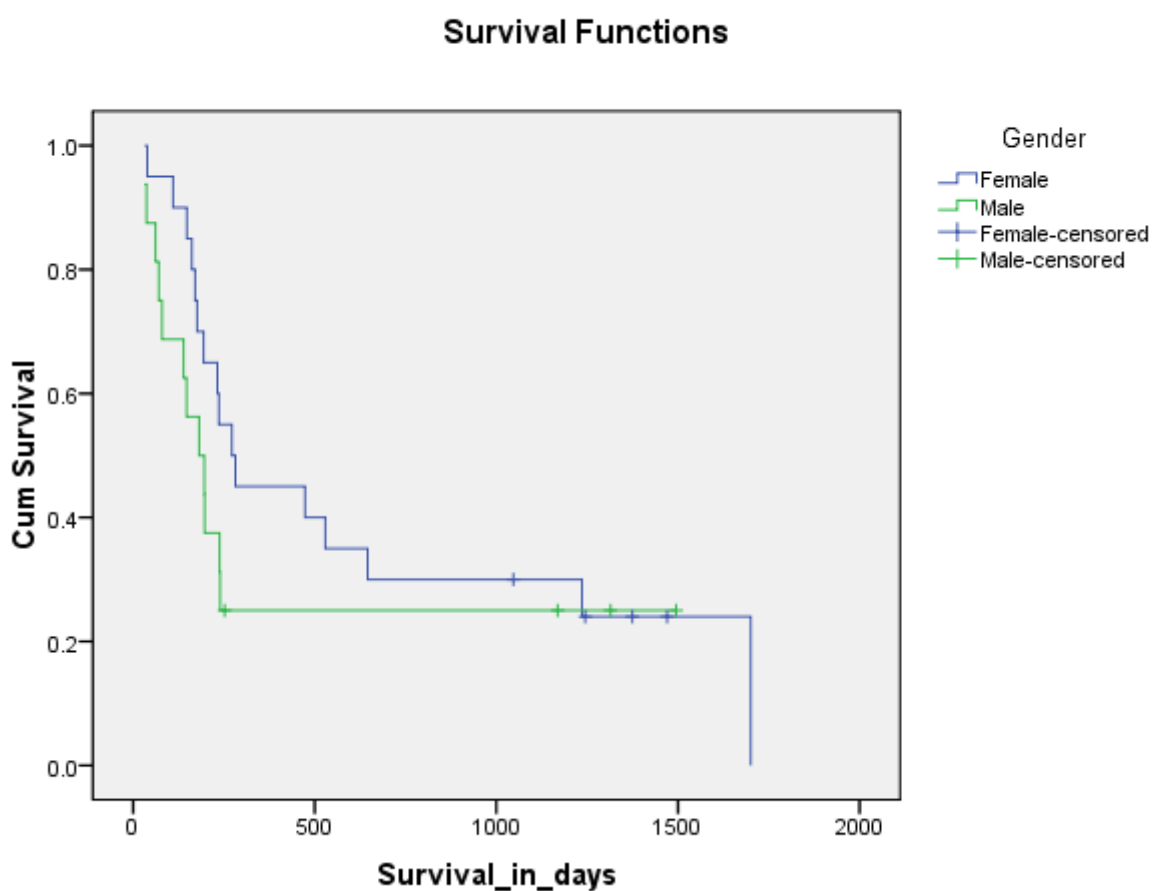
Figure 28. Gender and overall survival curve

There was no statistically significant correlation between the gender of the patients (M=15, F=21) and the overall survival ($p = 0.285$, log rank test).

3.8.5.2 Age (Figure 29)
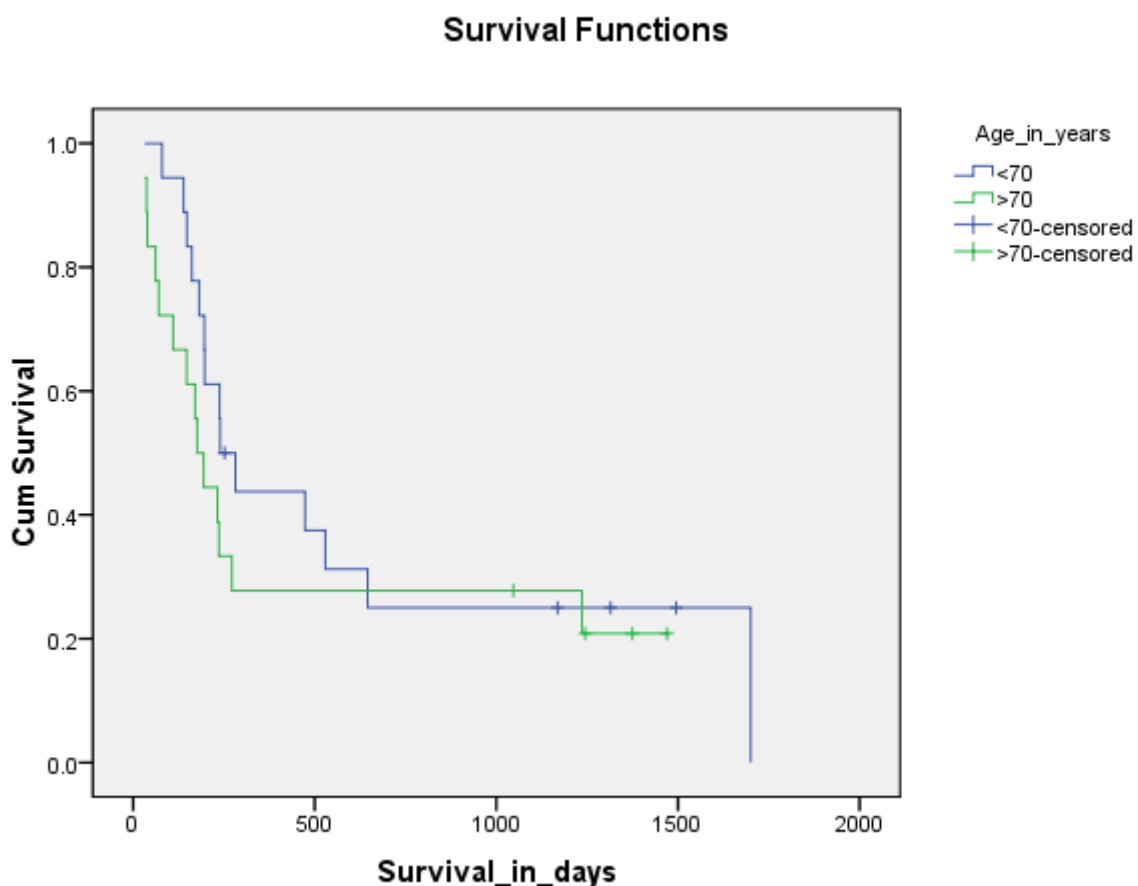
Figure 29. Age and overall survival curve

Age of the patient is an important decision maker in treating individuals with oesophageal cancer, with 70 years being considered as the age below which there is physiological reserve for a patient supportive enough of TSO; hence this age is taken as a parameter to evaluate the survival pattern, although not all patients are considered for surgery.

There was no statistically significant correlation between the age of the patients (age >70 years = 18, age < 70years = 18) at diagnosis and the overall survival ($p = 0.350$, log rank test).

3.8.5.3 Depth of tumour invasion (T stage) (Figure 30)
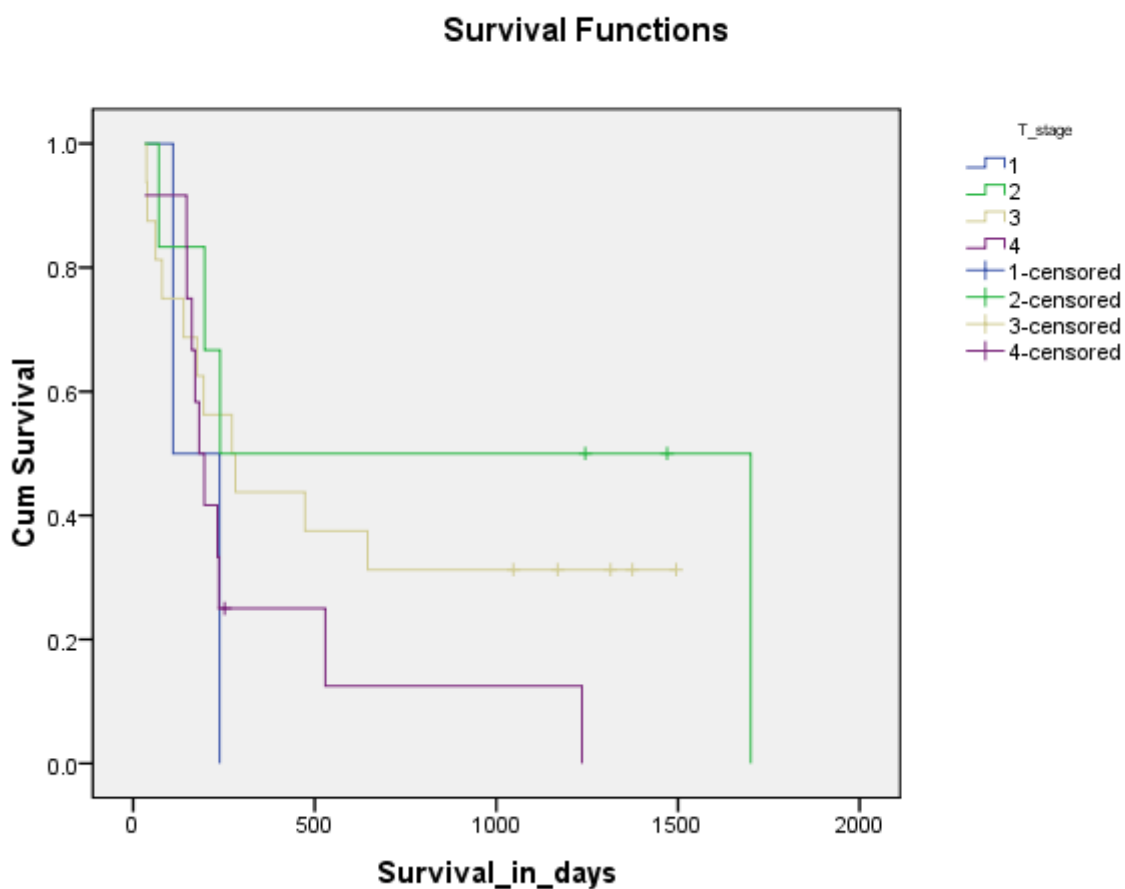
Figure 30. T stage and overall survival curve

There was a trend for better overall survival with early T stage (T1 and T2) disease (T1 = 2, T2 = 6, T3 = 16, T4 = 12 patients) but this did not reach statistical significance (p= 0.192, log rank test).

3.8.5.4 Lymph node (N stage) (Figure 31)
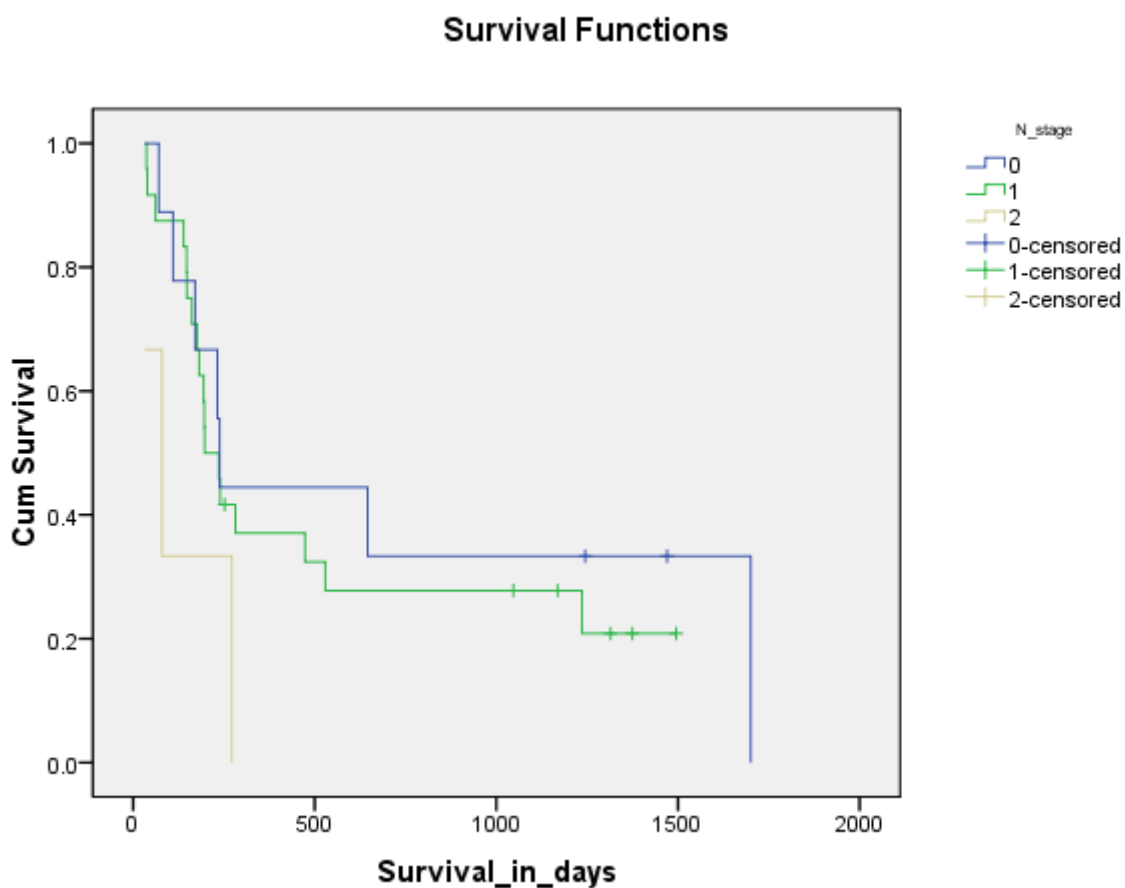
Figure 31. N stage and overall survival curve

Again, there was a trend for better overall survival with early N stage disease (N0 = 9, N1 = 24, N2 = 3 patients), but this did not reach statistical significance (p= 0.171, log rank test).

3.8.5.5 Metastasis (M stage) (Figure 32)

Survival Functions
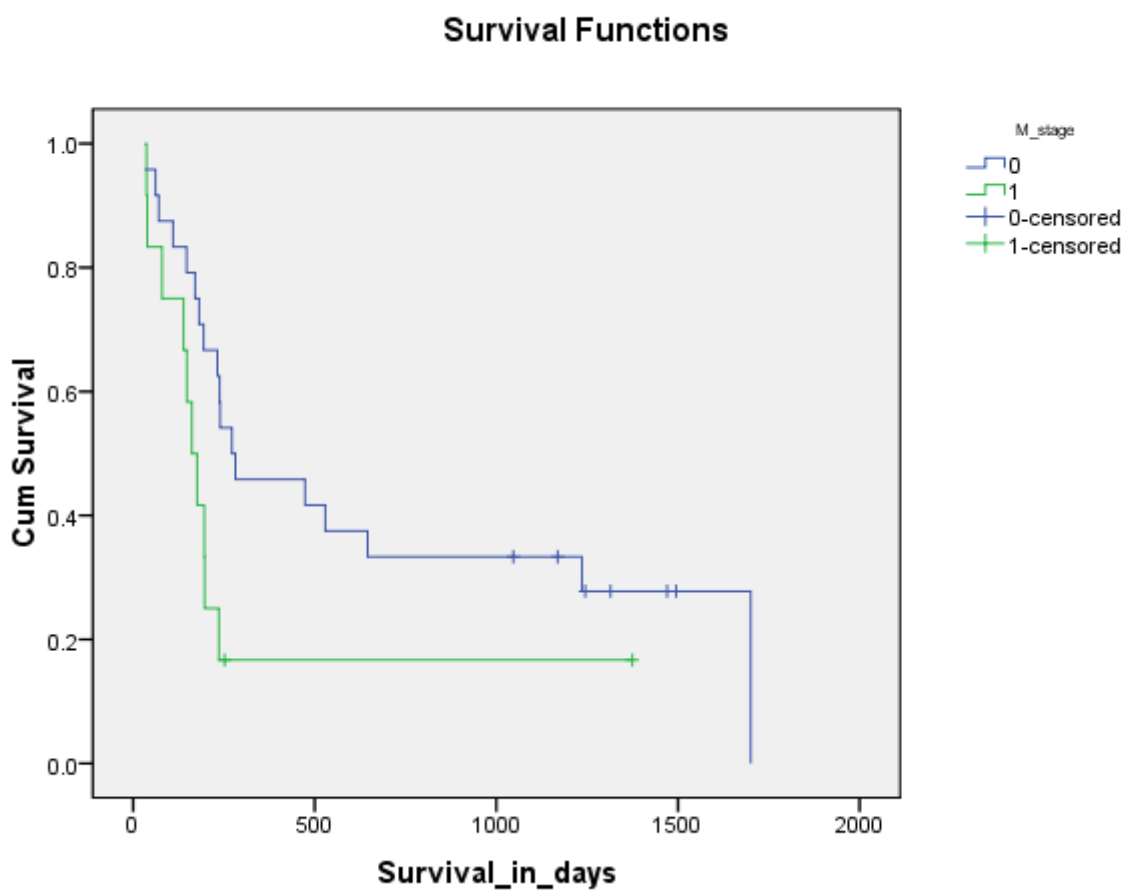
Figure 32. M stage and overall survival curve

There was significant survival advantage for those with no metastatic disease at the time of diagnosis (M0 = 24, M1 = 12 patients; p= 0.05, log rank test).

3.8.5.6 Integrated UICC TNM stage (Figure 33)
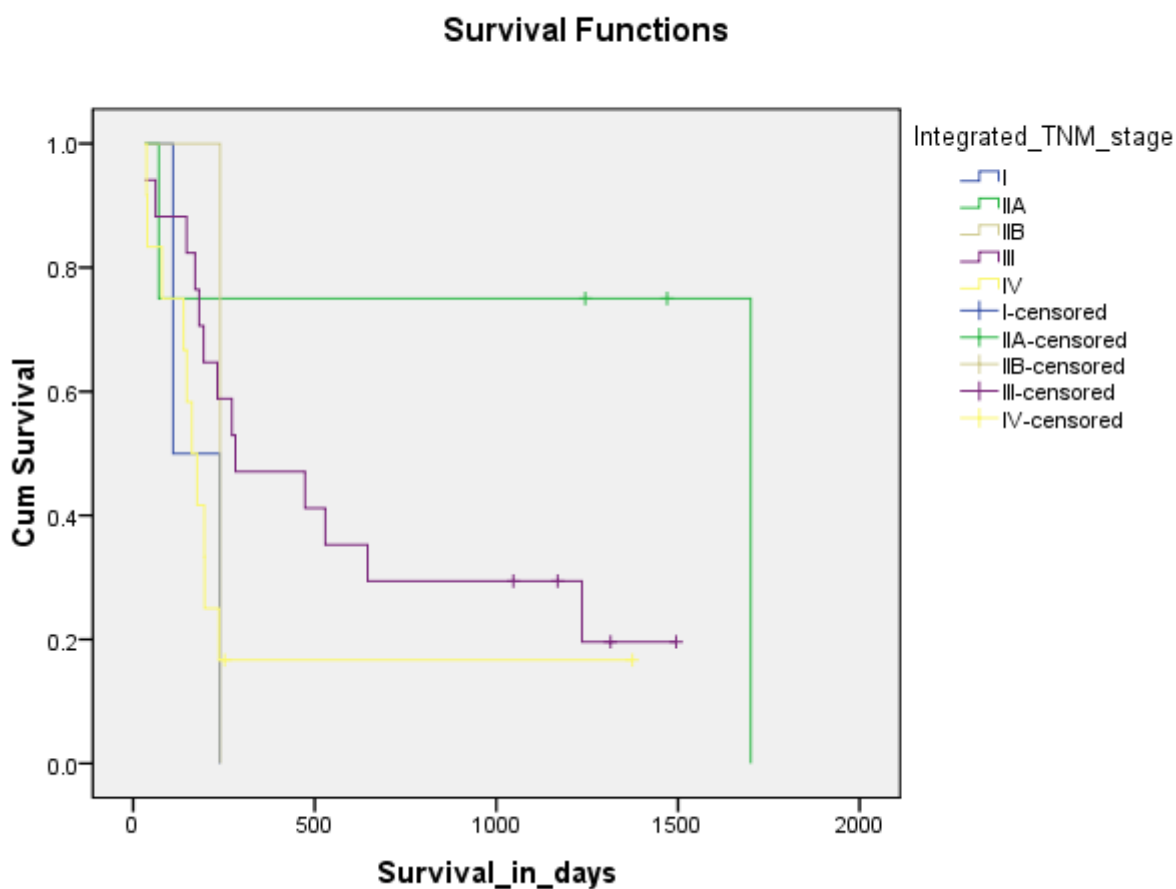
Figure 33. TNM stage and overall survival curve

There was a trend of survival advantage for patients with low stage disease (Early stage = 7, Late stage = 29 patients; p= 0.122, log rank test), but this was not statistically significant.

3.8.5.7 Degree of differentiation of tumour (Figure 34)
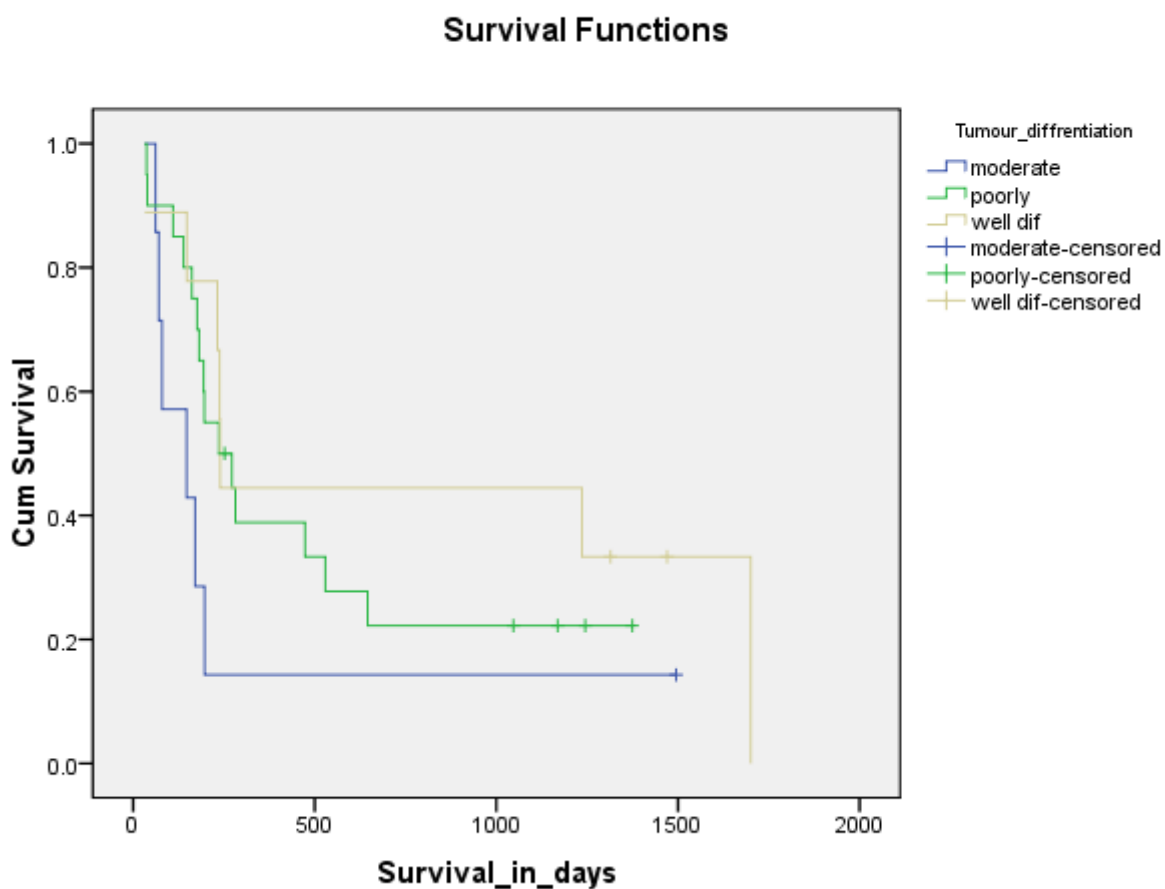
Figure 34. Differentiation of tumour and overall survival curve

There was a trend for survival advantage for patients with well differentiated tumour, but not at statistically significant level (Well differentiated = 9, moderately differentiated = 7, poorly differentiated = 20 patients; p= 0.194, log rank test).

3.8.5.8 Aim of treatment (curative vs. palliative) (Figure 35)
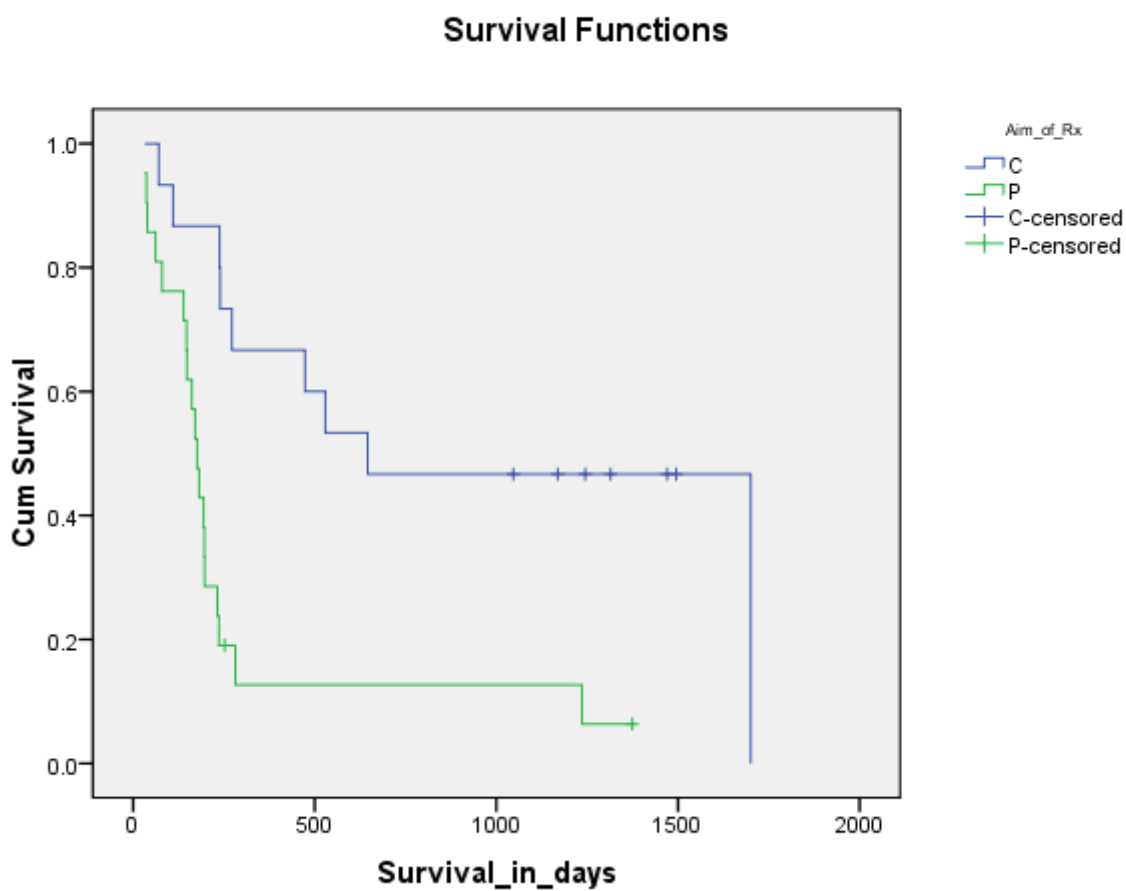
Figure 35. Aim of treatment and overall survival curve

There was significant survival advantage in patients treated with curative intention (Curative intention = 15, palliative intention = 21; p= 0.001, log rank test).

3.8.5.9 Multivariate analysis

On multivariate analysis, treatment aim as curative intent was the only statistically significant independent prognostic indicator.

3.8.5.10 Summary

The above results are summarised below as Table 10.

Table 10. Summary of Univariate analysis of factors associated with overall survival in patients with SCC of oesophagus:

Variable	Number of patients (36)	Median survival (in days)	P value
HIF 1α expression			0.908
Low	17	238	
High	19	196	

Gender			0.285
Male	15	182	
Female	21	272	

Age (years)			0.350
< 70	18	240	
> 70	18	176	

T stage			0.192
T1	2	110	
T2	6	242	
T3	16	272	
T4	12	182	

N stage			0.171
N0	9	238	
N1	24	197	
N2	3	80	

Variable	Number of patients (36)	Median survival (in days)	P value

M stage			0.051
M0	24	272	
M1	12	162	

Integrated UICC TNM stage			0.122
Stage I	2	110	
Stage IIA	4	1700	
Stage IIB	1	240	
Stage III	17	282	
Stage IV	12	162	

Tumour differentiation			0.194
Well differentiated	9	240	
Moderately differentiated	7	147	
Poorly differentiated	20	236	

Aim of treatment			0.001
Curative intent	15	646	
Palliative intent	21	176	

CHAPTER FOUR

DISCUSSION

4.1 General discussion

The present study is the first reported and published one (Munipalle et al, 2011; Appendix 9) on the role of HIF 1α in oesophageal squamous cell carcinoma in a UK population and second study in European population. Ling et al in 2006 reported the only other similar study on SCC of oesophagus in European population, carried out in Germany. This study group differs in patient characteristics and possible aetiology of the disease from previous studies, which were reported mostly on Asian populations (Kurokawa et al, 2003; Kimura et al, 2004; Matsuyama et al, 2005; Katsuta et al, 2005; Chen et al, 2009). This is important due to the recently available evidence that frequency patterns of HIF 1α polymorphism are different between Asian and European populations, as reported by Kim et al (2008) in their study on breast cancer. Whether these differences in genetic expression translate into clinical behaviour of the tumours is currently not known.

For staining the tumour tissues, previously validated technique and reagents including monoclonal antibodies were used. Our results showed that the strength of expression of HIF 1α obtained using this methodology was good and easily identifiable. The scoring pattern showed the level of expression of HIF 1α and the ratio between high and low expressing tumours to be comparable with previously reported studies (Kurokawa et al, 2003; Matsuyama et al, 2005; Katsuta et al, 2005). The agreement between the two independent observers (Cohen's Kappa 0.82, $p<0.01$) regarding the expression of HIF 1α can be rated as excellent to perfect according to various statistical authors (Koch & Landis, 1977; Fleiss, 1981). We have set the expression level of HIF 1α at 10% as the cut-off mark in our study. If the scores of the same sample from the two observers fell on either side of this cut off mark, then the results from particular sample were discussed between them to agree upon a mutually acceptable average score. The scores of three specimens were finalised in this manner. In each instance, the initial scores were found to be close to each other, even though falling on either side of the cut-off mark. This confirms that the scoring of expression of HIF 1α using similar technique of immunohistochemistry is repeatable between different observers.

The reliability of the laboratory tests was ensured by the inclusion of the control material during staining of the test slides. All the RCC specimen slides employed as positive controls stained positive during the tests, and hence there were no false

negative results. Similarly, all the tests where external and internal negative control were provided by TBS stained negative for the expression of HIF 1α, eliminating the possibility of false positives. Inclusion of the control material in the protocol had ensured good validity and reliability of the staining process. The use of control material was not explicitly reported in some of the published studies, and hence the occurrence of false positives and false negatives could not be ascertained from their reports in contrast to the present study.

HIF 1α was shown not be expressed in normal oesophageal epithelium, as concluded in many previous studies (Koukourakis, 2001; Sohda et al, 2004; Chen et al, 2009). The expression of HIF 1α is modulated and up regulated only in the hypoxic cells of oesophageal tumour than healthy epithelium, and any valid reason could not be justified to repeat similar process again on healthy epithelial cells. Hence the identification of HIF 1α in normal oesophageal epithelium was not included in the present study.

Researchers have reported significant differences in the expression pattern and levels of HIF 1α in previous studies on SCC. The reasons for this phenomenon are poorly understood. The HIF 1α levels are probably a reflection of the degree of hypoxia in the tumour tissue at the time of biopsy. It was shown that the degree of hypoxia increases towards the centre of the tumour, to facilitate tumour survival through recruitment of anaerobic mechanisms (Busk et al, 2009). Seeber et al (2010) showed that in endometrial carcinoma, the expression pattern of HIF 1α can be perinecrotic, diffuse or mixed. They postulated that the perinecrotic expression is hypoxia driven, while the diffuse pattern is not. So the area in a tumour that is biopsied would influence the level of expression of HIF 1α and subsequent identification. It would be difficult to standardise the area of biopsy across different studies so that the results could be compared.

In addition, different methods of identifying HIF 1α and scoring its expression were employed in various studies as mentioned in chapter 2. This may have resulted in different degrees of expression of HIF 1α in the SCC tissues studied. But it might be possible to standardise the process of quantifying the HIF 1α expression levels in the lines of some previous interesting studies. Ugurel et al in 2001 published the reliability of measuring various angiogenic factors namely angiogenin, VEGF, basic fibroblast growth factor and interleukin (IL-8) in the serum of 125 malignant

melanoma patients in comparison with 30 healthy volunteers. They found their levels to be correlating to stage of the disease, and being predictive of overall and progression free survival. Literature search failed to yield any such serological studies measuring HIF 1α levels in cancer patients. The reason for this could be the presence of HIF 1α within the nuclei of the cells, rather than as a serum factor.

ELISA method could also be employed for quantitative measurement of HIF 1α protein in solid cancers, which was not possible with immunohistochemistry alone. Formento et al in 2005 identified and validated ELISA technique to quantitatively measure HIF 1α levels in in-vitro tumour cell lines. Studies on this promising quantitative test may provide a platform to objectively measure HIF 1α expression levels in various human cancers without subjective bias.

Another interesting finding was the ethnicity related variations in the expression of HIF 1α. Ribeiro et al (2009) investigated the frequency of C1772T and G1790A alleles of HIF 1α gene in four distinct populations from Columbia, Portugal, Mozambique and Guinea-Bissau. They discovered statistically significant differences among these populations and suggested a role for this differential expression in tumour behaviour and aggressiveness. Currently no information is available information on the genetic make-up of HIF 1α among UK population and I suggest further studies on the aspect.

Genetic polymorphism of HIF 1α expression contributing to tumour characteristics was suspected for a long time - evidence is now available to support this theory. In addition to the study of Ling et al (2005) as described earlier, Shieh et al (2010) studied C1772T and G1790A polymorphisms of HIF 1α in oral SCC to identify the effect of these on tumour behaviour. They reported a relation between genetic polymorphism and the tumour characteristics. Similar polymorphic variations in HIF 1α genotype were identified in other solid organ cancers like renal cell carcinoma, stomach, liver, pancreas, cervix & uterus, breast, colorectal and prostate cancers. These findings support further studies on the genetic make-up of HIF 1α to evaluate the role of these variations contributing to its function.

4.2 Analysis of the results - HIF 1α and clinico-pathological variables

The patient group had 15 male and 21 female patients, with more preponderance of female patients compared with national figures; the male: female ratio with SCC was 1:0.95 as per the National audit 2010. The HIF 1α expression pattern showed no relation to the gender of the patients (p = 0.709). Similar results were showed by Kurokawa (2003), Sohda (2004), Katsuta (2005) and more recently Chen (2009). Kimura (2004) however reported significantly higher expression of HIF 1α among male patients in his series of SCC – 31 male patients out of 72 showed higher expression, in contrast to 1 female patient among 10 with higher expression. His group had 72 male and 10 female patients though, in contrast to the present study. Interestingly the statistics show that the survival rates in oesophageal cancer at the end of 5 and 10 years are less in men than women (www.cancerresearchuk.org). Possible correlation between the higher expressions of HIF 1α in men and their relatively worse survival needs further study. This may alter the management of the disease on a gender basis.

The patient's age at the time of diagnosis was in the range of 52 – 95 years, with a median of 76 years, confirming this as a disease of older adults. The national figures show the median age to be 72 years (National audit, 2010). Most of the studies on Asian populations that reported patient's median age quoted this as being the 60's (Kurokawa (2003), Sohda (2004) Matsuyama (2005). This might represent a different patient population. To study the role of age on HIF 1α expression, patient age of 70 years was taken as the cut-off mark in the present study. The expression of HIF 1α in the resulting two groups of patients, namely aged above and below 70 years was observed. Patient's age of 70 years was identified as a bench mark because during decision making regarding the fitness of a patient for TSO, 70 years was traditionally chosen as a limiting factor. Patients aged above 70 years were considered to have limited physiological reserve and hence a major operation like TSO was not routinely considered above this age due to possible high operative morbidity and mortality in this group (Elsayed et al, 2010). This practice may influence the outcome of the disease indirectly through the decision making as to the intention of treatment. There was significant higher expression in patients aged above 70 years (p = 0.022) compared to a younger population but the clinical significance of this is currently not known. It is important to identify through the

expression of HIF 1α if the characteristics of the disease vary in the elderly, as this may influence the current decision making regarding the management of this specific age group patients. The present study was the first reported one demonstrating the positive relation between the old age of the patient and expression of HIF 1α. Kurokawa's study (2003) showed higher expression in those aged above 60 years, but not to a significant level. In contrast, more recently Chen et al (2009) reported nearly significant low expression in patients aged above 60 years. The reason for a cut off value of 60 years was not supported in either paper. Further studies are recommended to evaluate the role of patient's age on the expression pattern of different molecular prognostic markers. If the association of age with the prognosis of the patient is more firmly illustrated, this evidence could play a vital role in treating the elderly with any stage of oesophageal cancer. To the author's knowledge, there were no reported studies that investigated the relation between patient's age at diagnosis and the survival, independent of the mode of treatment.

T stage disease pattern showed two patients with T1, six with T2, 16 with T3 and 12 with T4. Higher numbers of patients in the present study were of a late T stage compared with the studies of Kurokawa (2003) and Katsuta (2005). This might be due to the well established screening programmes for oesophago-gastric cancer detection in the Far East, resulting in identifying the cancer at an early stage. The expression of HIF 1α was not significantly different across the four T stages of the disease ($p = 0.408$). While the T stage was compressed into early (T1 and T2 together, with 8 patients) and late (T3 and T4 together, with 28 patients) stages, the expression pattern was again similar with no difference between these two stages. Findings from present study are in agreement with those of Sohda (2004), Matsuyama (2005) and Katsuta (2005). In contrast, Kimura (2004) and Chen (2009) reported lower expression in early T stage, while Kurokawa (2003) showed higher expression with less depth of invasion, similar to the standard early T stage. These conflicting results may demonstrate the non-uniformity of expression across different patient groups. It is currently not known if the expression pattern of HIF 1α can change as the disease progresses from early to late stage. There are practical and ethical issues involved in studying the progression of the disease on the expression pattern of HIF 1α though – in patients with treatable disease which responds well to treatment, it is not possible to study the progression. In patients with advanced

disease at the time of presentation, not much information could be gained by the follow up of the disease, and it might be unethical to subject them to further tests which are not likely to benefit them personally.

Tumour differentiation is considered as one of the indicators of inherent tumour aggressiveness with well differentiated tumours generally showing good prognosis. While majority of patients in the present study had poorly differentiated tumours (20 out of 36), the expression pattern of HIF 1α did not differ significantly across the groups (p = 0.221). Chen et al (2009) reported significantly higher expression as the tumour differentiation gets poorer.

The expression pattern was not influenced by lymph nodal status either. 24 patients had N1 status, followed by 9 patients with no lymph nodal involvement (N0) and 3 patients with N2 status. No significant differences were observed among these groups (p = 0.846), similar to the reporting of Sohda and Matsuyama. There were conflicting results from other studies. Katsuta and Chen described much higher expression in tumours with lymph nodal metastases, in contrast with Kurokawa who did not. One possible answer for these contrasting results could be explained by genetic polymorphism, which was the presence of genetic structure in different forms within the same species. It was observed that SCC tumours with certain HIF genotypes (C/T) were associated with higher lymph node metastases and this might be the reasons for the different results in the studies so far (Ling et al, 2005). He studied the single nucleotide polymorphism (C1772T) in HIF 1α gene and reported that genotype C/T was found to be associated with larger tumours and more frequent occurrence of lymphatic metastases.

Analysis of expression of HIF 1α as per the metastases stage (M) showed that there was difference between the two groups. Eight out of 12 primary tumours which metastasised showed higher expression, compared to 12 out of 24 primary tumours which were locoregionally confined. This may represent the inherent nature of some tumours to adapt to hypoxic environment better and hence spread beyond the primary tumour. The different levels of expression did not reach statistical significance though (p = 0.238), in agreement with Sohda. Again, Kurokawa reported that tumours with no evidence of metastatic spread showed significantly higher expression of HIF 1α in contrast to us. These finding show the inconsistencies in the expression of HIF 1α pertaining to various tumour characteristics. The presence of

occult metastases and micrometastases at the time of initial diagnosis is one of the main reasons for so called 'tumour recurrence' and the current diagnostic techniques are insufficiently powered to detect the presence of these. If HIF 1α expression could help identify the tumours which are likely to metastasise, patients with such primary tumours could be treated more aggressively with systemic therapies so as to minimise potential spread. Multicentre retrospective studies looking at the metastatic status of a large number of patients with SCC and the HIF 1α expression levels in the primary tumour may provide answers for this.

However, there was marked higher expression of HIF 1α in integrated TNM stage IV tumours compared to stage III, nearly reaching statistical significance (p = 0.067). This may again represent the trend of higher expression in more advanced or more aggressive tumours. Chen also reported similar pattern. It is not known at this stage if the expression pattern gets more prominent as the tumour advances, or the tumour advances due to the higher expression. The results from present study differed from previous studies of Kurokawa et al (2003), who showed significantly higher expression in early p (pathological) stage disease.

The aim of the treatment showed only a trend of difference in the pattern of expression of HIF 1α in the present study (p = 0.169). The aim of treatment for a cancer patient is described as with either curative intent or palliative intent, depending upon various disease and patient specific factors. Fifteen patients received treatment with curative intent while 21 were treated palliatively. It is interesting that HIF 1α expression was higher though not significantly in the group of patients treated with palliative intent, which may reflect the advanced nature of the disease and hence expected poorer prognosis.

The present study could not show statistically meaningful results concerning the expression levels of HIF 1α with two variables. One of them was the comparison of the expression levels of HIF 1α in lymph nodes with metastatic spread. Five operative specimens showed the involvement of metastatic lymph nodes all of which expressed HIF 1α, with one N1 node and one N2 node demonstrating high expression. Three N1 nodes lymph nodal specimens showed low levels of expression. Due to the small number of the specimens tested the results were insufficient for any statistical analysis.

We had similar results with resection margins of the tumour specimen. As the resected oesophageal specimen can be described as 'tubular', it has proximal, circumferential and distal margins. Presence of tumour in the resection margins represents the likelihood of residual tumour and subsequent high chances of local recurrence, and hence this parameter was included in the data. The National Audit 2010 reported that among 1907 patients who underwent TSO, the percentage of specimens where in the longitudinal and circumferential resection margins showed tumour presence was 6.4% and 29% respectively. In the present series, out of eight specimens from the patients who underwent TSO, one showed positive proximal margin and two showed positive circumferential margins on a similar trend. Two out of these three specimens showed high expression levels of HIF 1α while the other one showed low expression. Kurokawa analysed the expression pattern with relation to surgical margin among 130 SCC specimens and reported low expression in 6 out of 9 positive margins. While the involvement of the margins reflects more of a surgical technique, the tumour size also influences this which in turn is dependent on the inherent tumour biology.

Evaluating the expression of HIF 1α pertaining to lymphatic and vascular invasion by the tumour cells could be considered if more resected specimens were available. It is interesting to see that many previous studies (Kurokawa et al, 2003; Kimura et al, 2004; Katsuta et al, 2005) showed higher expression of HIF 1α associated with lymphatic invasion by tumour cells. Venous invasion also was reported to be associated with higher expression (Kimura et al).

The relation of HIF 1α expression with clinico-pathological variables was summarised in Table 5 (Chapter 3). Statistically significant higher expression was noticed in primary tumours of patients aged above 70 years. Only a trend of higher expression was noticed in tumours with stage IV disease compared to stage III disease, and in tumours treated with the aim of curative intent.

4.3 Analysis of results - HIF 1α and Survival (primary end point)

The recruited patients were followed up for a period ranging from 32 – 1700 days (1 - 56 months), with a median of 42 months. Five year study period was traditionally employed to identify the mortality figures from cancers studies and the present study nearly approached it, with the longest follow up being 4 year 8 months. The survival was reported as disease specific and overall survival.

Disease status during the study period separated the patient population into different groups with independent survival characteristics. 23 patients had persistent disease since the time of diagnosis with no evidence of cure (group A), while 8 had recurrence (group B) and 5 patients achieved complete cure with no evidence of recurrence (group C). From each of these groups respectively, 3, 3 and 2 patients were alive by the end of the study period. In group A, 13 showed high and 10 showed low expressions HIF 1α. There was no statistically significant difference in the survival pattern of patients with high and low expression and hence not related to the expression of HIF 1α ($p = 0.457$). In group B, five patients showed high and the rest low expressions of HIF 1α. Their survival pattern again was not different ($p = 0.216$). In group C, one patient showed high expression and the rest low expressions of HIF 1α. Again, the difference in the survival of this group was not statistically significant and independent of HIF 1α expression ($p = 0.299$).

The overall survival takes into consideration only the survival of the patient, irrespective of whether the death was caused by the cancer or any other unrelated causes. In many patients it was not possible to ascertain the actual cause of death. Overall survival is generally reported as the percentage of the patient population alive at a particular time since diagnosis. The overall survival rate in the study participant population at the end of one year from the time of diagnosis was 33.3%, 2 years was 25%, 3 years was 25% and 4 years was 22%. These rates were comparable with the hitherto reported UK survival figures (www.cancerresearchuk.org). Recently published results from the National Oesophago-gastric Cancer Audit 2010 show 2 year survival figures of 55% for patients treated with curative intent and 12% for those treated palliative. The present study comprised a mixed patient population with higher number of palliative group (23) compared to 13 patients in the curative intent group; so results were comparable to national figures.

Expression of HIF 1α showed a trend of influence on the overall survival of SCC patients in the present study. The median survival in the 17 patients with low expression of HIF 1α was 238 days, in comparison with 196 days in the 19 patients with high expression. However this difference was not statistically significant. The significance of the sample size in the present study might have influenced this result. For example, it was well established that 'T' and 'N' stages have a prognostic significance but this was not observed in our study likely due to small sample size. A bigger sample size could have possibly confirmed the role of these stages on survival. On similar lines, the role of HIF 1α could have been more clearly observed with a bigger sample size.

Our results were in agreement with many previous studies, which reported various levels of correlation between HIF 1α and survival. Koukourakis (2001), Kimura (2004), Katsuta (2005) and Chen (2009) showed a trend of poorer overall survival in patients with higher expression of HIF 1α but not at statistically significant level; Kurokawa (2003) reported significant poorer overall survival in patients with higher expression, but not as an independent prognostic indicator; Matsuyama (2005) reported statistically significant worse disease free survival associated with higher expression, but again not as an independent indicator.

Ling et al (2006) showed that HIF 1α mRNA or protein expression did not correlate with histomorphological regression or survival following neoadjuvant chemotherapy for SCC. Kimura (2004) eliminated the effect of neoadjuvant chemotherapy on survival by looking at the results of patients who were not given this treatment, and reported a contrasting significant survival advantage in the group with low HIF 1α expression.

Ogane (2010) published the only available direct evidence linking HIF 1α expression to prognosis in SCC. His group studied 96 operated specimens with pT1 disease and showed that HIF 1α expression was positively correlated to lymph node metastases. Moreover, both disease free and overall survival irrespective of the lymph node status were influenced by HIF 1α expression, with higher expression predicting poorer survival. However, all the patients had disease confined to T1 stage in their study, which is to be taken note of. His results could not be applied across the spectrum of pT disease stages, while many other studies included patients with all pT stage patients in their studies. Majority of the patients with T1 disease would be

treated with curative aim anyway and hence the need to identify a better prognostic indicator is more vital with more advanced disease. Many studies including the present one have included all the T stages of the disease for this reason (Koukourakis (2001), Kurokawa (2003), Kimura (2004), Katsuta (2005) and Chen (2009)).

The effect of HIF 1α expression on survival probably depends on the interplay between inherent malignant potential of the tumour and the balance between apoptotic and anti-apoptotic genes. Sowter et al (2001) demonstrated that the presence of cell death genes like Bcl2/adenovirus EIB 19kD-interacting protein 3 (BNIP3) and its analogue Nip3-like protein X were amplified in the perinecrotic areas of tumour by the presence of HIF 1α. This action of HIF 1α is in contrast to its other effects like escalating anaerobic metabolism. At the same time, the up-regulation of apoptotic factors like p53 is independent of HIF 1α regulation (Wenger et al, 1998). Hence, it is currently believed that there are a multitude of hypoxia responsive factors balancing the tumour survival and it is this interplay that determines a solid tumour's behaviour and survival pattern.

4.4 Analysis of results - Other clinico-pathological variables and survival (secondary end points)

The overall survival pattern in relation to various clinic-pathological variables was analysed. The overall survival was independent of the gender (p = 0.285) and age (p = 0.350) of the patients in the study. The trend of improved overall survival were noted in patients with initial T stages (T1 and T2; p = 0.192), negative or early nodal stages (N0 and N1; p = 0.171), and early stages of integrated TNM (I, IIA and IIB; p = 0.122) but these trends did not reach levels of statistical significance. Similar result was noticed with well-differentiated tumours (p = 0.194).

We however recognised two independent variables with significant impact on overall survival, namely status of metastases (M) and aim of treatment. M status showed significant prognostic value in determining the survival pattern of the patients in the study. Patients with no evidence of metastases at the time of diagnosis showed significant survival advantage compared to those with metastases at the time of primary diagnosis. The absence of detectable metastatic disease confirms that the disease is confined to loco-regional stage at the time of diagnosis; this might represent either earlier detection of the disease or better biological behaviour by the

tumour. This would explain the better survival rate in this group of patients. But the M status failed to show significance as an independent prognostic indicator.

Similarly, patients treated with curative aim had significantly better survival rates than those treated with palliative aim. Opting for treatment with curative aim implies favourable factors – that the tumour was in early stage, and the patient has good performance status and could tolerate the treatment. It was expected that the combined effects of these factors was likely to produce the better survival rates in that sub-group of patients. In fact, only the aim of treatment was the independent prognostic factor on multivariate analysis of the study results.

The survival analysis was summarised in Table 10 (Chapter 3). No difference correlating with the expression of HIF 1α was noticed with the disease specific survival; a trend of better overall survival was noticed in the low expression group but again this difference was not statistically significant ($p > 0.05$, log rank test). HIF 1α was not an independent prognostic factor for survival ($p > 0.05$). Absence of metastases at diagnosis ($p = 0.05$) and treatment with curative intent ($p = 0.001$) were statistically significant prognostic factors on univariate analysis; on multivariate analysis, treatment with curative intent was the only independent statistically significant prognostic factor ($p < 0.001$).

4.5 Limitations of present study

The present study had some limitations which affected the usefulness of the results. These are discussed below.

4.5.1 Sample size

The study results could have been more robust if a larger number of patients were available for the study. Even though all the eligible patients during the study period were considered for recruitment, due to the limited number of the patients affected by SCC the numbers were confined to 43. The general condition of some patients further limited the study population. Six patients were frail and their disease was advanced by the time of identification of their disease. This led to their exclusion from the study. As many of the SCC patients are elderly, this fact has to be considered in considering sample size on similar population in future.

A longer recruitment period would have increased the sample size. Due to limited resources available the current study was confined to the present numbers.

4.5.2 Non-consideration of adenocarcinoma

The present study did not include the identification of expression of HIF 1α in ACC of oesophagus as part of the current project. The methodology that was used in the study was based on previous experiments done in JCUH laboratory, to identify the feasibility of scoring the expression of HIF 1α using the Mouse monoclonal (HIF 1α 67-sup clone) antibody to HIF 1α (ab463, Novus Biologicals, UK). These initial experiments showed satisfactory levels of expression of HIF 1α in SCC tumour cells, but not in ACC. Out of eleven SCC tumours, only one did not show any expression of HIF 1α while 23 out of 28 ACC tumours failed to show any expression (Ms A Ahitan, JCUH, personal communication); In the five specimens, the expression was faint and could not be utilized for the identification of expression levels. Hence the assessment of HIF 1α in ACC using the tested methodology was considered unreliable and accordingly the ACC patients were not included in the study group. Takala et al (2010) also reported very high occurrence of HIF 1α in SCC than ACC of oesophagus (p=0.009). Various other authors studied the expression only in SCC of oesophagus, but did not explicitly mention the reasons for not including adenocarcinoma (Kimura, Katsuta, Yu).

The reasons for the failure of satisfactory assessment of HIF 1α expression in ACC of oesophagus were not totally understood. This might be due to the differences in the molecular pathways or the hypoxic environments in these two distinctly different tumours. The reagents and antibodies used to identify the expression itself might be a cause for the lack of expression. It is interesting to note that so far only one centre has reported on the satisfactory expression of HIF 1α in oesophageal ACC. Expression of HIF 1α and other hypoxia inducible proteins in gastric and gastro-oesophageal adenocarcinoma was reported by Griffiths et al in 2007. His team used a different antibody (610958, BD Biosciences) and these results were published after out laboratory work was completed. Hence similar methodology could not be employed for the present study.

CHAPTER FIVE

CONCLUSIONS

The methodology employed for the present study was valid and reliable. The results suggested no statistically significant role for HIF 1α expression in influencing survival of SCC patients within the limitations of the study. Hence the null hypothesis that there is no relationship between the expression level of HIF 1α and survival rates in the recruited patients with SCC of oesophagus is accepted.

HIF 1α expression was higher in patients aged above 70 years but the significance of this is currently unknown. Better overall survival was observed in patients treated with curative intent and with no evidence of metastatic spread at the time of diagnosis, with only the aim of treatment being the independent prognostic indicator.

CHAPTER SIX

FURTHER WORK

The present study identified certain areas where further research would be beneficial in extending the current knowledge about the role of HIF in human cancers, and oesophageal cancer in particular. HIF 1α is gaining more attention due to the availability of drugs which can influence the cancer biology by inhibiting HIF 1α (Semenza, 2010). A variety of anti-cancer therapies could be potentially developed by studying the role of HIF 1α further. I would recommend the following works to facilitate development of these areas.

A multicentre research project is recommended to further study the expression levels of HIF 1α in oesophageal cancer tissues. Such a project would recruit a larger patient population and a retrospective design would further expand the available tissue sample size. The statistical validity of the information obtained pertaining to HIF 1α expression and various clinicopathological parameters would be further enhanced. This knowledge would assist in answering the question about whether further research is needed into the role of HIF 1α in SCC of oesophagus.

Another vital area for further attention is the increasing incidence of oesophageal ACC throughout the Western world and majority of the newly diagnosed oesophageal cancers are ACC in nature. Currently the incidence of distal oesophageal and gastro-oesophageal junctional ACC is increasing faster than any other malignancy in the Western world (Blot et al, 1991; Demeester, 2006). The prognostic role of HIF 1α in ACC needs to be evaluated further in view of the significant impact it may have on public health. HIF 1α was detected in adenocarcinomas of various organs in other studies. These include Griffiths et al (2007, gastric & GOJ); Wu et al (2010, colorectal); Jung et al (2011, prostate), Wang et al (2011, pancreas), Gu et al (2010, lung), Aprahamian (2010, liver), Espinosa (2010, endometrium), Iwashita (2010, renal cell carcinomas) etc. These studies used different methods and reagents for this purpose. Their methods and reagents could be utilised to identify the best methodology to study HIF 1α expression and its clinical relevance in oesophageal ACC.

Obesity, gastro-oesophageal reflux and Barrett's metaplasia were identified to be the major risk factors to ACC of oesophagus (Vial et al, 2010; Wang et al, 2011). Hence it is vital to study further the role of HIF 1α in the aetiology of Barrett's dysplasia-ACC sequence as this might have huge implications in the management of the oesophageal cancer. Studies on the role of HIF 1α expression in the adenocarcinoma

sequence are recommended to be carried out on the local population to develop knowledge in this area.

The tissue sampling and scoring methods to evaluate the expression of HIF 1α could be standardised. This would assist in comparing the results from different studies and in providing a platform for meta-analysis. Automated methods are available for quantitative identification in immunohistochemistry and trials are recommended to study the utility of these techniques in measuring HIF 1α expression. This could help in easy processing of a large quantity of sample material while reducing the observer associated bias.

Age of the patient which was found to show interesting relation with the survival of the patient deserves to be studied further. Similarly, further research into the association between patient's age and various molecular prognostic indicators is recommended. This knowledge may influence the treatment options which currently consider patient's age as a factor in choosing them.

The possible role of HIF 1α polymorphisms in tumour behaviour and in causing differences in disease characteristics across different ethnic populations needs further study. This might help in targeting specific forms of HIF 1α which are responsible to cancer in a given population. The genetic make-up of HIF 1α within the UK population also needs further research.

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Appendix 1 – Copy of the South Tees NHS Trust R&D approval letter

South Tees Hospitals **NHS**
NHS Trust

Academic Division
Directorate of Research and Development
The Academic Centre
The James Cook University Hospital
Marton Road
Middlesbrough
TS4 3BW

Our Reference: RGW/TW/rgw299-2004 / 2004112

14th December 2004

Mr P C Munipalle
Clinical Research Fellow in Upper GI Surgery
Department of Upper GI Surgery
The James Cook University Hospital

Dear Mr Munipalle

Re: **Expression of Hypoxia Inducible Factor 1 alpha as a prognostic indicator
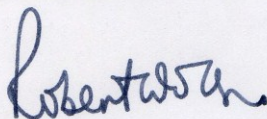of long term survival in oesophageal cancer.**

Many thanks for your letter addressing the issues raised from your project review at
the Research Approval Board meeting on 1st December 2004. I am now happy to
approve your project via Chairman's action.

May I take this opportunity to remind you that now your project has been approved by
R&D you will be able to submit your project for Local Ethics Committee approval. I
would be grateful if you could notify R&D when you receive Ethics approval by
forwarding a copy of the approval letter for our files

May I wish you well with the study and we look forward to hearing how it progresses
in due course.

Kind regards.

Yours sincerely

Professor R G Wilson
**Director of Research & Development/
Chairman Research Approval Board**

Professor Rob Wilson MD FRCS - Director of Research & Development Tel 01642 854149 E-mail r.wilson@ncl.ac..uk
Karen Stage - Research & Development Manager Tel 01642 854965 E-mail karen.stage@stees.nhs.uk

Appendix 2   Copy of the South Tees NHS Trust ethics approval letter

SL5 Favourable opinion at first review
Version 2, October 2004

NHS

South Tees Local Research Ethics Committee
1st Floor, Academic Centre
The James Cook University Hospital
Marton Road
Middlesbrough
Cleveland
TS4 3BW
Telephone: 01642 282451
Facsimile: 01642 854768

Email: carol.cheesebrough@stees.nhs.uk

04 January 2005

Mr Phanibhushana C Munipalle
Clinical Research Fellow
Dept of Upper GI Surgery
The James Cook University Hospital
Marton Road
Middlesbrough
Cleveland   TS4 3BW

Dear Mr Munipalle

Full title of study: *Expression of Hypoxia Induced Factor 1 alpha as a prognostic indicator of long term servival in oesophageal cancer*
REC reference number: *04/Q1003/91*
Protocol number:

The Research Ethics Committee reviewed the above application at the meeting held on 25 November 2004.

Ethical opinion

CF & PIS to include GP to be informed
CF & PIS to be re-written using standard format
Explain what the 'test' is
To be more explicit in how looking at survival data
A35 - indemnity needs to be included

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

An advisory committee to County Durham and Tees Valley Strategic Health Authority

SL5 Favourable opinion at first review
Version 2, October 2004

Document Type:	Version:	Dated:	Date Received:
Application	Version 3.0		16/11/2004
Investigator CV	Version 1		16/11/2004
Protocol	Version 1		16/11/2004
Covering Letter	Version 1	11/11/2004	16/11/2004
Sample Diary/Patient Card			16/11/2004
Participant Information Sheet			16/11/2004
Participant Information Sheet	V.2	06/12/2004	13/12/2004
Participant Consent Form	V.2	06/12/2004	13/12/2004
Participant Consent Form			16/11/2004
Response to Request for Further Information		10/12/2004	13/12/2004

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Notification of other bodies

The Committee Administrator will notify the research sponsor that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

04/Q1003/91	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely,

Carol - Co-ordinator

pp. **Dr John Drury**
Chair

E-mail: carol.cheesebrough@stees.nhs.uk

Appendix 3   Copy of the Teesside University ethics approval letter

UNIVERSITY OF
TEESSIDE

PRIVATE AND CONFIDENTIAL

Direct Line: 01642 384154

8th June 2005

Prof. Carolyn Summerbell
School of Health & Social Care
University of Teesside

Dear Carolyn

Study 051/05 – Expression of Hypoxia Inducible Factor 1 alpha as a prognostic indicator of long term survival in oesophageal cancer
Researcher: Mr. P. C. Munipalle Supervisor: Carolyn Summerbell

Thank you for submitting the above proposal for ethical approval. I can confirm that through Chairs Action the study can now proceed.

The School of Health & Social Care Research Ethics Committee wish you well with your study.

Yours sincerely,

Tricia Forster

<u>Tricia Forster</u>
Chair
Research Ethics Committee
School of Health & Social Care

DEAN: Professor PAUL KEANE
SCHOOL OF HEALTH & SOCIAL CARE

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Appendix 4 – Patient Information Sheet

PATIENT INFORMATION SHEET
(For patients being treated for cancer of the oesophagus)

(Version 2: 6 Dec 2004)

'Expression of Hypoxia Induced Factor 1 alpha as a prognostic indicator of long time survival in oesophageal cancer'

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

1. What is the purpose of the study?

Research is an important part of medical practice. Many treatments, operations and medicines which are life saving throughout the world were discovered through research, including most of the currently used treatments. Research is an ongoing process and aims at constantly improvising the patient care.

We are trying to find out if some of the chemical substances associated with cancer tissue tell us about the future course of the disease. This information may help us in knowing beforehand about the prognosis of a particular patient. In long run this information might be utilised to find more effective treatments for cancer.

2. Why have I been chosen?

As you are aware you are being treated for cancer of your gullet. We appreciate your help on carrying out research on the tissue samples removed from your gullet.
Other patients with similar disease and undergoing treatment will also be approached and requested for participation in this study.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

4. What will happen to me if I take part?

After any camera test or surgery the removed tumour tissue and normal tissue will be routinely sent for laboratory examination to know the stage of the cancer and to decide if you need any further treatment. We would like to subject a sample of these tissues for a special test as part of our research. During this test a special stain is used to detect the level of the above mentioned chemical in the tissues. In previously done studies the level of this chemical has been found to be representing the severity of some other cancers and we would like to know through this study if it happens in gullet cancer too.

This project involves you no more that another patient undergoing the same operation but not participating in research as we are only looking at the tissue, which is removed as part of your operation. That means your hospital stay, medication, investigations or future follow up with us/ your GP is not altered in any manner.

We would like to keep you under this study as long as possible with your permission. As we treat you for the cancer we will collect information about the progress and response of the disease. This information is used in this study.

At present we do not have sufficient information based on which your treatment may be changed and we are unable to give you more information about when this is likely to happen.

5. What do I have to do?

Your hospital stay, medication, investigations or future follow up with us/ your GP is not altered in any manner.
There are no life style or dietary restrictions on the participating patients. You can drive; drink and take part in sport and other social activities as per your wish. You can continue all your medications as usual.

6. What are the side effects of any treatment received when taking part?

Your will not be given any treatment as part of this research study and so we don't expect you to have any side effects from this study.

7. What are the possible disadvantages and risks of taking part?

Your participation in this research does not subject you to any additional risks on its own. The risks associated with the camera test/ operation are the same whether you participate in the research or no.

Also we will not be identifying any new diseases from this study.

8. What are the possible benefits of taking part?

There are no immediate benefits from taking part in this study.

Through this research we are trying to find out if some of the chemical substances associated with cancer tissue tell us about the future course of the disease and response to various treatments. This information may help us in knowing beforehand about the prognosis of a particular patient (and at present we do not have sufficient information to advice you about your particular case). In long run this information might be utilised to find other effective treatments for cancer of the gullet. However, this cannot be guaranteed.

9. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the disease, which might be applicable to you. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

10. What happens when the research study stops?

This will not make any difference to your clinical care.

11. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

12. Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital/ surgery will have your name and address removed so that you cannot be recognised from it.

Your own GP will be notified of your participation in the trial if you agree to this. This includes other medical practitioners not involved in the research but who may be treating you.

13. What will happen to the results of the research study?

We hope to share the findings of the study with other doctors and NHS managers by presenting the findings at meetings and by publishing scientific articles that describe the study and results. You will not be identified in any report/ publication. You may be informed of the results if appropriate to your care.

We cannot give you the likely date of the publication of results at this time.

14. Who is organising and funding the research?

The clinical care will be provided by the NHS, as it would normally be. The money for the laboratory tests will be provided by the Oesophageal Cancer Support Group.

15. Who has reviewed the study?

The Research Approval Board at the South Tees Hospitals NHS Trust and the South Tees Local research Ethics Committee approve all research studies that will be carried out in the trust hospitals.

16. Contacts for further information

Mr Y K S Viswanath and Mr P A Davis are the clinicians whom you can approach for further information about this study. The other members of the surgical team treating you may be involved in this project either directly or indirectly.

Kindly address your communication to Mr P C Munipalle, Clinical research Fellow in Surgery at The James Cook University Hospital:

Mr P C Munipalle
Clinical research Fellow in Upper GI Surgery
Surgical Directorate
The James Cook University Hospital
Marton Road
Middlesbrough TS4 3BW

Or you can discuss with him/ or one of the team members in person when you come for your operation/ camera test.

- Thank you for spending your valuable time on going through this leaflet. We will talk to you again at he time of your admission for your operation and answer any questions you might ask.

- If you are willing to participate in this research you will be asked to sign another form before we include you in our research project. A copy of the form is enclosed herewith and if you wish you can sign this form now and bring it with you to the hospital where we will collect this from you and give you a copy. You can keep this patient information sheet.

- If you wish so kindly discuss this with friends and family before agreeing to participate and signing the consent form.

- You have every right to decline to participate in this research project. You can withdraw your consent later at any stage even though you have consented now.

Thank you very much in anticipation of your co-operation.

Mr P C Munipalle

Appendix 5 – Consent Form

Centre Number:
Study Number:
Patient Identification Number for this trial:

<u>**CONSENT FORM**</u>

(Version 2: 6 Dec 2004)

Title of Project: 'Expression of Hypoxia Induced Factor 1 alpha as a prognostic indicator of long time survival in oesophageal cancer'

Name of Researcher: P C Munipalle

Please initial box

1.   I confirm that I have read and understand the information sheet dated 6 December 2004: version 2 for the above study and have had the opportunity to ask questions.   ☐

2.   I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.   ☐

3.   I understand that my participation in this project would be informed to my GP and other medical practitioners treating me.

    I give permission for these individuals to have access to my records.   ☐

4.   I agree to take part in the above study.   ☐

_____   _____   _____
Name of Patient   Date   Signature

_____   _____   _____
Name of Person taking consent   Date   Signature
(if different from researcher)

_____   _____   _____
Researcher   Date   Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 6 - Leica Processing Schedule

Equipment – Leica TP 1050 issue processing machines

Reagent	Station	Time	Temp	P/V	Drain	Stir
		Hr:Min	OC	P/V	Secs	
Formalin	1	00:30	45	P/V	120	ON
70% Alcohol	2	00:45	37	P/V	120	ON
90% Alcohol	3	00:45	37	P/V	120	ON
Abs Alcohol	4-7	01:15	37	P/V	120	ON
Abs Alcohol	4-7	01:15	37	P/V	120	ON
Abs Alcohol	4-7	01:15	37	P/V	120	ON
Abs Alcohol	4-7	01:15	37	P/V	120	ON
Xylene	8-10	01:15	37	P/V	120	ON
Xylene	8-10	01:15	37	P/V	120	ON
Xylene	8-10	01:15	37	P/V	120	ON
Wax	Left	01:00	62	P/V	120	ON
Wax	Middle	01:00	62	P/V	120	ON
Wax	Right	01:30	62	P/V	120	ON

Health and Safety

1. Wear safety spectacles. Nitrile gloves are recommended but due to the manual dexterity required, latex gloves are acceptable provided they are changed frequently.

2. Use ventilated bench when changing solutions

3. Spillage

CHEMICAL	WATER	CHEMICAL GRANS	Fo GRANS & RESP
Formaldehyde			X
Alcohol	X		
Xylene		X	
Wax	SET & SCRAPE		

4. Hazard

Formaldehyde: Toxic Harmful

Xylene: Flammable Harmful

Wax: Flammable

Appendix 7 - Staining protocol worksheet

Step	Reagent	Duration (Minutes)	Test	Control
1	Target retrieval			
2	TBS wash	5	_____	_____
3	Reagent 1 (Peroxidase Block)	5	_____	_____
4	TBS wash	5	_____	_____
5	Reagent 2 (Protein Block)	5	_____	_____
6	Reagent 3 (Primary Antibody) (or TBS for negative control)	60	_____	_____
7	TBS wash	5	_____	_____
8	TBS wash	5	_____	_____
9	TBS wash	5	_____	_____
10	Reagent 4 (Link Antibody)	15	_____	_____
11	TBS wash	5	_____	_____
12	TBS wash	5	_____	_____
13	TBS wash	5	_____	_____
14	Reagent 5 (Streptavidin-Biotin Complex)	15	_____	_____
15	TBS wash	5	_____	_____
16	TBS wash	5	_____	_____
17	TBS wash	5	_____	_____
18	Reagent 6 (Amplification reagent)	15	_____	_____
19	TBS wash	5	_____	_____
20	TBS wash	5	_____	_____
21	TBS wash	5	_____	_____

22	Reagent 7 (Streptavidin-Peroxidase)	15	_____	_____
23	TBS wash	5	_____	_____
24	TBS wash	5	_____	_____
25	TBS wash	5	_____	_____
26	Reagent 8 (DAB chromogen)	5	_____	_____
27	Rinse in distilled water			
28	Reagent 11 (Haematoxylin counterstain)		_____	_____
29	Rinse in distilled water		_____	_____
30	Mounting		_____	_____

Appendix 8 - HIF Expression Data

Participant	Observer 1 (D.S.)	Observer 2 (P.C.M.)	Averege score
1	0	0	0
2	39	37	38
3	0	0	0
4	12	8	10
5	41	58	49.5
6	12	6	9
7	0	0	0
8	21	57	39
9	29	41	35
10	0	0	0
11	0	2	1
12	34	33	33.5
13	42	32	37
14	19	16	17.5
15	0	0	0
16	36	24	30
17	4	3	3.5
18	28	13	20.5
19	0	1	0.5
20	5	4	4.5
21	14	8	11
22	0	0	0
23	4	2	3
24	0	7	3.5
25	12	35	23.5
26	32	29	30.5
27	44	47	45.5
28	0	0	0
29	41	34	37.5
30	15	13	14
31	6	4	5
32	1	1	1
33	24	38	31
34	17	19	18
35	2	5	3.5
36	38	41	39.5

Appendix 9 – Original Article published in 'Diseases of the Esophagus'

Title: **Prognostic value of Hypoxia inducible factor 1α in esophageal squamous cell carcinoma**

Authors: **P C Munipalle, Y K S Viswanath, P A Davis, D Scoones**

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                 Cleveland
                 UK

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Abstract:
Introduction
Hypoxia Inducible Factor 1α (HIF 1α) plays a major role in the pleitropic response observed secondary to hypoxic conditions in tumors. Its expression in the tumor cells has been correlated to tumor aggressiveness and prognosis in squamous cell carcinoma (SCC) of the esophagus in Far Eastern population[1], but limited information is available on the prognostic role of HIF 1α in SCC of esophagus in European population. This information may help in choosing appropriate therapeutic strategies and possibly developing a monoclonal antibody with therapeutic potential targeting the HIF 1α.
Methods
Tumor samples from 36 patients diagnosed with SCC of the esophagus were collected. Prepared tissue sections were stained with validated and specific monoclonal antibodies for HIF 1α and the expression was correlated with the disease pattern and survival.
Results
Out of 36 patients, 17 patients showed low and 19 high expression of HIF 1α. There was no difference in the disease free and overall survival between these two groups ($p>0.05$, log rank test). Regression analysis showed that HIF 1α was not an independent prognostic factor for survival ($p>0.05$).
Conclusion

HIF 1α did not show prognostic value in squamous cell carcinoma of esophagus in our study on European population, in agreement with previous studies. Novel strategies on the therapeutic manipulation of HIF 1α in cancer are to be explored further and may have a role to play in improving treatment outcome.

Key words: Esophageal squamous cell cancer, Hypoxia inducible factor 1α, prognostic factor

Introduction

Esophageal cancer is a disease on the rise yet with no major improvement in mortality rates, despite the advances in treatment[2]. Among Western Europe the incidence is highest in the North east of England in the United Kingdom, with five year survival of only 8%[3]. Survival may be improved by identifying potential sub-populations of patients with better prognosis and targeting them with multi-modality therapy where applicable[4]. This strategy can avoid the significant morbidity caused by the intensive neoadjuvant, adjuvant and radical surgery treatment strategies in those sub-populations with poorer prognosis, who are unlikely to benefit from such treatments.

The current best prognostic indicator in esophageal cancer is TNM clinical staging[5]. Other indicators which are recently under consideration are epidermal growth factor receptor (EGFR), Angiogeneic factors such as vascular endothelial growth factor, the tumor suppressor gene p53, cell cycle regulators, apoptotic factors and matrix metalloproteinases[4]. There is therefore a potential to identify more accurate prognostic indicators to aid in the management of esophageal cancer.

Hypoxia inducible factor 1α (HIF 1α) belongs to the PAS (Per, ARNT, Sim) subfamily of basic-helix-loop-helix (bHLH) family of transcription factors[6]. To date, the transcription of more than 70 genes are found to be directly regulated by HIF 1α. These genes are involved in processes that act to address the cellular hypoxia by decreasing oxygen dependence and consumption by cells, and by increasing the efficiency of oxygen delivery to cells. These processes include vasculogenesis and angiogenesis, metabolism, vasodilatation, cell migration, signaling and cell fate decisions. Many of these processes are implicated in solid tumor survival in a hypoxic environment. In fact, HIF 1α expression is shown to be of prognostic value in many human cancers[7, 8].

In the present study we examined the expression pattern of HIF 1α in squamous cell carcinoma (SCC) of esophagus in European population and investigated its prognostic value.

Material and Methods

The project was approved by the Research approval board & Research ethics committee of South Tees NHS Trust, UK and carried out at the James Cook University Hospital, Middlesbrough, UK. We examined tumor samples from 36 consenting patients diagnosed or treated with SCC of esophagus between June 2005 – August 2006. The follow up of surviving patients at the time of analysis in June 2009 was 34 - 48 months (median 40 months).

Histological diagnosis, grading and N staging was done on haematoxylin-eosin stained sections. Lymph node sections were stained where available. HIF 1α expression was estimated in the tissues as below:

Immunochemistry:

The sections were deparaffinised and rehydrated. Heat mediated epitope was used for antigen retrieval. Peroxidase was quenched with 3% Methanol in H_2O_2 for 5 minutes. Serum-free protein in Phosphate Buffered Saline was used for 5 minutes as protein block. Primary antibodies (Mouse monoclonal antibody to HIF 1α at 1:1000 concentration, Novus Biologicals, UK) were applied to the test slides for 60 minutes followed by TrisBufferSaline (TBS) wash. Sections were incubated with secondary antibody (Biotinylated rabbit anti-mouse immunoglobulins, Dako, UK) for 15 minutes followed by TBS wash. Sequential 15 minute incubations with Streptavidin-Biotin-Peroxidase Complex, amplification reagent, and Streptavidin-Peroxidase followed by TBS wash were done. The colour was developed by 15 minute incubation with DAB chromogen and the sections were weakly counterstained with haematoxylin.

Sections from a high grade Renal Cell Carcinoma with very high HIF 1α expression were used as positive controls. TBS was substituted for primary antibody as the negative control.

Expression of HIF 1α in the SCC cells was determined by the strength of the nuclear staining to HIF 1α antibody. Under $\times$ 400 magnification, the percentage of tumor cells in that field which stain positive for HIF 1α compared to those cells staining negative was visually scored. The expression of HIF 1α in all the slides was scored independently by the authors (P C M and D S) who were blinded to the staging of the tumors. The average score of expression is used to categorise the samples, based on previously reported studies[1]. The samples where $\geq$10% SCC cells showed positive staining were scored as 'High' expression and the samples with <10% SCC cells with positive staining were scored as 'Low' expression (Figure 1).

Patient demographics, disease specific and survival data were collected from cancer information system and case notes. Disease free survival was defined as the time interval from the end of primary therapy until the first evidence of progression of the disease. Overall survival was defined as the time interval from the end of primary therapy till the last known survival date or death.

Statistical analysis:

Cohen's kappa was used to measure the agreement between the two independent scorers. A value of 1 is considered as perfect agreement. Pearson's χ^2 test or Fisher's exact test were used for testing relationships between categorical variables. Survival curves were plotted using the method of Kaplan-Meier, and the log-rank test was used to determine the statistical significance. Cox proportional hazard regression model was used to assess the effects of patient and tumor variables on survival. Statistical analysis and graphic representation were performed using SPSS version 13 (SPSS, Chicago, Illinois, USA). $P < 0.05$ was considered significant.

Results

The study population comprised 15 males and 21 females, with a median age of 76 years (range 52 – 95). Seventeen patients expressed low levels of HIF 1α while 19 expressed high levels.

The correlation between various clinicopathological variables and the expression of HIF 1α in the tumor is summarised in Table 1.

Expression of HIF 1α was significantly higher in study population aged above 70 years (p=0.02). There was a trend towards higher expression in advanced stage of the disease, nearing statistical significance (p=0.06).

There was good agreement between the two independent observers regarding the reading of the expression of HIF 1α (Cohen's Kappa 0.82, p<0.01).

Survival analysis:

Eight of the 36 patients were alive at the end of the follow up period spanning 4 years, with an overall survival rate of 22% (Survival range 252 – 1495 days). The survival of the 28 patients who died in this period ranged from 32 – 1700 days. The median overall survival was 232 days after diagnosis. The survival rate at the end of 1 year was 33.3%, 2 years was 25%, 3 years was 25% and 4 years was 22%. The 17 patients with a low expression of HIF 1α had a mean (median) survival of 438 (238) days with a 4 year survival rate of 18 %. The mean (median) survival for the 19 patients with high expression were 533 (196) days with a 4 year survival rate of 26%. Figure 2 shows the Kaplan-Meier curve plotted for HIF 1α expression. The censored data corresponds to those still alive at the end of the study period.

There was no statistically significant correlation between the overall survival rate and the strength of expression of HIF 1α ($p = 0.908$, log rank test).

Table 2 shows the results of the Univariate analysis of various factors. The aim of treatment which reflects the stage of the disease and the status of metastases were the only significant risk factors. HIF 1α expression was not a risk factor.

Multivariate analysis showed only the aim of the treatment as independent prognostic factor (p<0.001).

Discussion

Our study is the first reported study on European population with patient and disease characteristics different from the previous studies.

As aggressive tumors grow rapidly and the angiogenesis cannot keep up with the demand for oxygen and nutrients, the central part could become hypoxic. The tumors recruit various mechanisms to adapt for this[9]. HIF 1α is a master regulator of this adaptive response and hence is integral in the malignant potential of tumors. HIF 1α has been shown as a key factor in regulation of VEGF and VEGFR and other angiogenic factors, which play a major role in tumor survival and spread. A variety of proteins targeted by HIF 1α are involved in tumor cell proliferation, survival, adhesion and mobility, implicating the role of HIF 1α in these processes. Genetic events facilitating tumor survival and spread like deficiency or mutation of tumor suppressor genes (VHL, p53 and PTEN), and amplification of oncogenes (Akt, Ras, ERK1/2) were shown to be associated with HIF 1α overexpression in previous studies[10]. Bos et al[11] showed that HER-2 immunoactivity and gene amplification, VEGF expression, and Ki-67 expression are correlated strongly with HIF 1α positivity in lymph node negative breast carcinoma.

Most cancers over-expressing HIF 1α are associated with increased mortality. These include breast, stomach, brain, ovarian, endometrial, cervical, prostate and colon cancers[7]. In head and neck and non-small cell lung cancers, studies have shown conflicting results, probably due to the interaction of HIF 1α with apoptotic genes. The effect of HIF 1α expression probably depends on the interplay between inherent malignant potential of the tumor and various genetic elements.

HIF 1α showed increased association with venous invasion, VEGF expression and tumor microvessel density in SCC of esophagus[1], suggesting more aggressive course of the disease in tumors with high expression of HIF 1α. In addition, HIF 1α probably plays a role in lymphatic spread of SCC through the induction of vascular endothelial growth factor C[12]. Our study has shown significantly higher expression of HIF 1α in the SCC tumors of patients aged above 70 years although this was not translated to decrease in survival. We noticed clear trend of higher expression in patients with advanced stage of SCC, which may suggest more aggressive disease. This knowledge may help in choosing appropriate treatment strategy for this sub-population, avoiding intensive therapies which can cause significant morbidity without much clinical benefit. Further studies evaluating the role of HIF 1α expression in influencing the treatment planning shall be considered.

HIF 1α expression in SCC of esophagus did not show independent prognostic value in predicting overall survival in many previous studies. Kurokawa et al[13] showed in their study that HIF 1α expression was a risk factor but not an independent prognostic factor of survival. High expression of HIF 1α in SCC was found to have a significant effect on disease free survival but not on overall survival[14]. A recent study by Yongshun et al[15] did not show significant role for HIF 1α in predicting survival. Ling et al [16] showed that HIF 1α mRNA or protein expression did not correlate with histomorphological regression or survival following neoadjuvant chemotherapy for SCC. Our results at the end of 4 year follow-up period suggest similar role for HIF 1α in the prognosis of SCC of esophagus.

Our study has shown better overall survival in patients treated with curative intent and with no evidence of metastatic spread at the time of diagnosis. The overall survival figures in our study population mirror those reported from other SCC treatment centers in Western world.

Expression of HIF 1α in esophageal cancer was shown as a predictor of response to Photodynamic therapy[17] and chemoradiotherapy[18]. Recently reported novel strategies on the role of therapeutic manipulation of HIF 1α in cancer[8, 19, 20] may encourage further studies in this area.

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Figure legends:

Figure 1: Expressions of HIF 1α expression in SCC of oesophagus (× 200, × 400). The arrow shows positively stained nucleus.

Figure 2: Kaplan-Meier survival curve of 36 patients stratified for HIF 1α expression.

Table 1: Correlation of HIF 1α expression with clinicopathological variables

Variable	Total	Low expression of HIF 1α	High expression of HIF 1α	p value
Gender	36	17	19	0.709
Male	15	7	8	
Female	21	10	11	
Age	36	17	19	0.022*
<70	18	12	6	
>70	18	5	13	
T stage	36	17	19	0.408
T1	2	2	0	
T2	6	2	4	
T3	16	8	8	
T4	12	5	7	
N stage	36	17	19	0.846
N0	9	4	5	
N1	24	12	12	
N2	3	1	2	
M stage	36	17	19	0.238
M0	24	13	11	
M1	12	4	8	
UICC stage	36	17	19	0.067
Stage I	2	2	0	
Stage IIA	4	0	4	
Stage IIB	1	1	0	
Stage III	17	10	7	
Stage IV	12	4	8	
Tumor grade	36	17	19	0.221
Well diff	9	2	7	
Moderately diff	7	4	3	
Poorly diff	20	11	9	
Aim of treatment	36	17	19	0.169*
Curative intent	15	9	6	
Palliative intent	21	8	13	

* Fisher's exact test

Table 2: Univariate analysis of factors associated with overall survival in patients with SCC of esophagus

Variable	No. of patients *(n = 36)*	Median overall survival (days)	p value
HIF expression			0.908
Low	17	238	
High	19	196	
Gender			0.285
Male	15	182	
Female	21	272	
Age (years)			0.350
<70	18	240	
>70	18	176	
T stage			0.192
T1	2	110	
T2	6	242	
T3	16	272	
T4	12	182	
N stage			0.171
N0	9	238	
N1	24	197	
N2	3	80	
M stage			0.051
M0	24	272	
M1	12	162	
Integrated TNM stage			0.122
Stage I	2	110	
Stage IIA	4	1700	
Stage IIB	1	240	
Stage III	17	282	
Stage IV	12	162	
Tumor grade			0.194

Well diff	9	240	
Moderately diff	7	147	
Poorly diff	20	236	
Aim of treatment			0.001
Curative intent	15	646	
Palliative intent	21	176	
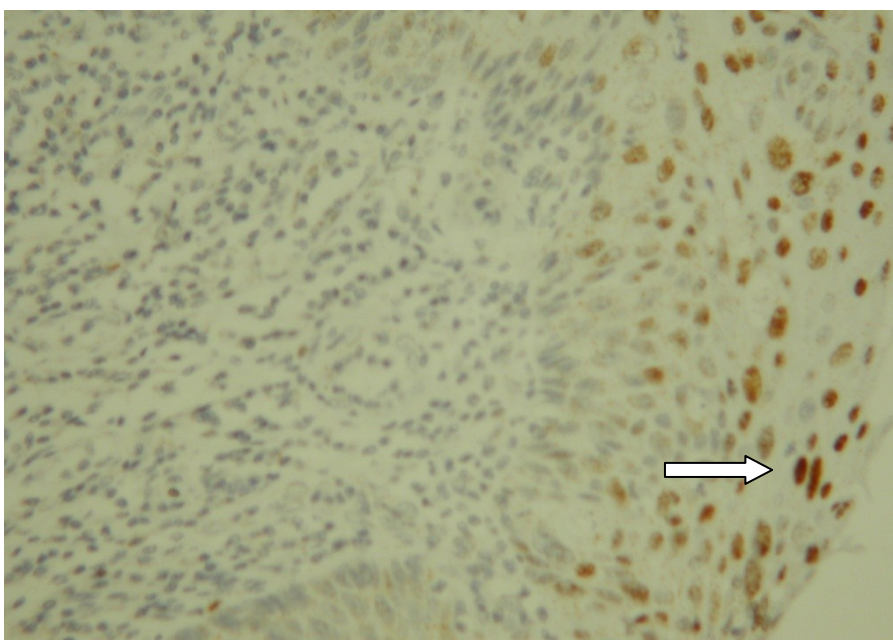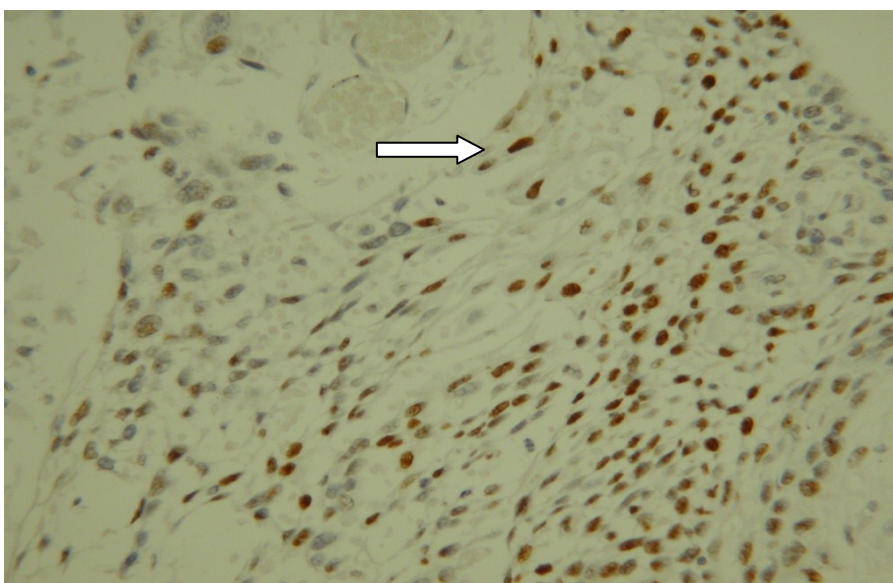
Well diff	9	240
Moderately diff	7	147
Poorly diff	20	236

Figure 1:

Figure 2:

Survival Functions
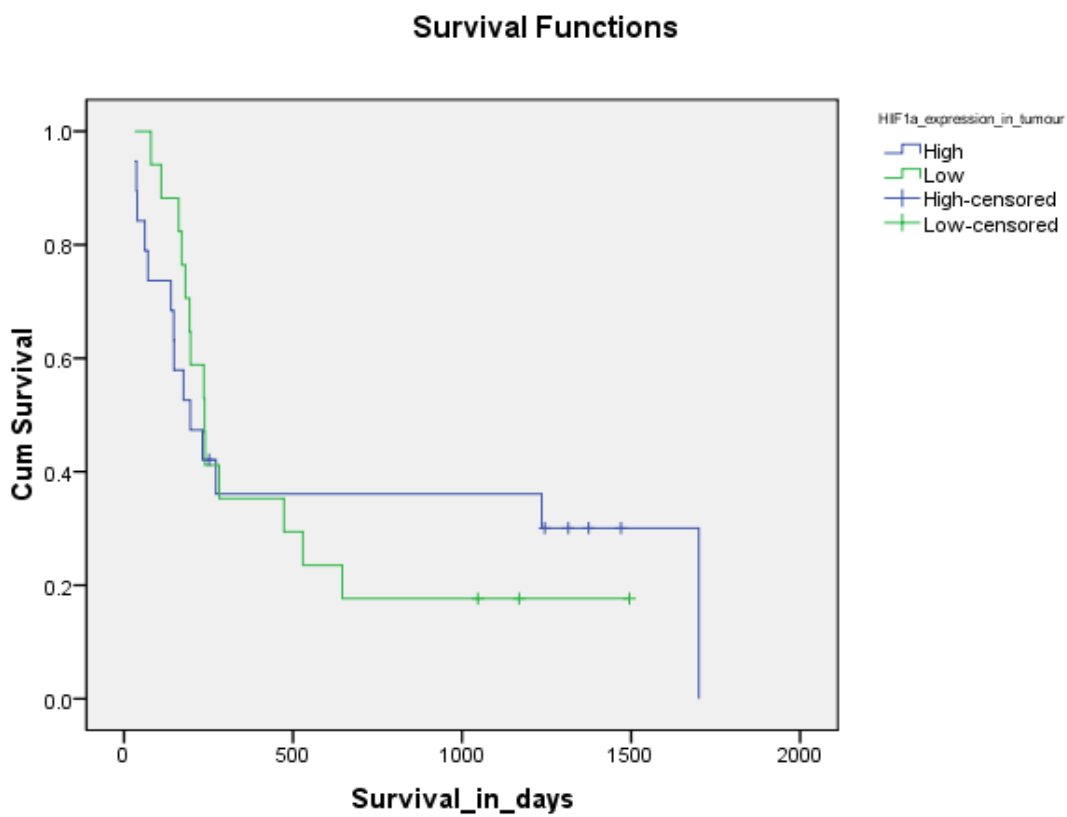
Appendix 10

Prognostic relevance of Hypoxia Inducible Factor (HIF 1 α) expression in squamous cell carcinoma of oesophagus

P C Munipalle, A Ahitan, P A Davis, YKS Viswanath

Upper Gastrointestinal Regional Audit Day, Wynyard, UK (2008) Oral presentation

Introduction

Hypoxia Inducible Factor (HIF 1 α) plays a major role in the pleitropic response observed in response to hypoxic condition of tumours. Its expression is correlated to the aggressiveness of various tumours but its prognostic relevance in squamous cell carcinoma of oesophagus is unclear. This information may help in choosing between therapeutic strategies such as intensive combined modality therapy vs. palliative therapy.

Methods

All the patients diagnosed with squamous cell carcinoma of oesophagus over a period of 3 years in our tertiary oesophago-gastric centre are recruited in this prospective study, with a median follow-up of 28 months. The cancerous tissue specimens are stained with specific monoclonal antibodies for HIF 1 α and the expression is correlated with the disease pattern.
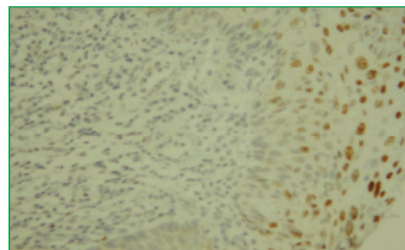
Results

Based on the pattern of HIF 1 α expression, 36 patients are divided into low (n=17) and high (n=19) expression groups. There is no difference in the disease free and overall survival between these two groups ($p>0.05$, log rank test). Multiple regression analysis showed that HIF 1 α is not an independent prognostic factor for squamous cell carcinoma of oesophagus ($p>0.05$).

Conclusion

HIF 1 α did not show promising prognostic relevance in this study. To improve the results in oesophageal cancer there is a constant need to identify better prognostic indicators through further molecular studies.

Appendix 11

Association of Surgeons of Great Britain and Ireland International Surgical Congress, Glasgow, UK (May 2009) Poster presentation