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ISOLATED TUMOUR CELLS IN OESOPHAGEAL CANCER: APPLYING THE SENTINEL LYMPH NODE CONCEPT

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THESIS ABSTRACT

INTRODUCTION: Accurate staging of oesophageal cancer is critical in predicting prognosis and tailoring therapy. However, the current TNM based staging system is suboptimal because it combines patients with very different outcomes into each disease stage. Our aims are to identify pathological factors or molecular markers that can significantly improve the accuracy of the oesophageal cancer staging system by both a retrospective database review as well as detailed analysis of oesophageal cancer specimens. The benefit of incorporating sentinel lymph node biopsy with oesophageal resection will also be determined.

METHODS: 240 patients (mean age, 62 yrs) were identified from an Oesophageal Cancer database between 1997 and 2007. We re-examined all pathology slides from the original resection to identify significant prognostic factors, and to determine suitable paraffin blocks for the remaining parts of the study. Tissue microarrays were constructed from 89 paraffin blocks for HER2 gene amplification by silver-enhanced *in situ* hybridization (SISH). Incidence of HER2 positivity, and correlation to clinicopathological variables were determined. Of the original 240 patients, we identified 119 patients who were classified as node-negative. Additional sections with immunohistochemistry (IHC) staining were performed on the relevant paraffin blocks. The yield of occult tumour deposits was determined along with their prognostic significance. Thirty-one consecutive oesophageal cancer patients underwent resection and sentinel lymph node retrieval. Endoscopic peritumoural injection of ^{99m}Tc antimony colloid was performed, and sentinel lymph nodes were identified and sent off separately for serial sections and IHC.

RESULTS: The 5-year overall survival rate was 36% (median, 24 months). Only histological grade and refined nodal status were found to be independent prognostic factors. True HER2 gene amplification was detected in 14 (16%) oesophageal cancer specimens. No significant associations were found among gene amplification, clinicopathological factors, or survival. Of 119 node negative patients, 31 patients (26%) were found to have occult tumour deposits with serial sections and IHC. Five-year survival rates were 60% for patients who remained node-negative, 33% for patients with isolated tumor cells, 40% for patients with micrometastases, and 0 for the patient with a metastasis (P=0.02). At least one sentinel lymph node (median, 3) was identified in 29 of 31 patients (success rate, 94%). In 28 of 29 patients, the sentinel lymph node accurately predicted findings in non-sentinel nodes (accuracy, 96%).

CONCLUSIONS: A staging model in oesophageal cancer which incorporates refined nodal status and histological grade appears to be more accurate than the current TNM staging system. While molecular targeting may be possible for approximately 16% of oesophageal adenocarcinoma patients, HER2 oncogene amplification was not associated with any affect on survival in this study. Almost one third of all node negative patients had occult tumour deposits in their nodes that were missed on their original pathology. Surprisingly, even those with isolated tumour cells had a signficiantly worse prognosis than those without. Sentinel lymph node biopsy seems to be feasible and accurate in predicting overall nodal status. It improves staging accuracy and should therefore become standard of care in the surgical treatment of patients with oesophageal cancer.

THESIS DECLARATION

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CHAPTER 1: INTRODUCTION

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1.1 CANCER OF THE OESOPHAGUS

1.1.1 Epidemiology

Oesophageal cancer is the 5th and 7th most common cancer in males and females, respectively, and is one of the most aggressive tumours. Over the last decade, the incidence of oesophageal adenocarcinoma has increased by 2.1% per year in the United States (2.6 per 100,000), while oesophageal squamous cell carcinoma has decreased in incidence by 3.6% per year (1.8 per 100,000)[1,2*]. While this increase has been seen predominantly in the United States, the United Kingdom and Australia, there is disquieting evidence for an increasing incidence of oesophageal adenocarcinoma in some Asian populations as well, including Singapore and Japan[3]. In Australia, the incidence of adenocarcinoma has increased fourfold since the 1970s (4.8 per 100,000), a rate of increase which is faster than that of any other cancer. Hence, oesophageal adenocarcinoma now accounts for more than 80% of oesophageal cancer in Australia, while squamous cell carcinoma of the oesophagus accounts for the rest.

1.1.2 Aetiology

Oesophageal adenocarcinoma and squamous cell carcinoma exhibit many differences in aetiology[3-9]. Risk factors can be sub-divided into 3 categories: predisposing conditions, lifestyle/habits, and environmental/dietary factors.

1.1.2.1 Predisposing Conditions

Oesophageal adenocarcinoma targets Caucasian men with an incidence eight times higher than in Caucasian women, and five times greater than in African American men[3]. In the

*Bibliography for Chapters 1 & 7 begins on p. 209.

United States, the incidence of oesophageal adenocarcinoma among white men has increased to 3.2-4.0 per 100,000 persons over the past two decades[4]. Symptomatic gastro-oesophageal reflux disease is the strongest known risk factor for oesophageal adenocarcinoma. People with frequent symptoms have a 4-fold or higher reported relative risk of developing cancer. However, symptomatic reflux is infrequent or absent in 40-48% of those who develop oesophageal adenocarcinoma[3]. Two other predisposing conditions have been associated with an increased risk of oesophageal cancer: hiatal hernia (likely from increased gastro-oesophageal reflux) and achalasia[5]. Patients with achalasia (a motility disorder of the oesophagus) have a 10-fold increased risk of developing either type of oesophageal cancer compared to the rest of the population. This is thought to be due to stasis and fermentation of food in the dilated oesophagus[5].

1.1.2.2 Lifestyle/Habits

Both the intensity and duration of smoking increase the risk of developing squamous cell carcinoma, while only duration of smoking increases the risk of oesophageal adenocarcinoma. Alcohol consumption is also a well-established risk factor for squamous cell carcinoma. It is estimated that 90% of oesophageal squamous cell cancers are attributable to tobacco and alcohol in more developed countries[6]. One study showed no association between alcohol consumption and oesophageal squamous cell cancer below 170 g/week, however there was a 3% increase in the risk of squamous cell carcinoma for each 10 g/week of alcohol thereafter[6]. The development of oesophageal squamous cell carcinoma depends on the cumulative quantity of tobacco and alcohol, and the effects of these two are synergistic[6]. No association between alcohol consumption and oesophageal adenocarcinoma has been found[7]. As well, the consumption of hot or very hot tea (>60°C), common in various Eastern European and Asian regions, increases the risk of squamous cell carcinoma through recurrent thermal injury to the oesophageal mucosa[8].

1.1.2.3 Environmental/Dietary Factors

Obesity [as measured by body mass index (BMI)] has been shown to increase the risk of oesophageal adenocarcinoma[7]. People who are overweight (BMI = 25.0-29.9 kg/m^2) have a 1.5 to 1.8 relative risk of developing oesophageal adenocarcinoma[3]. Those who are obese (BMI > 30 kg/m^2) have a relative risk of between 2.4 and 2.8[3]. This increased risk is particularly relevant in those individuals with an abdominal/visceral pattern of obesity[7], as visceral fat may induce an altered inflammatory state (T lymphocyte activation) and potentially drive tumourigenesis[9]. An additional risk factor for both types of oesophageal cancer is a diet low in fruit and vegetables, and this is thought to account for up to 15% of new cases annually in the US population[3,6].

1.1.3 Barrett's Oesophagus

Barrett's oesophagus is the presence of intestinal-type metaplasia of the oesophageal mucosa. This is currently defined as endoscopically visible columnar metaplasia within the tubular oesophagus, irrespective of length[7]. It was convincingly linked with adenocarcinoma of the oesophagus in 1975, and it is thought by many to act as a precursor to this particular type of cancer via a sequence of low and high-grade dysplasia to invasive adenocarcinoma[3].

The true incidence and prevalence of Barrett's oesophagus in the general population is unknown. However, in two endoscopic studies performed in Italy and Sweden, 1.3% and 1.6% of adults, respectively, were found to have Barrett's oesophagus[3]. Recent studies suggest that most individuals with Barrett's oesophagus do not develop oesophageal adenocarcinoma in their lifetime. Meta-analyses estimate the incidence of oesophageal adenocarcinoma among those with Barrett's oesophagus to be 6-7 per 1000 person-years[3].

1.1.4 Classification System for Oesophageal Adenocarcinoma

It is important to define oesophageal adenocarcinomas of the gastro-oesophageal junction because there is disagreement about whether they are all of oesophageal or gastric origin. The morphological classification of Siewert and Stein, published in 1998[10], is probably the most widely used classification system for junctional adenocarcinomas. This system is based on the precise anatomical location of the tumour (Figure 1.1).

Figure 1.1 Classification of adenocarcinoma of the gastro-oesophageal junction[10]

Type 1:	Adenocarcinoma of the lower oesophagus: the tumour centre or two thirds of the tumour mass lies >1cm above the anatomical GOJ
Type 2:	True adenocarcinoma of the cardia: the tumour centre or two thirds of the tumour mass lies within 1cm above and 2cm below the anatomical GOJ
Type 3:	Subcardial adenocarcinoma: the tumour centre or two thirds of the tumour mass lies >2cm below the anatomical GOJ

The authors' base this classification on a combination of preoperative endoscopic and radiological findings, the intra-operative appearance, and pathological examination of the resected specimen. Controversy persists particularly regarding Type 2 tumours of the gastric cardia. Siewert and colleagues believe these should be staged as gastric carcinomas and treated similar to Type 3 tumours by extended total gastrectomy[11]. Other groups believe they are more like Type 1 tumours, and should be staged and treated as oesophageal cancers[12-14]. Our group agrees that Type 2 tumours are best resected by oesophagectomy, and are amenable to chemoradiotherapy, rather than chemotherapy alone. It is for these reasons that Type 2 tumours are included in the work that follows in oesophageal cancer.

1.1.5 Treatment Outcomes

The overall prognosis for oesophageal cancer remains poor, even though operative and non-operative therapies have undergone great advances over the past few decades. Overall survival for all patients presenting with oesophageal cancer is approximately 10%. This is because despite treatment improvements, the majority of patients continue to present with in-operable disease. Only 40% of patients are suitable for surgical resection and even in this select group, over half have lymphatic metastases at the time of surgery[15,16].

1.2 STAGING IN OESOPHAGEAL CANCER

1.2.1 TNM Staging System

Accurate staging of oesophageal cancer is critical in predicting prognosis and tailoring therapy. The modern era of TNM based staging for oesophageal cancer was revised by the American Joint Committee in Cancer (AJCC) and the International Union Against Cancer (UICC) in 2002[17,18]. This system uses T (tumour depth), N (regional lymph node status), and M (metastatic disease) to stage each type of cancer (Figure 1.2).

Figure 1.2 AJCC/UICC: TNM staging for oesophageal carcinoma (6th Edition)[17,18]

Stage	Tumour (T)	Nodes (N)	Metastasis (M)
0	is	0	0
I	1	0	0
IIA	2 / 3	0	0
IIB	1 / 2	1	0
III	3 / 4	1	0
IVA	any	any	1a*
IVB	any	any	1b**

Tis- carcinoma in situ (high grade dysplasia)

T1- invading mucosa / submucosa

T2- invading muscularis propria

T3- invading adventitia

T4- invading adjacent structures

***M1a-** celiac node metastasis from a lower third tumour

****M1b-** non-regional lymph node or other distant metastasis

Since the inception of this thesis, the 7th edition of the AJCC/IUCC *Cancer Staging Manual* has been published. This new edition includes many of the conclusions of the work that follows (in particular Chapter 2) regarding the importance histopathologic cell type and histologic grade, as well as the number of positive lymph nodes (a new pN classification)[19]. The impetus for these changes was in part due to the growing body of literature concerning the inadequacies of the (then) current staging system.

1.2.2 Additional Prognostic Factors

Using the TNM staging system, patients diagnosed with node-negative oesophageal cancer (Stage I or IIA) should have close to 100% disease-free survival at 5 years. Unfortunately this is not the case, as the 5-year survival in these patients is only 50%. How can we improve the TNM staging system?

Several potential methods exist. For example, one could stratify patients by subdividing T1 tumours into T1a (intramucosal) and T1b (submucosal) lesions. Other methods include subdividing N stage depending on the number of positive lymph nodes, incorporating the presence or absence of extracapsular invasion of the lymph node (when positive), and examining the circumferential margin of the tumour. Finally, if receiving neoadjuvant therapy, patients could be stratified according to the magnitude of tumour response to neoadjuvant chemoradiotherapy.

1.2.2.1 Stratifying pT Stage

With the introduction of minimally invasive techniques, much more attention has been focused on early oesophageal cancer, and the potential importance in differentiating intramucosal lesions (pT1a) from submucosal tumours (pT1b). Large Japanese studies of early squamous cell carcinoma demonstrate that 0-12% of intramucosal tumours exhibit lymph node spread compared to 26-46% of submucosal lesions[20-22]. In early oesophageal adenocarcinoma, combining the results of five studies, a total of 5/155 (3%) intramucosal and 48/216 (22%) submucosal tumours had lymph node metastases[23-27].

It therefore appears that the incidence of lymphatic metastases in early oesophageal cancer is less in patients with adenocarcinoma compared to those with squamous cell carcinoma[28]. This may be due to the protective effect of chronic inflammation in the submucosal layer

secondary to gastro-oesophageal reflux. The inflammatory response may obliterate submucosal lymphatic channels and delay lymphatic dissemination in patients with oesophageal adenocarcinoma. However, it is not yet clear whether stratifying pT patients into pT1a (intramucosal) and pT1b (submucosal) will improve the accuracy of the TNM staging system.

1.2.2.2 Stratifying pN Stage

The literature suggests that lymph node metastasis is the most important prognostic determinant in patients with operable oesophageal cancer. It has been consistently found to be an independent prognostic factor[29,30]. The 6th edition AJCC classification defines the pathological nodal status based merely on the presence (N1) or absence (N0) of lymph node metastasis. However, distinct survival curves can be produced on the number (0, 1 to 2, or ≥ 3), as well as the ratio (0, < 0.15, or ≥ 0.15), of metastatic to total resected lymph nodes[31-34]. There is increasingly worse survival in patients with 3 or more involved lymph nodes compared to patients with less than 3 metastatic lymph nodes, as found by Wijnhoven *et al* in their publication in the *Annals of Surgery* in 2007[35].

Further, a few investigators have shown that capsular invasion of the lymph node is an independent negative prognostic factor[36-38] (Figure 1.3). Lerut and colleagues[36] found that in patients with only one involved lymph node, the 5-year survival associated with extracapsular tumour extension was significantly worse (33.3%) than it was compared to intracapsular nodal involvement only (85.7%). They concluded that the number and characteristics of lymph nodes should be incorporated into the next edition of the AJCC *Cancer Staging Manual*[36].

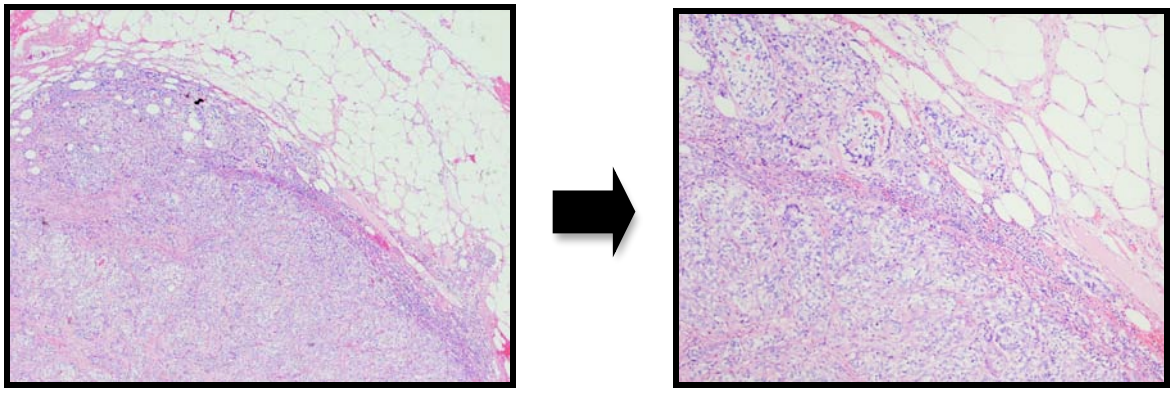


Figure 1.3 Extracapsular lymph node invasion of a lymph node with Hematoxylin & Eosin staining (x 100, x 40).

1.2.2.3 Circumferential Margin

A positive circumferential resection margin (CRM) is defined as the presence of tumour within 1 mm of the surgically cut surface of the adventitial tissue. Dexter *et al* found a significant decrease in 5-year survival from 39 to 21 months when tumour cells were detected within 1 mm of the radial margin[39]. Similarly, Griffiths *et al* found a median survival of 37 months (range 28-47) in patients with a negative CRM, and 18 months (range 13-23) in those with a positive CRM, respectively[40]. However, it is not yet clear whether a positive radial margin is an independent prognostic factor in oesophageal cancer.

1.2.2.4 Pathological Response to Chemoradiotherapy

The treatment response following neoadjuvant therapy has been classified in various ways. Swisher *et al* published one of the more recent classifications in 2005[34]: complete eradication (no residual tumour cells, P0), partial response (1 to 50% residual cancer, P1), and little response or chemoradiotherapy-resistance (>50% residual cancer, P2). Using this classification system, they found a significant difference in survival between complete responders (3-year survival=74%), partial responders (3-year survival=54%), and poor responders (3-year survival=24%)[34] following neoadjuvant therapy and oesophageal

resection. It is therefore possible that the extent of pathological response to neoadjuvant therapy is an independent prognostic factor, and should be incorporated in the TNM staging system to better predict patient outcome.

1.2.3 Lymphatic Spread in Oesophageal Cancer

1.2.3.1 Lymphatic Drainage Pathways

Lymphatics in the submucosal layer form a complex interconnecting network that extends the length of the oesophagus, intermittently piercing the muscularis propria to drain into regional nodes in the peri-oesophageal tissue. It is important to note that as soon as cancer cells breach the basement membrane (of the mucosa), tumour may then spread via the lymphatics. As discussed in the previous section, the risk of lymphatic involvement increases once the submucosa is penetrated. Lymph may also drain by direct connections to the thoracic duct.

1.2.3.2 Pattern of Lymph Node Dissemination

Lymph node spread from gastric adenocarcinoma and oesophageal squamous cell carcinoma has been studied in depth in Japan, and much of the current nomenclature is based on these findings. The nodal tiers for oesophageal tumours are based on their anatomical location (abdominal, thoracic, and cervical), and subdivided according to the frequency of nodal metastases at each site. This has then been correlated to the need for radical resection with increasing levels of lymphadenectomy[41].

There is, however, evidence of significant differences in the pattern of lymph node dissemination between oesophageal adenocarcinoma and squamous cell carcinoma. Dresner *et al* found that left gastric, left and right paracardial nodal stations (in the abdomen), and para-oesophageal nodes (in the mediastinum) were most frequently

involved in 104 patients with Type 1 adenocarcinoma of the lower oesophagus[16]. And in 78% of node-positive tumours, both abdominal and mediastinal nodal stations were involved. This pattern differed from that of 48 Type 2 tumours of the gastric cardia, which spread less frequently to mediastinal nodes[16].

To establish whether a sequential pattern of lymph node spread exists, Matsubara *et al* studied early lymphatic dissemination for patients with squamous cell carcinoma of the oesophagus[42]. In 46% of lymph node positive cases, disease was limited to a solitary node and these patients had an excellent 5-year survival of over 60% following radical surgery. Although some involved nodes were anatomically distant from the primary tumour, this was considered to be a result of direct longitudinal lymphatic spread. A number of other findings support the concept of sequential lymphatic spread. Dresner *et al* found that distant nodal stations were only positive when nodes within the first tier were also involved[16]. Van de Ven *et al*[43] found that 90% of patients with node-positive adenocarcinoma had an involved node within 3 cm of the primary tumour, and Feith *et al*[44] found skipping of regional lymph node stations (i.e. positive "distant" nodes in the absence of positive "regional" nodes) in less than 5% of patients.

1.2.4 Extent of Lymphadenectomy

Cancers at any site in the oesophagus have the potential for malignant cells to lodge in regional lymph nodes from the neck to the upper abdomen. In the West, most cancers occur in the lower esophagus and in the region of the cardia. For these cancers, malignant cells may be found in cervical nodes in about 30% of cases in squamous cell cancers[41], and in about 15% of cases in adenocarcinoma[45]. Furthermore, a recent publication has suggested that the more lymph nodes that are removed the better the 5-year survival, with this effect holding up to greater than 40 nodes removed[46]. Self-evidently, radical

lymphadenectomy procedures remove more nodes than a non-radical lymphadenectomy[47] (Figure 1.4).

Figure 1.4 Terminology for lymphadenectomy in oesophageal cancer[47]

NOTE:
This figure is included on page 27 of the print copy of the thesis held in the University of Adelaide Library.

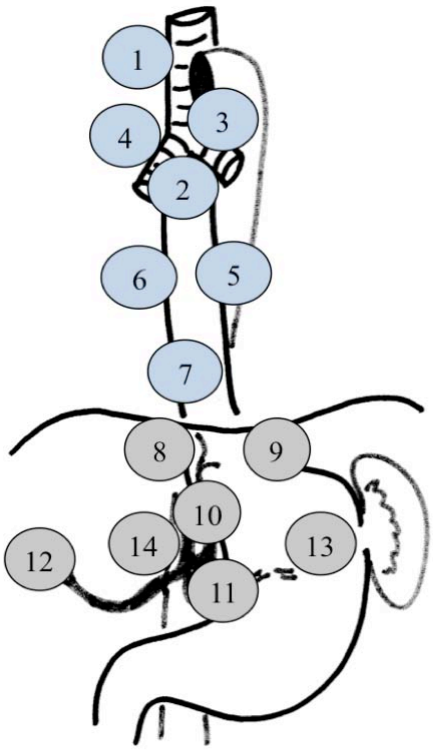
As for many solid organ tumours, there is controversy about the extent of lymphadenectomy for oesophageal cancer. This is because there is a lack of high-level evidence to support any type of radical lymphadenectomy. There has been one randomized controlled trial published for adenocarcinoma involving the oesophagus[48]. Patients were randomised to have a transhiatal oesophagectomy with conservative lymphadenectomy (n=106), against a transthoracic oesophagectomy with infra-carinal two-field lymphadenectomy (n=114). Overall 5-year survivals for each group were not significantly different (34% and 36%, respectively) and, as expected, patients with no involved nodes did not benefit from a more radical lymphadenectomy[48]. However, it is interesting to note that in the subgroup of patients with 1-8 involved lymph nodes, a locoregional disease-free survival advantage was present if operated on via the

transthoracic route[48]. For squamous cancer there have been two small, randomized controlled trials published. One study[49] found a significant difference in 5-year survival rates, and the other did not[50].

There is no doubt that for patients with oesophageal cancer, the presence and number of metastases in lymph nodes greatly worsens a patient's prognosis. There is also no doubt that the best operation to be certain that a patient is node negative is a three-field lymphadenectomy. That is purely in staging terms. But in the absence of any high-level evidence supporting the more radical procedure, many centres (like ours) continue to perform conservative lymphadenectomy with oesophageal resection. An important end-point of the work that follows will be to determine whether a conservative lymphadenectomy provides less prognostic information and worse overall survival for the patient compared to a two-field lymphadenectomy (Figure 1.5).

Figure 1.5 Lymph nodes removed in a two-field (A) vs conservative (B) procedure

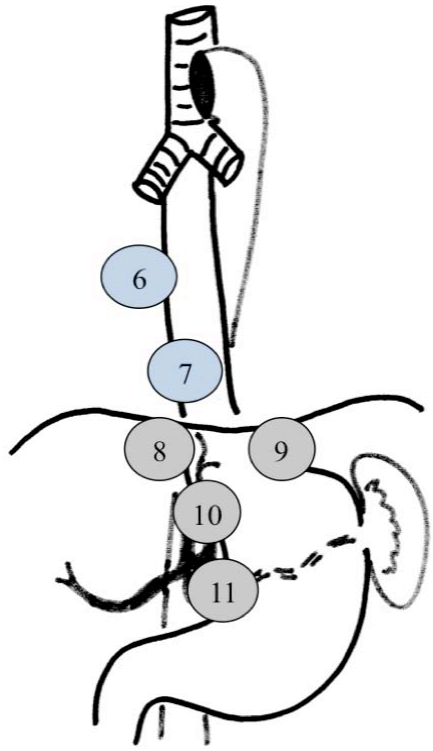
A



Mediastinal Nodes
1 Paratracheal
2 Carinal
3 Left bronchial
4 Right bronchial
5 Para-aortic
6 Middle paraesophageal
7 Lower paraesophageal

Abdominal Nodes
8 Right paracardial
9 Left paracardial
10 Left gastric
11 Lesser curve
12 Common hepatic
13 Splenic artery
14 Celiac axis

B



Mediastinal Nodes
1 Paratracheal
2 Carinal
3 Left bronchial
4 Right bronchial
5 Para-aortic
6 Middle paraesophageal
7 Lower paraesophageal

Abdominal Nodes
8 Right paracardial
9 Left paracardial
10 Left gastric
11 Lesser curve
12 Common hepatic
13 Splenic artery
14 Celiac axis

1.2.5 Molecular Markers

Molecular markers have been described as the Holy Grail of prognostic indicators. They have the potential to identify patients with a high risk of metastatic relapse, despite favourable staging by standard histopathologic means. Tumour profiling with cDNA microarrays allows the simultaneous expression analysis of thousands of genes from tumour specimens that can then be used to determine their prognostic potential[51].

A malignant tumour comprises cells with increased proliferative activity, prolonged lifespan, and metastasizing capacity. These cells are caused by an accumulation of mutations in the genome (genomic instability). The targets of genomic instability can be found in 4 classes of genes[52]:

1. ***Proto-oncogenes***: dominant genes found in normal cells that perform a regulated role in activation of cell proliferation or inhibition of apoptosis (cell death). Upon activation through mutation, these genes turn into oncogenes with continuous stimulation of cell proliferation.
2. ***Tumour suppressor genes***: recessive genes in normal cells that inhibit cell proliferation or stimulation of cell apoptosis. Both gene copies need to be lost for these cells to lose their suppressive function.
3. ***Mismatch repair genes***: recessive genes that repair DNA sequence mistakes during DNA replication. Inactivation of both copies of the gene results in defective DNA repair, and therefore increases in mutations. Tandem repeat DNA sequences (microsatellites) are vulnerable to DNA replication mistakes, therefore microsatellite instability is an example of a defect in DNA repair.
4. ***Mitotic checkpoint genes***: genes involved in ensuring the proper separation of chromosomes during cell division.

Activation of proto-oncogenes and inactivation of tumour suppressor genes form the key elements of tumour development. To date, there are no proto-oncogenes or tumour

suppressor genes that are activated or inactivated in all cancers.

Molecular markers that have been identified as having prognostic potential in oesophageal cancer include TGF-α (transforming growth factor-α), HER2/*neu*, COX-2 (cyclooxygenase-2), and p53 tumour suppressor gene[52]. Molecular markers will hopefully lead to more accurate differentiation in the aggressiveness and ultimately, the treatment, of oesophageal cancer. Markers need to be correlated to clinical outcome data in order to determine their prognostic significance, and we have chosen to examine the potential of HER2/*neu* using tissue microarrays on our oesophageal cancer specimens.

1.3 OCCULT TUMOUR DEPOSITS

1.3.1 Introduction

The not infrequent observation of later tumour recurrence in patients who have seemingly had a complete resection of their tumour suggests that clinically undetectable tumour deposits must be present. And the fact that lymph nodes are a frequent site of tumour recurrence indicates that this compartment must be an important site for occult disease. Recent studies indicate that 1 to 17% of histologically negative lymph nodes and 11 to 50% of pathologically node negative patients have nodal metastases that are missed by routine pathologic examination[53].

1.3.2 Definitions

The Union Internationale Contre le Cancer (UICC) has published guidelines on distinguishing micrometastases (metastases not visible on conventional histological analysis) and isolated tumour cells (Figure 1.6)[54]:

Figure 1.6 UICC Definitions for Occult Tumour Deposits[54]

Micrometastatic disease in the lymph nodes can be detected with the use of immunohistochemical (IHC) techniques and reverse transcriptase-polymerase chain reaction techniques. These techniques can detect the presence of 1 tumour cell in approximately 105 normal cells[51]. The markers used to detect micrometastases include cytokeratins, mucins (cell surface glycoprotein), and molecular markers. Monoclonal antibodies directed against epithelial-cell proteins are most appealing because epithelial elements are not usually present in lymph nodes. It is also important to note that IHC techniques are more reliable than non-morphologic methods (i.e. polymerase chain reaction) because they have a lower false-positive rate at present[54].

Two epithelial antibodies have been shown in prior studies to possess a high specificity in lymphatic tissue for the detection of epithelial tumour cells: anti-EpCAM epithelial antibody Ber-EP4, and monoclonal epithelial antibody AE1/AE3. The monoclonal antibody AE1/AE3 is much more widely used because it recognizes a broad range of keratin subtypes expressed in oesophageal carcinomas[55]. Using the epithelial antibody AE1/AE3 (Figure 1.7), investigators have found between 30-40 % of pN0 patients have occult micrometastatic tumour deposits[56-60]. Bonavina *et al*[56], in a small group of patients,

assessed whether the rate of metastatic lymph nodes detected could be increased by routinely performing additional serial sectioning or IHC staining. They concluded that it could and that IHC staining was the most cost-effective method to increase detection rates.

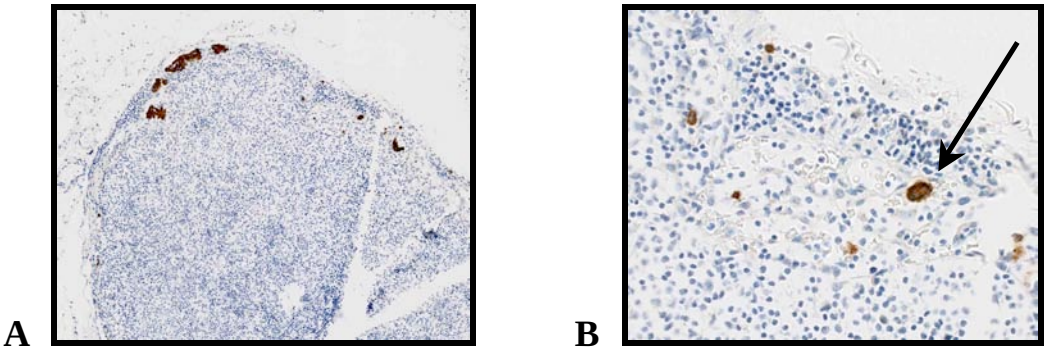


Figure 1.7 A micrometastasis (A) and an isolated tumour cell (arrow, B) in an oesophageal cancer lymph node, using IHC with AE1/AE3 (x 400).

1.3.3 Importance of Occult Tumour Deposits

A total of eleven studies have examined the correlation of lymph node micrometastasis and survival[56-66]. Eight studies[56-63] have shown a significant correlation between lymph node micrometastasis and decreased survival. However, three studies[64-66] have not shown a correlation and herein lays the controversy as to whether immunohistochemical techniques should be used routinely for examining the lymph nodes from oesophageal cancer specimens. Why the discrepancy in results? The average sample size in these studies is 62, which is a reasonable number. The problems rest with differing methodologies, and lack of definitions. Three studies performed only one additional section to look for micrometastases[60,65,66]. Assuming a lymph node is on average 1 cm in diameter, they could have easily missed a micrometastasis less than 2 mm in diameter. Only two studies differentiate between a micrometastasis and an isolated tumour cell[60,63]. They both found no decreased survival with the detection of isolated tumour cells, but they did find a

significant decrease in 5-year survival with the detection of micrometastases. All other studies failed to differentiate between isolated tumour cells and micrometastases making their final analysis meaningless.

The importance of free lying isolated tumour cells has not been determined. It seems highly likely that different methods for detecting and defining lymph node micrometastases, which is the area addressed by this study, are responsible for the controversies in this area.

1.4 THE SENTINEL LYMPH NODE CONCEPT

1.4.1 Introduction

While including IHC staining in routine lymph node analysis for oesophageal cancer specimens may certainly provide significant prognostic data for the patient, it is not cost-effective since, at the present time, therapy is not based on the presence or absence of isolated tumour cells in lymph nodes. Therefore, performing a detailed analysis of each resected lymph node is simply impractical and is not accepted as the standard of care for conventional clinical practice. The *sentinel lymph node concept* relates to the preferential lymphatic drainage of a primary tumour to one or more regional lymph nodes[67,68]. Identification and excision of these lymph nodes along with the specimen (and non-sentinel lymph nodes) allows for closer scrutiny of the sentinel lymph nodes with both serial sectioning and IHC. Unlike breast cancer and melanoma, identification of one or more sentinel nodes will not change the extent of nodal dissection (at this point in time). However, we expect that a more intensive review of such nodes for micrometastases will improve staging accuracy and might guide the use of postoperative therapies in the future.

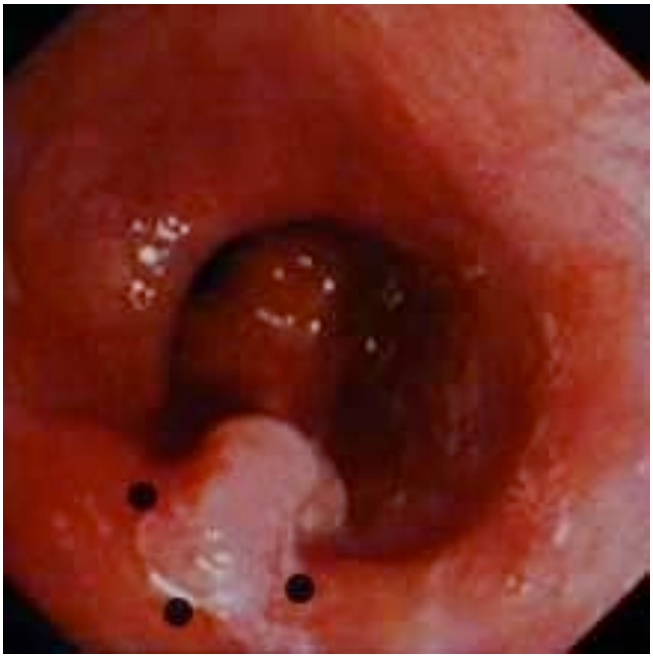
1.4.2 Mapping Techniques

The most commonly used radionucleotide agent in Europe for the detection of sentinel lymph nodes in breast cancer is technetium (^{99m}Tc) nanocolloid. It is popular due to its size of less than 80 nanometres, and its half-life of 6 hours. Colloid particles between 4 to 100 nm in size are necessary to translocate from the interstitial injection site to lymphatic channels, and to be retained within the first lymph node(s) encountered along such pathways[69,70]. However, the type of radiocolloid available for clinical use is strongly dependent on that particular country's legislation[69]. Filtered ^{99m}Tc-sulphur colloid is used routinely in North America, ^{99m}Tc-albumin nanocolloid in Europe, ^{99m}Tc-tin fluoride colloid in Japan, and ^{99m}Tc-antimony trisulfide colloid in Australia[71]. This has important implications for both this study and in interpreting the literature. ^{99m}Tc-tin colloid with a particle size of 100 nm results in a long period of tracer deposition in the lymph which allows surgeons in Japan to perform a lymphoscintigraphy 24 hours prior to surgical resection[72]. The remaining 3 radiocolloids have smaller particle sizes, with a median transit time of 10 minutes to sentinel nodes, and a half-time for washout of activity in the node(s) between 4-8 hours[69,71]. As a consequence, surgery must be planned shortly after peritumoural injection (or in the case of antimony colloid, a much higher dose must be given). This leaves little time for preoperative lymphoscintigraphy, and in the current study, peritumoural injection has been undertaken immediately prior to oesophagectomy.

Technetium (^{99m}Tc) antimony trisulfide colloid is injected immediately prior to surgery with the patient anaesthetised on the operating table with a double-lumen endotracheal tube in situ. The operating surgeon performs a video-endoscopic examination of the tumour (Olympus series) and, using a 25-gauge endoscopic injection needle (US Endoscopy Group), 4 aliquots of 0.5 millilitres (corresponding to 40-50 MegaBecquereles) is injected submucosally at the margins of the tumour. Peritumoural injection can only occur

proximally in the presence of a stricture (Figure 1.8).

Figure 1.8 Correct technique for peritumoural injection of radiocolloid



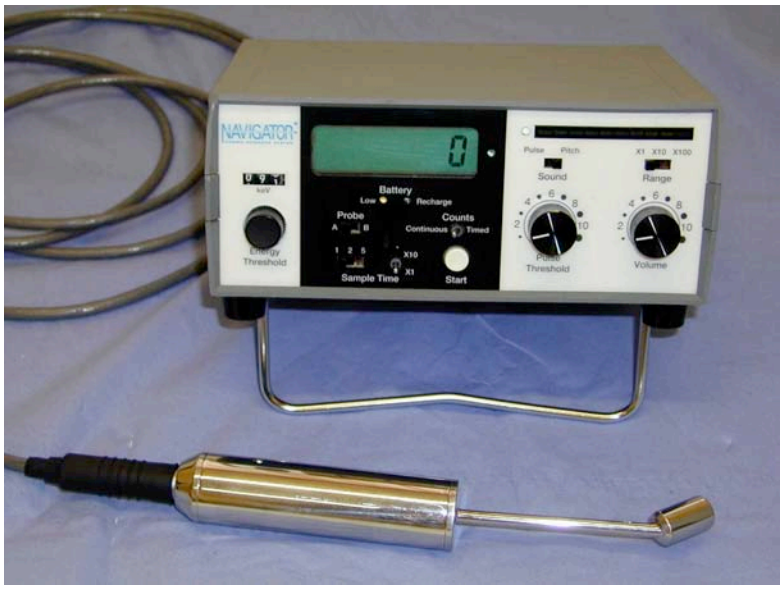
1.4.3 Definition of a Sentinel Lymph Node

A Navigator^{TM} Gamma Guidance System (United States Surgical Corporation) is used to detect 'hot' sentinel nodes containing technetium (^{99m}Tc) antimony trisulfide colloid (Fig 1.9). This gamma probe uses a solid-state cadmium telluride detector system and gives a quantitative reading of activity with an audible count-rate indicator. It has been shown to effectively and reliably detect sentinel nodes in many studies, mostly involving breast cancer[73].

In studies using radioactive tracer rather than aqueous dye, there is no standard criteria for defining hot nodes or sentinel lymph nodes[74,75]. Many investigators have defined a sentinel node as any node with an intraoperative activity more than twice that of surrounding tissue (sentinel node:background ratio of 2:1 or 3:1 *in-vivo*), or any node with

a postoperative gamma probe reading of more than 10 times background activity (sentinel node:background ratio of 10:1 *ex-vivo*)[69,76]. It is important to note that the absolute reading obtained from a lymph node is a function of the dose of radioactive colloid, time from injection, background interference, detection sensitivity of the probe, and precise positioning of the probe[74]. There is some evidence the false-negative rate can be lowered by treating all nodes with greater than 10% of the radioactivity of the 'hottest' node as sentinel nodes[75]. As such, the most recent EANM-EORTC guidelines for sentinel node diagnostics in melanoma (published in 2009[71]) state that a sentinel node is the hottest node plus any other hot nodes containing more than 10% of the activity in the hottest node in the lymphatic basin. This is the definition used in our study.

Figure 1.9 Navigator™ gamma guidance system



1.4.4 Pathological Examination of a Sentinel Lymph Node

After surgical resection, each lymph node station is dissected off the specimen on the back table and sent off to Pathology. Specimens are sent either fresh or in formalin, depending

on the preference of the pathologist. Sentinel lymph nodes are also identified and sent in separate containers (Figure 1.10).

Figure 1.10 Labelled pots with separate lymph node stations for pathological analysis

There are no internationally-recognized protocols for sentinel lymph node analysis so the number of sections and choice of cytokeratin agent is dependent upon both the laboratory and the pathologist. In this project, designated sentinel lymph nodes are analyzed with a minimum of three serial sections and immunohistochemisty, using the monoclonal antibody AE1/AE3 (as discussed in section 1.3.2[55]), if negative on initial analysis[77-79].

1.4.5 Sentinel Node Biopsy in Oesophageal Cancer

Although there are preliminary reports from several centres demonstrating the feasibility of identifying sentinel lymph nodes in squamous cell carcinoma of the oesophagus[80], and in adenocarcinoma of the oesophagus[72,76,81], further studies validating the technique are needed. Lamb *et al*[76] have performed the largest study to date. They reported a 96% accuracy rate in identifying the sentinel lymph node (with both H&E and IHC) in patients with operable oesophageal or gastric adenocarcinoma. This study has cleared the way for

further studies looking at the sentinel lymph node concept in upper gastrointestinal cancers. Further, there are no published studies that focus on the prognostic significance of nodal staging based on focused analysis of the sentinel lymph nodes in oesophageal cancer[82].

1.5 AIMS

1. To examine the prognostic value of the following variables following oesophagectomy for oesophageal cancer on overall survival:
   a. Sub-division of T1 lesions into T1a (intramucosal) and T1b (submucosal) lesions
   b. Refinement of lymph node status into N1a (< 3 metastatic lymph nodes) and N1b (≥ 3 metastatic lymph nodes)
   c. Presence or absence of lymph node invasion (extra-capsular tumour extension)
   d. Presence or absence of a positive circumferential resection margin
   e. Degree of tumour response to neoadjuvant therapy (if applicable)

2. To determine the prognostic value of HER2/*neu* gene amplification and overexpression in oesophageal adenocarcinoma.

3. In resection specimens classified as lymph node-negative on conventional histological analysis, to determine the incidence and prognostic value of:
   a. immunohistochemically (IHC)-identified micrometastases and
   b. isolated tumour cells

   in the histologically negative nodes.

4. To validate the described technique for identification of sentinel lymph nodes in patients with cancer of the oesophagus and gastro-oesophageal junction, and confirm its accuracy in predicting nodal involvement of non-sentinel lymph nodes.

CHAPTER 2: IMPROVING THE ACCURACY OF TNM STAGING IN ESOPHAGEAL CANCER: A PATHOLOGICAL REVIEW OF RESECTED SPECIMENS

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2.2 ABSTRACT

Background: Controversy exists over the 6th edition of the International Union Against Cancer (UICC) TNM staging system for esophageal cancer. Inclusion of additional information such as the number of metastatic lymph nodes and extracapsular lymph node invasion may improve the current staging system, and lead to optimization of patient treatment. **Methods**: All patients in Adelaide who underwent resection for esophageal cancer between 1997 and 2007 were identified from a prospective database. Two independent observers then re-examined all pathology slides from the original resection. Univariate and multivariate analysis was performed to identify significant prognostic factors. The goodness of fit and accuracy of additional prognostic factors were assessed, and the staging system was modified according to this information. **Results**: 240 patients (mean age, 62 yrs) met the inclusion criteria. The 5-yr overall survival rate was 36% (median, 24 months). Only histological grade and a refined pN-stage were found to be independent prognostic factors, which could then be used to improve current TNM staging. Subdivision of pN-stage into 3 groups (0, 1-2, and > 2 positive nodes) showed significant differences in 5-yr survival between all 3 groups: 53% vs. 27% vs. 6%, respectively ($P<0.01$). The optimal staging model was the same for patients who received neoadjuvant therapy and surgery (n=116), and those who underwent surgery alone (n=124).

Conclusion: A staging model which incorporates a refined pN-stage and histological grade appears to be more accurate than the current UICC-TNM staging system. This staging model is still applicable in patients who receive neoadjuvant therapy.

2.3 INTRODUCTION

The incidence of esophageal adenocarcinoma has increased 6-fold over the past 25 years, and this rate of increase is faster than for any other malignancy in the Western world.[1] Accurate staging of esophageal cancer is critical in determining prognosis and in tailoring therapy. The modern era of TNM based staging for esophageal cancer was introduced by the American Joint Committee in Cancer (AJCC) in 1988, and then revised in 2002. This system represents a worldwide benchmark for reporting the extent of malignant disease and for providing accurate prognostic information.[2] However, in its present form, it is suboptimal because it combines patients with very different outcomes into each disease stage. There is also confusion as to its relevance in patients who have received neoadjuvant therapy prior to surgical resection.[3]

Recent publications examining the TNM staging system for esophageal cancer support the inclusion of sub-dividing T1 tumors into T1a (intramucosal) and T1b (submucosal) tumors, and refining nodal status depending upon the number and/or ratio of lymph nodes containing metastatic disease.[4-8] Other proposed prognostic factors that might improve the current staging system are tumor differentiation, extracapsular lymph node invasion, tumor length, an incomplete circumferential resection margin, and the presence of either vascular or perineural invasion.[4,9-16]

A retrospective review of all our esophageal cancer pathology specimens in our city was performed to evaluate the independent prognostic nature of all of these recently reported variables. Our aim was *not* to simply determine which factors had prognostic value, but to single out those factors which could significantly improve the accuracy of the current TNM staging system. As a secondary aim, the influence of neoadjuvant therapy on the

ability of these factors to predict outcome, and its influence on apparent TNM stage was also determined.

2.4 MATERIALS AND METHODS

2.4.1 Patient Selection

Surgical specimens were identified from an Adelaide-wide Esophageal Cancer Surgery audit database, held at the Royal Adelaide Hospital in Adelaide, Australia. Since July 1997, prospective follow-up data has been collected and stored in this database. In addition, the original pathology reports and operation records were reviewed. We included all patients with a "surgical resection" of either invasive squamous cell carcinoma or adenocarcinoma. Twelve cases were excluded from our study: 8 did not contain invasive carcinoma (i.e. carcinoma in situ or high-grade dysplasia), 2 were not classified as either squamous cell carcinoma or adenocarcinoma (1 collision tumor, 1 adenosquamous tumor), and 2 patients did not give consent for their slides to be reviewed. All operations were performed or closely supervised by one of 5 surgeons (G.G.J., D.I.W., P.G.D., Justin Bessell, Philip Game). The study was approved by the Research Ethics Committee at the Royal Adelaide Hospital and by the Flinders Clinical Research Ethics Committee, Bedford Park, South Australia.

2.4.2 Preoperative Staging and Surgery

Preoperative clinical staging had included upper gastrointestinal endoscopy, computed tomography scans (chest, abdomen, pelvis), and diagnostic laparoscopy (if gastroesophageal junction or cardia tumors). Since 2002, most patients also underwent positron emission tomography and endoscopic ultrasonography. Selected patients (T2 or greater) were treated with neoadjuvant therapy according to surgeon preference.

Neoadjuvant therapy consisted of 2 cycles of cisplatin (80 mg/m^2 on day 1) and 5-FU (800 mg/m^2 continuous infusion for 5 days), plus 15 fractions of radiation therapy (over 3 weeks) to a total of 40 to 50 Gray.

Patients underwent surgical resection 5 to 6 weeks after completion of neoadjuvant therapy. Esophagectomy was usually performed by a 2-surgeon synchronous Ivor-Lewis technique via a right antero-lateral thoracotomy and an upper midline laparotomy, as described previously.[17,18] In selected patients, a transhiatal (cervico-abdominal) or 3-stage (cervico-thoraco-abdominal) esophagectomy was performed. A standard or non-radical lymph node dissection (removal of all nodes adjacent to the tumor) was performed in all patients, regardless of operative technique. Continuity of the gastrointestinal tract was restored by either a handsewn or stapled end-to-side esophago-gastrostomy, depending on surgeon preference.

2.4.3 Pathology

Specimen identification numbers were obtained from the database, and corresponding complete sets of slides were then retrieved from one of 4 pathology laboratories: ClinPath Laboratories, IMVS, South Path Laboratories, and Adelaide Pathology Partners. A single pathologist reviewed the slides of all specimens in a blinded fashion (A.R.R.), along with an upper gastrointestinal surgeon (S.K.T.). No additional slides were cut from paraffin blocks nor were immunohistochemical techniques used.

The stage of esophageal cancer was confirmed (using current AJCC staging), including the sub-division of pT1 into pT1a (intramucosal) and pT1b (submucosal) tumors. Lymph node metastases were classified as intracapsular or extracapsular. The extension of cancer cells through the lymph node capsule into the perinodal fatty tissue was defined as

extracapsular lymph node invasion (LNI).[13] Care was taken not to confuse this with cancer cells in afferent lymphatic vessels. A positive circumferential resection margin (CRM) was defined as the presence of tumor within 1 mm of the surgically cut surface of the adventitial tissue.[4,19,20] When present, perineural invasion, vascular invasion, and Barrett's epithelium (intestinal-type metaplasia with the presence of goblet cells) was documented.

The treatment response following neoadjuvant therapy was classified according to Swisher *et al.*'s system[21]: complete eradication (no residual tumor cells in either the esophagus or lymph nodes, pCR), partial response (1 to 50% residual cancer, pPR), and little response or chemoradiotherapy-resistance (>50% residual cancer, pLR). Patients with T0N0 (i.e., pCR) were classified as stage 0. Patients with T0N1 disease were assigned as stage IIB.[3]

The grade of tumor differentiation was assessed based on the preoperative biopsy result as some patients had received neoadjuvant therapy. We classified all tumors as well/moderately, or poorly/un-differentiated, recording the poorest grade within the biopsy.[4] Tumor length and the total number of lymph nodes was obtained from original pathology reports as this could not be determined from the slides alone. The lymph node ratio (LNR) was calculated as the number of positive lymph nodes divided by the total number of resected lymph nodes. When there was a difference between the original pathology report and the current review, we took the current assessment for consistency.

2.4.4 Statistical Analysis

Overall survival was calculated from the date of operation to July 30, 2007 (if alive) or to the date of death (as recorded from the South Australian Cancer Registry) according to the Kaplan-Meier method. Overall survivals were compared to prognostic factors with the log-rank test. Multivariate analyses were performed by Cox regression for selected

prognostic factors that were significant on univariate analysis (all prognostic factors were not included to avoid over-fitting and colinearity). Subset analyses were performed for patients who had not received neoadjuvant therapy, and subsequently, for those who had received therapy. The optimal cutoff point for both the number of metastatic nodes and the lymph node ratio as predictors of survival were determined by using a scatter plot of both these variables versus Martingale residuals of the Cox model. A smoothed fit of the scatter was then applied to detect the optimal cutoff point.

To compare the "goodness of fit" of different staging models (including the independent prognostic factors following Cox regression), the Bayesian Information Criterion (BIC) was calculated for each scenario. A lower value for the BIC represents a better fit.[22] Harrell's C was calculated to determine which staging scenario was most accurate in predicting survival. A value closer to 1 indicates better diagnostic accuracy.[22] Statistical significance was set at the 5% level. Calculations were performed using SPSS 12.0.1 (Chicago, Illinois, USA), and Stata version 9.1 (College Station, TX).

2.5 RESULTS

2.5.1 Patients

There were 281 patients who underwent a surgical resection between July 1997 and January 2007 identified from the database. Of these, 240 patients met inclusion criteria for this study. The mean age was 62.3 years (95% CI 61.0-63.6 years). There were 188 men (78%) and 52 women (22%). One hundred and twenty-four patients (52%) underwent neoadjuvant therapy. Patients' and tumor characteristics are listed in Table 1.

2.5.2 Outcome of Surgery

Esophagectomy was performed using the Ivor-Lewis technique in 70% of patients, using a three-stage or McKeown technique in 26%, and using a transhiatal technique in 8%. The mean lymph node harvest was 7 (range 0-37). Six patients (2.5%) had a positive proximal margin, 11 (4.6%) had a positive distal margin, and 85 (35%) had a positive circumferential resection margin. The median length of stay was 14 days (range, 8-103 days). The 30-day mortality rate was 6.3% (15 patients). Complete follow-up was available for all 240 patients with an overall 5-year survival rate of 36%, and a median survival of 23.8 months (95% CI 15.3-32.3 months).

2.5.3 Prognostic Factors

Table 1 and 2 show survival according to patient and tumor characteristics, tumor stage, and additional pathologic criteria on univariate analysis. Tumor length >5 cm, an increasing grade of undifferentiation, histological type of adenocarcinoma, extracapsular lymph node invasion (LNI), the presence of vascular invasion or perineural invasion, the presence of Barrett's esophagus, and a positive circumferential resection margin (<1 mm) all were associated with a significant survival disadvantage on univariate analysis. Although not included in Table 1, a positive proximal or distal margin was also associated with significantly poorer survival (P=0.043 and P<0.001, respectively).

Patients with intramucosal (pT1a) tumors did not have a significantly improved 5-year survival compared to pT1b (submucosal) tumors (P=0.526) although an increasing depth of tumor invasion (pT-stage) overall was significantly associated with poorer survival. The number of positive lymph nodes as well as the lymph node ratio were highly significant independent prognostic factors. To determine the optimal cutoff point for the

number of positive lymph nodes, the Martingale residuals of the Cox model were first calculated and then plotted against the number of positive lymph nodes. The Lowess smoothed line crossed the line of zero residual at 1 positive lymph node. Hence, the best cutoff for predicting death is none versus one or more positive lymph nodes. We therefore opted to use the Rice classification of zero (pN0), 1 or 2 positive lymph nodes (pN1a), and 3 or more positive (pN1b) lymph nodes for our refined pN-stage. The same calculation was performed for lymph node ratio, and the best cutoff for predicting death is a lymph node ratio ≥ 0.1. We therefore examined patient survival based on a lymph node ratio of zero or ≥ 0.1. The overall survival according to TNM stage and number of involved lymph nodes is demonstrated in Figures 1 and 2, respectively.

Table 3A shows the results of the multivariate analysis after the inclusion of selected significant prognostic factors on univariate analysis. pT-stage, a refined pN-stage, and grade of differentiation remained significant. A positive circumferential resection margin did not retain its significance.

2.5.4 Influence of Neoadjuvant Therapy

One hundred and twenty-four patients (52%) had neoadjuvant therapy, and the results of this subset analysis are listed in Table 4. Twenty-five patients had a complete response (pCR) to chemo-radiotherapy with no residual tumor found. Seventy-nine patients had a partial response (pPR: 1-50% residual tumor), and 29 patients had little pathological response (pLR: >50% residual tumor). There was no significant difference in 5-yr survival between patients who received neoadjuvant therapy and those who did not ($P = 0.125$). Figure 3 demonstrates the significant difference in 5-yr survival between pCR patients compared to pPR and pLR groups: 63% vs. 43% vs. 22%, respectively ($P = 0.042$).

There was a significantly lower circumferential resection margin involvement if the patient had received neoadjuvant therapy compared with surgery alone (22% vs. 50%, respectively) (P<0.001). Similarly, patients with neoadjuvant therapy had lower rates of extracapsular lymph node invasion in their metastatic lymph nodes (44% vs. 72%, respectively) (P=0.004). A refined pN-stage was the only independent prognostic factor on multivariate analysis (Table 3B).

2.5.5 No Neoadjuvant Therapy

Univariate and multivariate analyses were repeated in the subset of patients who did not have neoadjuvant chemo-radiotherapy (n=116) (Table 5). Prognostic factors with a significant survival benefit were the same as those presented in Tables 1 and 2 for the whole study group with the exception of histological type, and tumor length (P=0.85 and P=0.17, respectively).

2.5.6 Staging System

Goodness of fit of the UICC-TNM staging system was compared to a refined UICC-TNM with the addition of independent prognostic variables. Table 6 shows the accuracy of various staging models in predicting survival after esophagectomy. The UICC-TNM staging system with the addition of pN1a (1 or 2 positive lymph nodes) and pN1b (>2 positive lymph nodes) and grade of differentiation was the most accurate model. Goodness of fit analyses in patients who did not receive neoadjuvant therapy were very similar. This refined system also applied to patients who had undergone neoadjuvant therapy.

2.6 DISCUSSION

Over the past decade, there has been a surge in the number of publications addressing various prognostic factors in esophageal cancer and/or proposed modifications to the current AJCC staging system for esophageal cancer. The existing TNM system is inadequate because it is based primarily on information from patients with squamous cell carcinoma of the upper and middle esophagus, it classifies lymph node involvement beyond the regional lymph nodes as M1 disease, and it does not stratify according to the number of positive lymph nodes.[20,23] The increased attention in the literature to this problem reflects the rapidly increasing incidence of esophageal adenocarcinoma, estimated to be, within the United States, 3.8 per million in 1973-1975 to 23.3 per million in 2001.[1]

In choosing to review all esophageal cancer specimen slides, we were able to include all possible prognostic factors that have been reported as having independent prognostic ability in the recent literature. In contrast to many of these articles, our principal aim was to identify which independent prognostic factors could increase the accuracy of our current staging system, not simply determine independent prognostic ability. An ideal staging system is one in which the stages demonstrate monotonic, distinctive, and homogeneous survival.[5] The various prognostic factors were tested with this in mind.

The median lymph node number in this series was 7 and reflects at least two things. First, all the surgeons involved share the view that optimal therapy for esophageal cancer involves removal of the cancer and surrounding tissues and adjacent lymph nodes en bloc, and that systematic lymphadenectomy does not confer a survival advantage for patients.[24] We accept that this view is not shared universally. Nevertheless, the 5-year survival rate of 36% is acceptable and consistent with many other studies.[6,9,15,19,25] Second, during much

of this period the pathologists involved have varied greatly in the assiduousness of their search for lymph nodes. Whether the removal of more nodes, or using increased numbers of lymph node sections, or using immunohistochemistry, would have altered our findings are questions we are currently addressing with further research.

Consistent with prior publications, we found tumor length >5 cm, grade of tumor differentiation, presence of either vascular invasion or perineural invasion, presence of Barrett's esophagus, extracapsular lymph node invasion, and a positive circumferential resection margin to be independent prognostic factors for survival in patients who have undergone an esophagectomy.[4,5,7,9,12-15,19,20,26-28] In addition, we found that pT-stage, pN-stage, an increasing number of lymph nodes, and lymph node ratio to be significant prognostic predictors of survival. However, only three prognostic factors remained significant on multivariate analysis: grade of differentiation, number of positive lymph nodes, and tumor depth. And only two additional prognostic factors were found that would improve the accuracy of the current TNM staging system: grade of differentiation and number of positive lymph nodes. Based on our findings, we believe a revised TNM staging system should incorporate these two prognostic factors regardless of whether the patient has received neoadjuvant therapy.

In addition, the best cutoff for the number of metastatic lymph nodes in our study was 0, 1-2, and 3 or more lymph nodes. There is great variation in the numbers used to refine the current pN-stage. In particular, some studies have described a cutoff of 3 or more positive lymph nodes[5,6], 4 or more positive lymph nodes[4,7,23,29-31], 5 or more positive lymph nodes[8,32], and combinations of 6, 7 or 8 or more positive lymph nodes[11,16,33,34]. It is impossible to directly compare results between centers with this lack of uniformity. It is also unclear on what basis other groups chose a particular cutoff point. Lymph node ratio

was also found to be a significant prognostic factor. However, the number of metastatic lymph nodes was more discriminatory in improving the accuracy of the TNM staging system.

Some other findings are worthy of discussion. Well and moderately differentiated tumors were combined into one category as were poorly and un-differentiated tumors. This is a modification of the grading system described by Dickson *et al.*, where they designated specimens as G1 (well differentiated), G2 (moderately differentiated), or G3 (poorly and un-differentiated). In their study, G1 and G2 patients had similar 3-yr survival (33% vs. 29%, respectively).[12] Thus, we combined these two categories. Some studies have found this to be a highly significant factor worthy of inclusion to the current staging system[12,35], while others have not.[4,5] This discrepancy could be due to an inaccurate grading of differentiation following neoadjuvant therapy, inter-pathologist variation, or limited sampling. We found grade of differentiation (based on preoperative biopsy) to be a very strong predictor of survival in patients with esophageal cancer, and as stated above, it significantly strengthened the current staging system.

Histological type was also found to be significant on univariate analysis. Khan *et al*. found that patients with adenocarcinoma fared worse than those with squamous carcinoma, a similar finding to the present study.[35] The reason for this is unclear and could be that tumors in the mid-esophagus present earlier than those in the lower esophagus or gastroesophageal junction. As well, 67% of patients with a squamous carcinoma received neoadjuvant therapy compared with only 47% of adenocarcinoma patients and it remains possible that neoadjuvant therapy has greater benefit in patients with a squamous cell cancer.[36] No significant survival difference was found between intramucosal (pT1a) and submucosal (pT1b) tumours similar to the findings of Ellis *et al*.[29] It is possible this is a

type II statistical error due to the low number of intramucosal tumors in our dataset since others have found a significant survival difference between pT1a and pT1b tumors.[5,6,32]

In our analysis, no intramucosal tumor was associated with positive lymph nodes, similar to the findings of Hagen *et al*. in which only one of 16 patients with an intramucosal tumor had a single positive lymph node.[32] In contrast, 17% of submucosal tumors (3 of 18 specimens in the no neoadjuvant group) were associated with nodal metastases. Submucosal tumors were found to have a similar survival to tumors invading the muscularis propria (pT2), supporting Rice *et al.*'s suggestion to group these patients together into Stage II, and to isolate intramucosal tumors into Stage I.[5,6]

Consistent with other publications and a meta-analysis by Wind *et al.*, extracapsular lymph node invasion was found to be an independent prognostic factor in patients with metastatic lymph node disease.[4,9,10,13,19,26,37,38] Sixty-eight of 112 patients (61%) with positive lymph nodes had evidence of extracapsular lymph node invasion which is similar to the recently reported pooled incidence of 57% (95% CI: 53-61%) for patients with esophageal cancer.[13] With the exclusion of the 3 studies which included vessel invasion and isolated deposits in their definition of extracapsular lymph node invasion, our incidence of 61% was identical to that of the remaining 3 papers (61%; 95% CI: 55-67%). Extracapsular lymph node invasion identifies a subgroup of patients with a significantly worse 5-year survival, 16% vs. 31% respectively.

No correlation was seen between extracapsular lymph node invasion and histological type (53% vs. 62% for squamous cell carcinoma and adenocarcinoma, respectively). However, patients who did not receive neoadjuvant therapy had significantly higher rates of extracapsular lymph node invasion in their metastatic lymph nodes. While it has been

suggested that comparable survival exists in patients without positive lymph nodes and patients with only intracapsular lymph node invasion[10,13], our results tend not to support this view. Node-negative patients in our review had a 5-year survival rate of 53% compared to 31% in those with intracapsular lymph node invasion, although this difference was not significant. We find it difficult to agree with the statement that "lymphatic dissemination does not essentially deteriorate prognosis, provided that the lymph node capsule remains intact."[13] Further, while extracapsular lymph node invasion is an independent prognostic factor for survival and its presence should be reported by pathologists, it is not as important as the number of positive lymph nodes in improving the accuracy of the current staging system.

Eighty-five specimens (35%) had a positive circumferential resection margin on slide review, however the rate of circumferential resection margin involvement was significantly lower if the patient had received neoadjuvant therapy compared with surgery alone (22% vs. 50%, respectively). These results are similar to those reported in the literature in patients treated with surgery alone (47-55%), and confirm Sujendram *et al.*'s findings of significantly lower circumferential resection margin involvement (31%) in patients treated preoperatively with chemotherapy.[14,19,20] Consistent with the findings of Khan *et al.*[39], radial margin status was not significant on multivariate analysis nor did it improve the accuracy of the current staging system. This is probably because 68% of patients with a positive radial margin were node-positive, and conversely, 65% of patients with a negative margin were node-negative. Therefore, the survival advantage conferred by having a negative circumferential resection margin does not seem to be due to a wider excision but by an association with negative lymph nodes.

Barrett's esophagus was present in 62% of specimens of adenocarcinoma. Its presence conferred a significant survival benefit such as that described in three recent papers.[4,27,28] The improved survival noted in patients with Barrett's esophagus was directly related to more favorable tumor characteristics (decreased length, better differentiation, and smaller pT-stage and pN-stage). As described by Portale *et al.*, the non-Barrett's group may represent tumors in whom metaplasia is no longer apparent or it may be that the cancers developed without Barrett's mucosa being present.[28]

The effect of neoadjuvant therapy on the prognostic ability of the above factors and on the overall pTNM staging system was evaluated as a secondary aim. Close to one third of these patients (29%) achieved a complete pathological response consistent with other reports on esophageal adenocarcinomas.[21,40,41] We performed an external validation of Swisher *et al*.'s system for estimating treatment response following neoadjuvant therapy by categorizing all specimens into three groups depending upon degree of treatment response. A significant improvement in 5-year survival was seen as the degree of response increased (63% vs. 43% vs. 22%, respectively). Consistent with Rizk *et al*., pT-stage and degree of treatment response were not as significant as pN-stage (including number of lymph nodes) in predicting survival in this patient subset.[3] Therefore, our patients with neoadjuvant therapy appeared to behave as per their downstaged 'stage', a finding reported by others[7,8,21], and thus a refined TNM model incorporating the number of metastatic lymph nodes and the grade of differentiation is applicable in both those patients who receive neoadjuvant therapy and those who do not.

2.7 CONCLUSION

Although many independent prognostic factors were found that could predict improved 5-year survival in patients who have undergone an esophageal resection, only two factors improved the accuracy of the current TNM staging system: grade of differentiation and number of positive lymph nodes (0, 1-2, >2 nodes). These results: 1) add further weight to the necessity of a refinement in nodal staging, 2) discount the ability of many independent prognostic factors to increase the accuracy of the current TNM model, and 3) are applicable to patients who received neoadjuvant therapy and those who were treated with surgery alone. We therefore submit that a revised TNM staging system should incorporate grade of differentiation and number of positive lymph nodes and furthermore, this should be irrespective of neoadjuvant therapy.

2.8 ACKNOWLEDGMENTS

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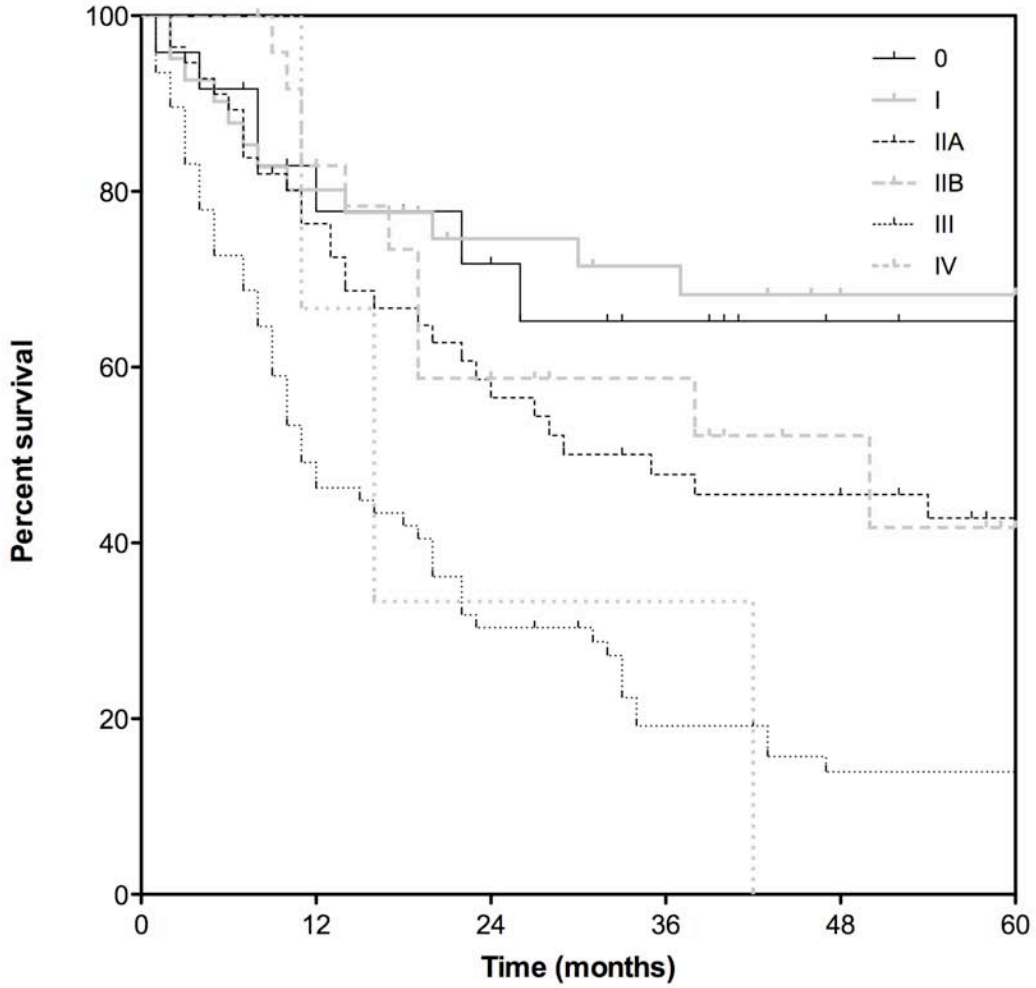
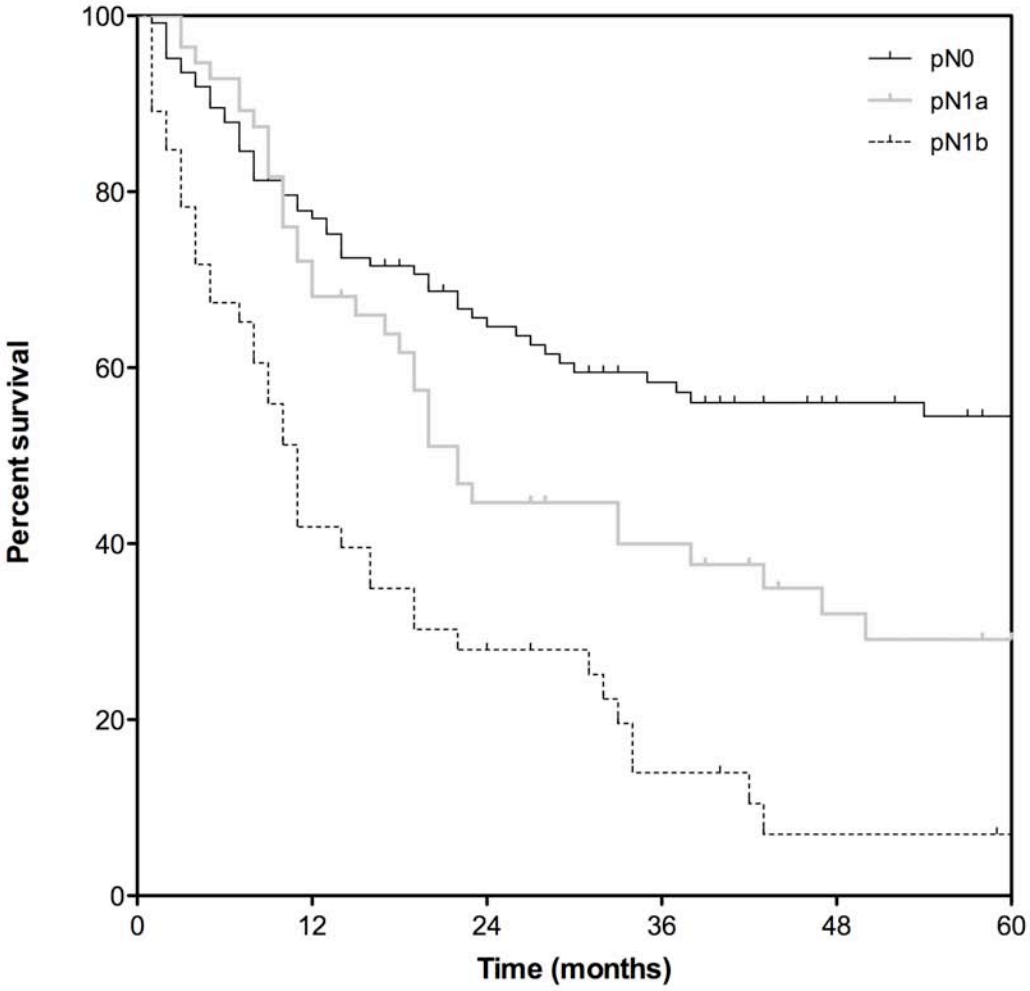


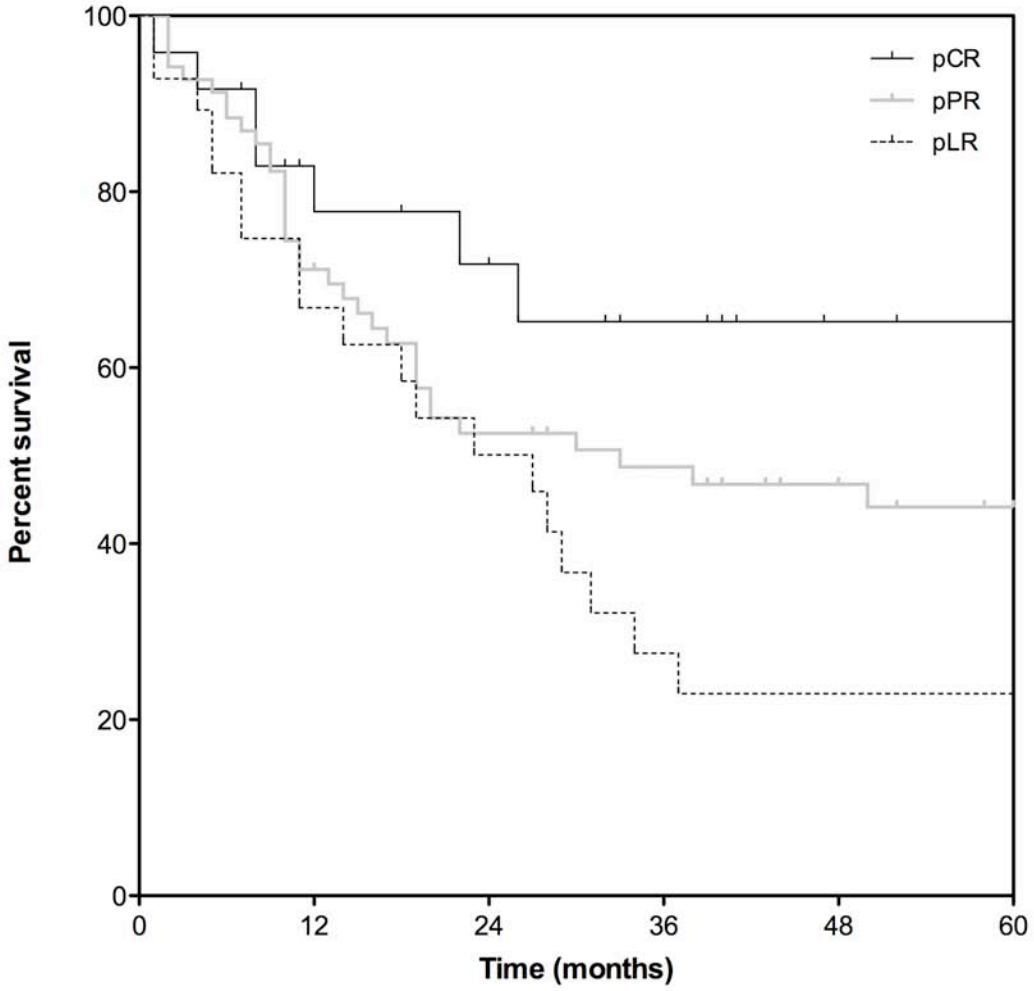
No. at risk						
0	24	16	12	8	4	3
II	41	31	24	22	17	16
IIA	56	40	28	21	20	14
IIB	25	19	12	9	5	2
III	77	34	21	12	8	8
IV	3	2	1	1	0	0

Figure 2.1. Overall 5-year survival according to pTNM-stage for 240 patients who underwent esophagectomy for esophageal cancer. There was a significant difference in survival between the subgroups (*P*<0.001).



No. at risk						
pN0	124	88	65	51	41	33
pN1a	56	36	21	17	11	9
pN1b	46	18	12	5	2	1

Figure 2.2. Overall 5-year survival for 240 patients who underwent esophagectomy for esophageal cancer according to number of involved lymph nodes. There was a significant difference in survival between patients with 0 positive nodes (pN0), 1 or 2 positive lymph nodes (pN1a), and more than 2 positive lymph nodes (pN1b) (*P*<0.001).



No. at risk						
pCR	24	16	12	8	4	3
pPR	69	44	30	25	19	15
pLR	28	16	12	6	5	5

Figure 2.3. Overall 5-year survival according to treatment response after neoadjuvant therapy for 124 patients who underwent esophagectomy for esophageal cancer. There was a significant difference in survival between patients with no residual tumor (i.e. complete response, pCR) compared to those with evidence of residual tumor (partial response, pPR; little response, pLR) (*P*=0.042).

Table 2.1. Survival According to Patients' and Tumor Characteristics (n = 240) on Univariate Cox Regression

Variable	No. Patients (% of 240)	Median Survival (mo)	5-yr Survival (%)	*P*
Age, y				0.099
< 70	177	29.8	37	
> 70	63	12.8	32	
Sex				0.93
Male	188	26.2	36	
Female	52	20.6	36	
Neoadjuvant therapy				0.125
No	116	20.6	31	
Yes	124	29.8	41	
Tumor length, cm				0.026*
0-5	195	28.2	40	
> 5	45	19.8	20	
Tumor location				0.521
Upper/middle	27	79.5	51	
Lower	68	32.1	44	
GEJ	145	22.1	29	
Grade of differentiation				<0.001*
Well/moderate (G1 + G2)	79		56	
Poor/undifferentiated (G3 + G4)	148	23.6	24	
Histology				0.018*
Adenocarcinoma	191	22.7	33	
Squamous cell	49	38.1	49	
Barrett's epithelium[a]				0.009*
No	64	20.0	13	
Yes	106	33.0	42	
Vascular invasion				<0.001*
No	95		53	
Yes	128	16.4	23	
Perineural invasion				<0.001*
No	123	38.1	49	
Yes	74	11.1	15	
Positive CRM[b]				<0.001*
No	155	50.0	48	
Yes	85	12.8	32	

[a]Data given for adenocarcinoma patients only
[b]CRM – circumferential resection margin

Table 2.2. Survival According to Tumor Pathology and pTNM stage (n = 240) on Univariate Cox Regression

Variable	No. patients	Median Survival (mo.)	5-Yr Survival (%)	*P*
pT-stage				
T0	51			
T1a	12		92	0.268
T1b	39	79.5	53	0.526
T2	33	27.6	42	0.216
T3	110	16.8	25	0.006*
T4	17	8.2	0	<0.001*
pN-stage				<0.001*
N0	128	24.4	53	
N1	112	3.2	18	
pM-stage				0.415
M0	237	22.8	36.7	
M1	3	16.8	0	
Stage groups (UICC 2002)[a]				<0.001*
0[b]	25		63	
I	44	88.8	64	
IIA	56	29.8	43	
IIB[c]	26	50.1	42	
III	86	10.7	12	
IV	3	16.8	0	
Extracapsular lymph node invasion				<0.001*
No	44	33.5	31	
Yes	68	10.1	16	
No. positive lymph nodes				
0	128	24.4	53	
1-2	60	20.6	27	0.010*
>3	52	9.8	6	<0.001*
Lymph node ratio				<0.001*
0	129	64.5	51	
>0.10	106	14.5	16	

[a]Stage I: T1N0M0; Stage IIA: T2-3N0M0; Stage IIB T1-2N1M0; Stage III: T3N1M0/T4anyNM0; Stage IV: anyTanyNM1
[b]Stage 0 = no residual tumour in specimen
[c]Stage IIB includes patients who were T0 (no residual tumour) and node-positive

Table 2.3A. Independent Prognostic Factors for Survival after Resection for Esophageal Cancer from Multivariate Cox Regression (n = 227 complete cases)

Prognostic Factor	Hazard Ratio	95% CI	*P*
pT-stage			
T0	1.00		
T1a	0.39	0.05 – 3.23	0.38
T1b	1.58	0.62 – 4.02	0.34
T2	1.52	0.59 – 3.96	0.39
T3	2.23	0.91 – 5.47	0.08
T4	3.22	1.10 – 9.38	0.03*
pN-stage			
N0	1.00		
N1a	1.40	0.90 – 2.17	0.13
N1b	2.25	1.42 – 3.57	0.001*
Grade of differentiation			
Well/moderate (G1 + G2)	1.00		
Poor/undifferentiated (G3 + G4)	1.71	1.13 – 2.59	0.008*
Positive CRM[a]			
No	1.00		
Yes	1.07	0.69 – 1.67	0.76

Table 2.3B. Independent Prognostic Factors for Survival after Resection for Esophageal Cancer for those who received neoadjuvant therapy from Multivariate Cox Regression (n = 112 complete cases)

Prognostic Factor	Hazard Ratio	95% CI	*P*
pT-stage			
T0	1.00		
T1a	2.54	0.15 – 43.42	0.52
T1b	3.65	0.45 – 30.00	0.23
T2	1.87	0.22 – 15.99	0.57
T3	3.94	0.50 – 31.02	0.19
T4	3.93	0.36 – 42.69	0.26
pN-stage			
N0	1.00		
N1a	1.61	0.83 – 3.10	0.16
N1b	3.75	1.64 – 8.60	0.002*
Grade of differentiation			
Well/moderate (G1 + G2)	1.00		
Poor/undifferentiated (G3 + G4)	1.47	0.83 – 2.62	0.19
Positive CRM[a]			
No	1.00		
Yes	0.72	0.35 – 1.47	0.39
Response to neoadjuvant therapy			
Complete	1.00		
Partial	0.62	0.06 – 5.33	0.62
Little	0.63	0.05 – 5.95	0.63

[a]CRM – circumferential resection margin

Table 2.4. Subset Analysis of Survival in Patients with Neoadjuvant Therapy (n=124)

Variable	No. Patients (% of 124)	Median Survival (mo)	5-yr Survival (%)	*P*
pT-stage				
T0	29		63	
T1a	4	88.0	75	0.668
T1b	21	38.0	40	0.187
T2	18	27.0	45	0.354
T3	49	19.0	33	0.032*
T4	3	31.0	0	0.114
pN-stage				0.016*
N0	79	79.0	54	
N1	45	19.0	17	
Extracapsular lymph node invasion				0.001*
No	25	33.0	30	
Yes	20	11.0	0	
No. positive lymph nodes				
0	79	79.0	54	
1-2	30	20.0	23	0.222
>3	15	14.0	9	0.001*
Lymph node ratio				0.006*
0-0.09	80	79.0	54	
>0.10	42	19.0	13	
Tumor length, cm				0.157
0-5	109	30.0	59	
> 5	15	20.0	18	
Grade of differentiation				0.084
Well/moderate (G1 + G2)	44	64.0	52	
Poor/undifferentiated (G3 + G4)	70	22.0	34	
Barrett's epithelium				0.498
No	49	23.0	35	
Yes	53	30.0	43	
Vascular invasion				0.211
No	69	31.0	45	
Yes	44	19.0	34	
Perineural invasion				0.097
No	77	31.0	45	
Yes	23	19.0	21	
Positive CRM[a]				0.184
No	97	37.0	46	
Yes	27	19.0	27	
Response to neoadjuvant therapy				
Complete	25		63	
Partial	70	30.5	43	0.220
Little	29	23.8	22	0.042*

[a]CRM – circumferential resection margin

Table 2.5. Subset Analysis of Survival in Patients with No Neoadjuvant Therapy (n=116)

Variable	No. Patients (% of 116)	Median Survival (mo)	5-yr Survival (%)	*P*
pT-stage				
T1a	8		100	
T1b	18		72	0.123
T2	15	38.0	41	0.028*
T3,T4	75	12.0	15	<0.001*
pN-stage				<0.001*
N0	49		51	
N1	67	11.0	17	
pM-stage				0.546
M0	113	20.0	32	
M1	3	16.0	0	
Extracapsular lymph node invasion				0.030*
No	19	34.0	32	
Yes	48	8.0	11	
No. positive lymph nodes				
0	49		51	
1-2	30	20.0	29	0.025*
>3	37	9.0	7	<0.001*
Lymph node ratio				0.001*
0-0.09	49	38.0	46	
>0.10	67	10.0	16	
Tumor length, cm				0.168
0-5	86	22.0	35	
> 5	30	13.0	21	
Grade of differentiation				<0.001*
Well/moderate (G1 + G2)	35		62	
Poor/undifferentiated (G3 + G4)	78	11.0	17	
Barrett's epithelium				0.040*
No	45	16.0	19	
Yes	60	32.0	41	
Vascular invasion				<0.001*
No	26		75	
Yes	84	12.0	17	
Perineural invasion				<0.001*
No	46		55	
Yes	51	10.0	13	
Positive CRM[a]				<0.001*
No	58	65.0	53	
Yes	58	9.0	13	

[a]CRM – circumferential resection margin

Table 2.6. Goodness of fit (BIC) and predictive accuracy (Harrell's C) of prognostic variables for esophageal cancer. Variables excluded from the models were not statistically significant after the other variables were included.

Model	Harrell's C	BIC
pT stage	0.626	1385
+ pN0,1 (UICC 2002)	0.649	1383
+ No. positive nodes (0,1-2,>3)	0.659	1382
+ Histological grade (well/mod, poor/undiff)	0.681	1294

CHAPTER 3: HER-2/*neu* GENE AMPLIFICATION IN ESOPHAGEAL ADENOCARCINOMA AND ITS INFLUENCE ON SURVIVAL

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Statements of authorship appear on pages 76-77 in the print copy of the thesis held in the University of Adelaide Library.

3.2 ABSTRACT

Introduction: HER-2/*neu* (c-*erb*B-2, HER2) gene amplification and protein overexpression have been associated with poor prognosis in several solid tumors, including breast and gastric cancer. Its incidence and significance in esophageal adenocarcinoma is unknown. **Methods**: Tissue microarrays were successfully constructed from 89 paraffin-embedded archival specimens of esophageal adenocarcinomas for HER2 gene amplification by silver-enhanced *in situ* hybridization (SISH). No patients had undergone neoadjuvant therapy. Protein overexpression was tested with immunohistochemistry (IHC) using automated immunostaining (Ventana Benchmark). Incidence of HER2 positivity, correlation to clinicopathological variables in esophageal cancer patients, and concordance between SISH and IHC were determined. **Results**: True HER2 gene amplification was detected in 14 (16%) esophageal cancer specimens, and 92% of those with high-level HER2 amplification showed positive HER2 protein overexpression. No significant associations were found among gene amplification and clinicopathological factors. Five-year survival rates were 57% for esophageal cancer patients with HER2 amplification compared to 32% without, but the difference in overall survival was not significant (P=0.37). The correlation between SISH and IHC was statistically significant (P<0.0001). **Conclusion**: While molecular targeting may be possible for approximately 16% of esophageal adenocarcinoma patients, HER2 oncogene amplification did not influence survival in this study.

3.3 INTRODUCTION

Targeted molecular therapy in upper gastrointestinal cancer has become an increasingly popular topic over the past few years. In part, this is due to rapid advances in our capability to characterize tumor biology. Another consideration is our less-than-satisfactory ability to predict a particular tumor's response to neoadjuvant therapy. Esophageal adenocarcinoma is an example of an aggressive cancer in which only one third of patients present with resectable disease. And of this select group, the average 5-year survival is only 35 to 45%.[1] The addition of neoadjuvant therapy has significantly improved 5-year survivals, but much improvement is still needed. Targeted molecular therapy may help in this regard.

Human epidermal growth factor receptor gene HER-2/*neu* (also known as c-*erb*B-2, now HER2) was recognized as an important prognostic factor in breast cancer in 1987.[2,3] However, its role in other solid tumors is controversial.[4-9] The published frequency of HER2 overexpression in esophageal cancer ranges from 11 to 73%.[10] Reports evaluating its significance are also varied in their conclusions. Nevertheless an international randomized Phase III trial, evaluating the survival benefit in gastric or gastro-esophageal junction cancer patients of the humanized anti-HER2 monoclonal antibody (Trastuzumab), has just been published.[11]

The aims of our study were 1) to determine the frequency of HER2 gene amplification and overexpression in esophageal adenocarcinoma; 2) to evaluate the association of HER2 gene amplification with patient and tumor characteristics and patient survival; and 3) to examine the correlation between amplification and expression of HER2 using silver-enhanced *in situ* hybridization (SISH) and immunohistochemistry (IHC).

3.4 MATERIALS AND METHODS

3.4.1 Patient Selection

All patients who had undergone a surgical resection for invasive upper gastrointestinal adenocarcinoma were identified from an Adelaide-wide Esophageal Cancer Surgery audit database, held at the Royal Adelaide Hospital in Adelaide, Australia. Since July 1997, prospective follow-up data has been collected and stored in this database. Esophagectomy was performed by a 2-surgeon synchronous Ivor-Lewis technique via a right antero-lateral thoracotomy and an upper midline laparotomy, as described previously.[12] A conservative lymph node dissection (removal of all nodes adjacent to the tumor) was performed in all patients, regardless of operative technique.[13] Patients who underwent neoadjuvant therapy were excluded from this study to obtain a homogeneous cohort of patients in terms of treatment and to circumvent possible stage migration following chemoradiation therapy. The study was approved by the Research Ethics Committee at the Royal Adelaide Hospital, Adelaide, South Australia.

3.4.2 Tissue Microarrays

In a previous study[1], we re-examined 240 esophageal cancer pathology specimens to determine which variables could improve the accuracy of the TNM staging system. During this project, we also selected appropriate paraffin blocks for construction of tissue microarrays which were used in this study. To increase our sample size, additional esophageal adenocarcinoma patients after January 2007 (up until December 2009) were included and appropriate paraffin blocks were selected for review. Specimen identification numbers were obtained from our database, and the designated paraffin blocks were then retrieved from one of 3 pathology laboratories: ClinPath Laboratories, Institute for Medical and Veterinary Science, and Adelaide Pathology Partners. Tissue microarrays

were constructed with 2 cores, each 1.0 mm in diameter, from 2 paraffin blocks (i.e. 4 cores/patient). Representative cores of tumor were selected by A.R.R. based on each block's corresponding hematoxylin and eosin (H&E) stained sections. Other studies have demonstrated the reliability of tissue microarrays in the evaluation of HER2 gene amplification in solid tumors including breast carcinomas.[14]

3.4.3 Double-Staining for HER2 Amplification and AE1/AE3 Cytokeratin Expression

Tissue microarray sections (4 μm) were cut, mounted on Superfrost Plus coated slides, labeled and then placed on a fully automated immunohistochemistry (IHC) staining and In Situ Hybridization (ISH) Ventana Benchmark XT (Roche Diagnostics) instrument. The sections were incubated with ISH-protease 3 (Roche Diagnostics) for 8 min, washed with reaction buffer (Roche Diagnostics) followed by denaturation of tissue DNA at 95 °C. The DNA probe for Human Epidermal Growth Factor Receptor 2 (HER2) (Roche Diagnostics), labeled with Dinitrophenol (DNP), was then added and hybridization occurred for 6 hours. Rabbit anti-DNP (Roche Diagnostics) was used to detect the labeled probe followed by visualization with *ultra*View silver *in situ* hybridization (SISH) detection kit (Roche Diagnostics) in accordance with the manufacturer's standard procedures.[15]

The section was then washed in reaction buffer followed by addition of Cell Conditioning 1 (CC1) solution (Roche Diagnostics) for 30 minutes. CC1 was removed, washed, and the primary mouse monoclonal epithelial antibody AE1/AE3 (Dako, Carpinteria, CA) for IHC was then added for 36 min whilst the slide was heated to 37°C. The monoclonal antibody AE1/AE3 is widely used because it recognizes a broad range of keratin subtypes expressed in esophageal carcinomas.[16] The *ultra*View™ Universal Alkaline Phosphatase RED kit

(Roche Diagnostics), used in accordance with the manufacturer's recommendations, was used to detect the location of the primary antibody AE1/AE3 followed by counterstaining with hematoxylin 11 (Roche Diagnostics).

3.4.4 Evaluation of HER2 Gene Amplification

Evaluation of SISH hybridization was performed with conventional light microscopy by a histopathologist (A.R.R.) and a medical scientist (R.D.). Both were blinded with respect to patient identification, tumor characteristics on conventional histopathology, and HER2 protein expression. Gene amplification was assessed as per the Australian HER2 Advisory Board criteria for single HER2 probe testing: diploid = 1 to 2.5 copies/nucleus in more than 50% of tumor cells; polysomy = 2.5 to 4 copies/nucleus in more than 50% of tumor cells; equivocal amplification = >4 to 6 copies/nucleus in more than 50% of tumor cells; low-level amplification = 6 to 10 copies/nucleus in more than 50% of tumor cells; high-level amplification = >10 copies/nucleus in more than 50% of tumor cells. When using the Chromosome 17 probe, the classification of not amplified was when the HER2/Chromosome 17 ratio was <1.8; equivocal >1.8 and <2.2; and amplification was >2.2. HER2 and Chromosome 17 assays were performed on contiguous sections allowing for the identification and exclusion of chromosome 17 polysomy.[2,15]

3.4.5 Staining for HER2 Protein with Immunohistochemistry

Sections (4 μm) of tissue microarrays were cut, mounted on coated slides, labeled, and then placed on the Ventana Benchmark XT (Roche Diagnostics) for detection of the HER2 oncoprotein. The sections were de-waxed then subjected to pre-treatment with CC1 for 30 minutes. Sections were then washed with reaction buffer followed by incubation with the rabbit monoclonal primary antibody HER-2/neu (Clone 4B5, Roche Diagnostics) for 28

minutes. On board detection using *ultra*View™ Universal DAB kit (Roche Diagnostics), used in accordance with the manufacturer's recommendations, was used to detect the location of the primary antibody HER2 followed by counter stain with hematoxylin 11 (Roche Diagnostics).

3.4.6 Evaluation of HER2 Protein Expression

Evaluation and scoring of HER2-protein expression was performed according to the Dako HercepTest™ scoring system for breast cancer. This scoring system has been validated for use in gastric cancer with minor modifications:[3,17] 0/negative = staining or membranous reactivity in <10% of cells; 1+/negative = faint membranous reactivity in >10% of cells or cells with reactivity only in part of their membrane; 2+/equivocal = weak/moderate complete or basolateral membranous staining in >10% of tumor cells; 3+/positive = strong complete or basolateral membranous staining in >10% of tumor cells.

3.4.7 Statistical Analysis

The presence of HER2 gene amplification and/or protein overexpression was correlated with clinical outcome. Overall survival was calculated from the date of operation to July 15, 2010 (if alive) or to the date of death (as recorded from the South Australian Cancer Registry) according to the Kaplan-Meier method. Fisher's exact tests were used to compare variables between the two HER2 amplification groups (present/not present). Survival was compared between the groups using a log rank test. Differences in survival between the HER2 groups were assessed using a log-rank test. Correlation between SISH and immunohistochemistry was calculated using the Kendall Tau-b correlation coefficient.[18] Statistical significance was set at the 5% level. Calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA).

3.5 RESULTS

3.5.1 Patients

There were 336 patients who underwent a surgical resection for esophageal cancer between July 1997 and December 2009 identified from the database. The 30-d mortality rate was 4.8%. Of these, 140 met inclusion criteria of an esophageal adenocarcinoma and no chemoradiotherapy prior to surgical resection. A further 51 patients were excluded for various reasons, and we were left with a study population of 89 patients (Figure 1).

Patients' and tumor characteristics are listed in Table 1. The mean age was 63.9 years (95% CI 61.7-66.1 years). There were 74 men (83%) and 15 women (17%). The median time of patient follow-up was 20.6 months (627 days). Complete follow-up was available for all 89 patients with an overall 5-year survival rate of 35%, and a median survival of 22.1 months.

3.5.2 HER2 Amplification or Overexpression

Fourteen esophageal cancer patients had HER2 gene amplification (Figure 2). Similar numbers of patients had weak/moderate or strong membrane staining for HER2 protein overexpression (Table 2). HER2 amplification was seen more commonly in pT1 (25%) and pT4 tumors (27%) versus pT2 (9%) and pT3 (11%) tumors but this difference was not significant (P=0.25). The presence of low or high HER2 amplification did not influence any other patient or tumor characteristic (Table 3). Five-year survival rates were 57% (median, 68.9 months) for esophageal cancer patients with HER2 amplification compared to 32% (median, 20.6 months) without, but the difference in overall survival was not significant (P=0.37) (Figure 3). Similarly, in the Barrett's cancer subset of patients, there was no significant difference in overall survival between groups (P=0.29).

3.5.3 Correlation between HER2 Amplification and Overexpression

When SISH results were compared with HER2 immunohistochemical (IHC) data, eleven of twelve cases (92%) with high-level gene amplification showed positive 3+ protein expression (Table 4). The remaining case was negative for protein expression. One of two low-level gene amplification cases was equivocal (2+) on IHC testing, while the other was negative (1+). None of the diploid nor Polysomy 17 cases showed equivocal or positive protein expression. We did not classify any cases in the equivocal category for HER2 amplification using SISH. Overall, there was a significant correlation between SISH and immunohistochemistry for HER2 gene amplification and expression ($P<0.0001$). The correlation coefficient between SISH and IHC was 0.636 (moderate/strong association) ($P<0.0001$).

3.6 DISCUSSION

Close to 16% of our esophageal cancer patients had HER2 gene amplification and overexpression in their primary tumor. The previously quoted range of 11-73% for HER2 overexpression largely originates from studies conducted in the 1990s, and using primarily immunohistochemistry.[19-25] Some of these older studies concluded that HER2 protein overexpression corresponds with poor survival.[19,20] But more recently, studies have examined the frequency of HER2 gene amplification in esophageal adenocarcinoma at the DNA level using either polymerase chain reaction (PCR) or some form of *in-situ* hybridization (ISH).[7,10,26-29] Our results correspond to these latter studies (except one[26] with a small sample size of 25) in which frequencies of HER2 amplification are consistently lower and range from 12-24%.

Unlike one study in esophageal adenocarcinoma[10], upon which current Herceptin-based trials seem to be based, we found no correlation between the presence of HER2 amplification and patient survival. Nor was there any correlation between HER2 amplification and clinicopathological factors. Brien *et al* evaluated HER2 amplification with FISH in 63 Barrett's adenocarcinoma patients, and although they found no significant association between HER2 amplification and clinicopathological factors, they reported a significant association between its presence and poorer survival.[10] However, in this study, a low threshold of 4 or more signals (rather than the currently accepted threshold of 6 or more signals[15]) per nucleus was used to determine the presence of HER2 amplification.[10] In addition, patients with chromosome 17 polysomy were not excluded. Aneuploidy of chromosome 17, usually involving an increase in the number of chromosomal copies (i.e. polysomy), has been reported in approximately one third of breast cancers. However, increased protein expression at the significant 3+ level does not seem to result from this

mechanism because HER2 appears to remain normally regulated.[26] Some investigators have suggested that controversy regarding the role of HER2 amplification and its affect on survival might be explained by the failure to distinguish between true HER2 gene amplification and chromosome 17 polysomy.[26,30]

In esophageal adenocarcinoma at least, our results seem to be the norm rather than the exception.[22-25,27,29] The lack of any apparent effect of HER2 amplification on patient survival is supported by the absence of any association between HER2 amplification and known poor prognostic pathological factors (i.e. pT-stage, pN-stage). Results of the ToGA (Trastuzumab for Gastric Cancer) trial suggested that HER2-positive patients with junctional gastro-esophageal cancers were potential responders to anti-HER2 monoclonal antibody-based therapy.[11] However, even the authors of this trial point out that the survival benefit seen in the HER2-positive group may have been due to the presence of HER2 overexpression alone rather than the result of HER2-targeted therapy.

Support for HER2 amplification as a prognostic and predictive factor in gastric adenocarcinoma is also controversial with several studies showing a significant association[31], and others not.[32] The most recent of these encompassed 924 gastric cancer cases and is the largest study to date showing that HER2 expression is not related to patient prognosis.[33] Unfortunately, the authors did not confirm their results with *in situ* hybridization techniques. Similarly, the importance of HER2 amplification and expression in esophageal squamous cell carcinoma remains unclear. Soares *et al* found that 37% of patients were HER2-positive with immunohistochemistry, while only 19% of these were HER2-positive by FISH criteria. Those positive on FISH were shown to have significantly poorer survival.[5] However, Gibault *et al* reported overexpression of HER2 in only 2.8% of

patients with esophageal squamous cell cancer, and they concluded that HER2 “appears to be of poor interest” as a potential therapeutic target in this type of esophageal cancer.[34]

Aside from methodological factors (discussed in greater detail below), we may not have found a survival advantage in HER2-negative cases due to the clonal divergence of primary tumors and disseminated tumor cells (DTCs). Klein *et al* recently reported that HER2 gene amplification was not conserved between primary tumors and DTCs (i.e. neither the presence nor absence of HER2 amplification in the primary tumor was predictive for the HER2 status in DTCs of the same patient). More importantly, they found that HER2 amplification in the primary tumor did not affect survival, while HER2 amplification in DTCs led to significantly shorter survival suggesting an increased dependence on HER2 signaling in the latter group.[35] This too is controversial however with Reichelt *et al* reporting the opposite finding.[27] They found perfect correlation of HER2 amplification using FISH between the primary tumor and lymph node/distant metastases, and no effect on overall survival.

There are several limitations to our study. Perhaps foremost, our negative findings may relate to sample size (type II statistical error). Our initial submission to the journal described the results of 70 esophageal adenocarcinoma patients. We reported a *P* value of 0.06 when comparing survival rates between those with HER2 amplification and those without (67% vs. 28%, respectively). Upon request by the journal, we re-analyzed failed SISH specimens in an attempt to increase our sample size. With a new total of 89 patients, we found similar differences in survival (57% with HER2 amplificaiont vs. 32% without) but a much less convincing *P* value of 0.37 suggesting that HER2 amplification has no influence on survival (at least in the negative sense).

Second, it is possible that by excluding patients who received neoadjuvant therapy, we created a selection bias favoring less advanced tumors. However, 65% of the patients in our study had advanced tumors (pT3 or pT4) due to the more infrequent use of neoadjuvant therapy in the late 1990s. And in our previous study[1], we found no significant difference in survival between 116 patients treated with surgery alone, and 124 patients treated with neoadjuvant therapy and surgery (5-year survival rates of 31% vs. 41%, respectively) (P=0.125). Further studies are needed which include patients who have received neoadjuvant therapy as well as those with metastatic disease.

As stated above, many prior studies have used immunohistochemistry (IHC) alone to determine HER2 expression in upper gastrointestinal cancer. However, IHC is susceptible to inter-observer variability and variations in testing protocols (such as insufficient or prolonged formalin fixation).[2,29] As well, a number of studies in breast cancer have indicated that gene amplification is a more accurate predictor of survival than gene expression.[31,36] Fluorescence *in situ* hybridization (FISH) was included in the diagnostic algorithms for HER2 positivity in breast cancer to reduce inter-observer error and confirm cases with equivocal HerceptTest staining (2+). However, FISH is a costly technology requiring both a fluorescence microscope and digital photography, and fluorescent signals will deteriorate over a few weeks.[2,31] In addition, a recent study by Rauser *et al* highlighted the unreliable detection of low-level HER2 amplification in Barrett's cancer using standard FISH in thin (4 μm) tissue sections.[37]

Bright-field *in situ* hybridization such as silver-enhanced *in situ* hybridization (SISH) used in our study is gaining popularity as it requires only a light microscope, and it is fully automated and rapidly performed. Staining remains stable for a long period and it is relatively easy to interpret.[2] An additional advantage over chromogenic *in situ*

hybridization (CISH) is that HER2 and chromosome 17 assays can be performed on contiguous slides allowing for exclusion of polysomy rather than locus-specific amplification.[2,31] High concordance has been found between FISH and SISH in breast cancer studies (>95%), and high inter-observer concordance exists with SISH (93-95%).[2,36] We found high concordance between IHC and SISH in this study.

3.7 CONCLUSION

HER2 gene amplification and overexpression was present in 16% of esophageal adenocarcinomas. It did not appear to influence survival. Although a subset of esophageal adenocarcinoma patients may meet the criteria for anti-HER2 monoclonal antibody therapy, it is too early to suggest that such therapy may decrease disease-free recurrence rates and increase long-term survival. Future studies should employ reproducible methodology using *in situ* hybridization techniques. As well, research into targeted molecular therapies will have to take into account characteristics of both the primary tumor and disseminated tumor cells.

3.8 ACKNOWLEDGMENTS

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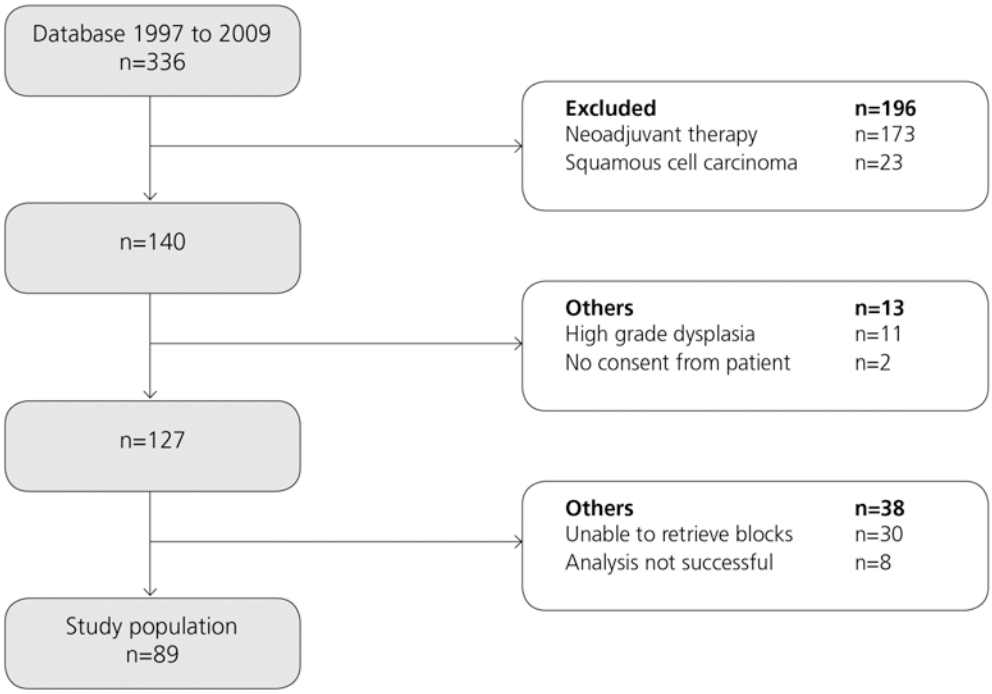

Figure 3.1. Study population.

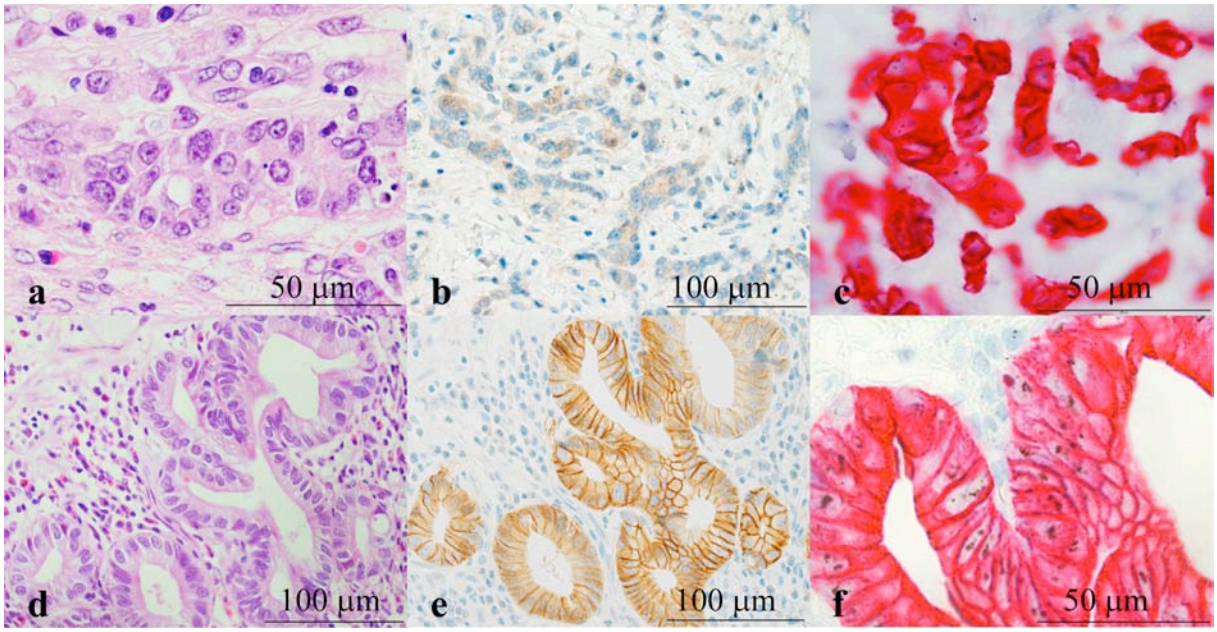

Figure 3.2. Formalin-fixed, paraffin-embedded esophageal adenocarcinoma tissue microarrays. Representative specimen (a, hematoxylin & eosin stain) showing no HER2 protein expression (b, AE1/AE3 immunohistochemical stain), and no HER2 gene amplification (c, silver-enhanced *in situ* hybridization). Second example (a, hematoxylin & eosin stain) showing 3+/positive HER2 protein expression (b, AE1/AE3 immunohistochemical stain), and high-level HER2 gene amplification (c, silver-enhanced *in situ* hybridization).

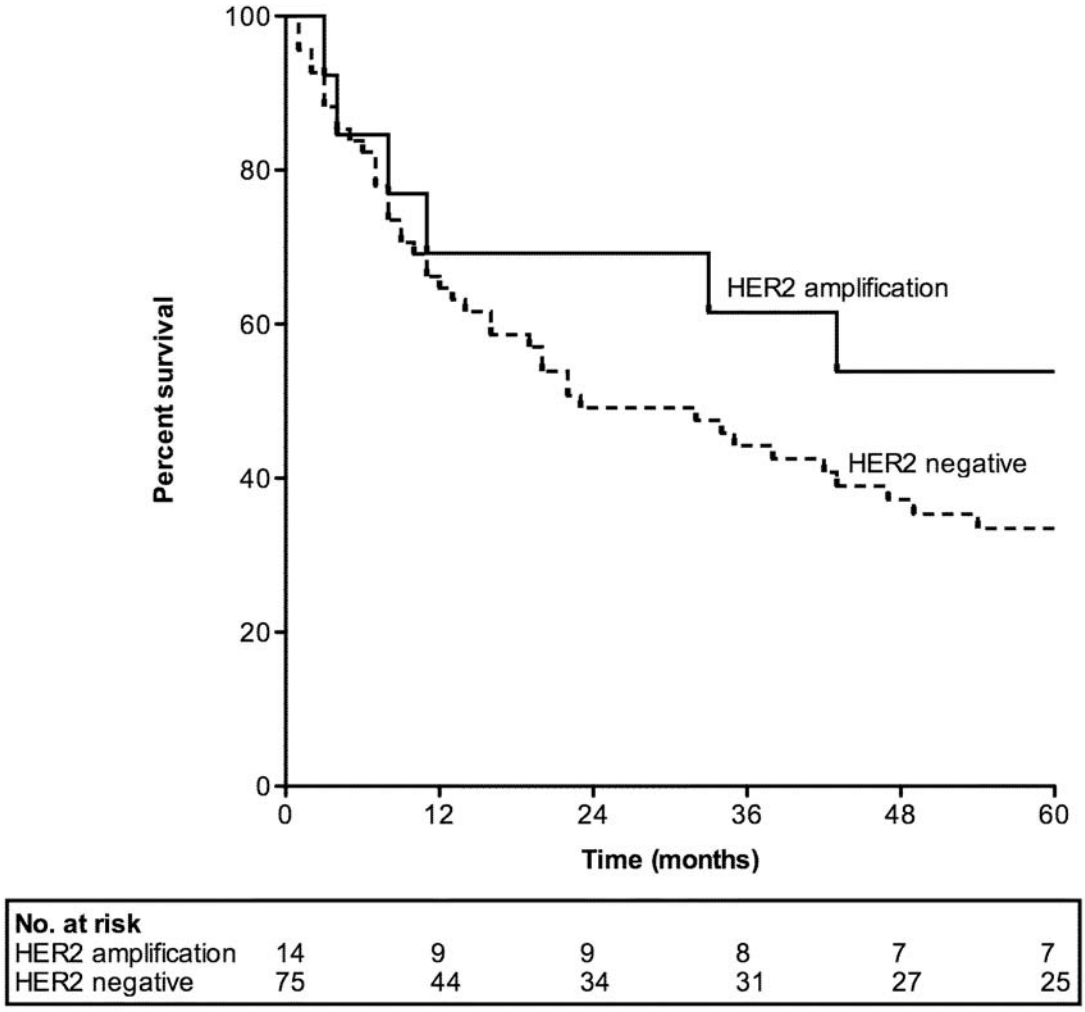

Figure 3.3. Overall 5-year survival according to the presence or absence of HER2 gene amplification for 89 patients who underwent surgical resection of esophageal adenocarcinoma. Although there was a difference in 5-year survival rates between these 2 groups : 57% vs. 32%, it was not significant (*P*=0.37).

Table 3.1. Patient and tumor characteristics

Variable	Esophageal Cancer[a] (n=89)
Age, y	
< 70	58 (65)
> 70	31 (35)
Sex	
Male	74 (83)
Female	15 (17)
Tumor location	
Lower 1/3 esophagus	23 (26)
GOJ[b]	66 (74)
Grade of differentiation	
Well/moderate (G1 + G2)	26 (29)
Poor/undifferentiated (G3 + G4)	61 (69)
Unknown	2 (2)
pT-stage	
T1	20 (23)
T2	11 (12)
T3	47 (53)
T4	11 (12)
pN-stage	
N0	37 (42)
N1	52 (58)
pM-stage	
M0	86 (97)
M1	3 (3)
Stage groups (UICC 2002)[c]	
I	19 (21)
IIA	17 (19)
IIB	5 (6)
III	45 (51)
IV	3 (3)
Radial margin	
Negative	43 (48)
Positive	45 (51)
Not assessable	1 (1)
Vascular invasion	
No	24 (27)
Yes	64 (72)
Unknown	1 (1)
Perineural invasion	
No	37 (42)
Yes	43 (48)
Unknown	9 (10)
Barrett's oesophagus	
No	38 (43)
Yes	51 (57)

[a]Esophageal cancer : all adenocarcinoma
[b]GOJ = gastro-esophageal junction
[c]Stage I: T1N0M0; Stage IIA: T2-3N0M0; Stage IIB T1-2N1M0; Stage III: T3N1M0/T4anyNM0; Stage IV: anyTanyNM1

Table 3.2. Incidence of HER2/neu amplification and immunohistochemical expression in esophageal adenocarcinoma

	Esophageal cancer (n = 89)
Gene amplification[a]	
No amplification	53 (59.5)
Polysomy 17	22 (25)
Low amplification	2 (2)
High amplification	12 (13.5)
Immunohistochemical expression[b]	
0	63 (71)
1+	14 (16)
2+	1 (1)
3+	11 (12)

[a]No amplification = <2.5 signals/nucleus; polysomy 17 = 2.5-5 signals/nucleus; low amplification = 6-10 signals/nucleus; high amplification = >10 signals/nucleus
[b]0 = negative, 1+= faint or incomplete membrane staining; 2+ = weak/moderate membranous staining; 3+ = strong membranous staining

Table 3.3. Association between patient and tumor characteristics and HER2/neu amplification in esophageal adenocarcinoma (n = 89)

Variable	No. Patients	HER2/neu + n (%)	*P* value
Age, y			
< 70	58	7 (12)	0.23
> 70	31	7 (23)	
Sex			
Male	74	12 (16)	1.0
Female	15	2 (13)	
Tumor location			
Lower 1/3 esophagus	23	2 (9)	0.51
GOJ[a]	66	12 (18)	
Grade of differentiation			
Well/moderate (G1 + G2)	26	4 (15)	1.0
Poor/undifferentiated (G3 + G4)	61	9 (15)	
Unknown	2	1 (50)	
pT-stage			
T1	20	5 (25)	0.25
T2	11	1 (9)	
T3	47	5 (11)	
T4	11	3 (27)	
pN-stage			
N0	37	5 (14)	0.77
N1	52	9 (17)	
pM-stage			
M0	86	13 (15)	0.41
M1	3	1 (33)	
Stage groups (UICC 2002)[b]			
I	19	4 (21)	0.48
IIA	17	1 (6)	
IIB	5	1 (20)	
III	45	7 (16)	
IV	3	1 (33)	
Radial margin			
Negative	43	7 (16)	1.0
Positive	45	7 (16)	
Not assessable	1	0 (0)	
Vascular invasion			
No	24	5 (21)	0.33
Yes	64	8 (13)	
Unknown	1	1 (100)	
Perineural invasion			
No	37	7 (19)	0.53
Yes	43	5 (12)	
Unknown	9	2 (22)	
Barrett's oesophagus			
No	38	6 (16)	1.0
Yes	51	8 (16)	

[a]GOJ = gastro-esophageal junction
[b]Stage I: T1N0M0; Stage IIA: T2-3N0M0; Stage IIB T1-2N1M0; Stage III: T3N1M0/T4anyNM0; Stage IV: anyTanyNM1

Table 3.4. Comparative data for SISH HER-2/neu gene copy status and HER-2 IHC (amended HercepTest) in esophageal adenocarcinoma

	IHC[a] 0 (n=63)	IHC 1+ (n=14)	IHC 2+ (n=1)	IHC 3+ (n=11)
Diploid (n = 53)	44	9	0	0
Polysomy 17 (n = 22)	18	4	0	0
Low amplification (n = 2)	0	1	1	0
High amplification (n = 12)	1	0	0	11

SISH = silver *in situ* hybridization; IHC = immunohistochemistry
[a]0 = negative, 1+= faint or incomplete membrane staining; 2+ = weak/moderate membranous staining; 3+ = strong membranous staining

CHAPTER 4: ISOLATED TUMOR CELLS IN ESOPHAGEAL CANCER: IMPLICATIONS FOR THE SURGEON AND THE PATHOLOGIST

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NOTE:
Statements of authorship appear on pages 106-107 in the print copy of the thesis held in the University of Adelaide Library.

4.2 ABSTRACT

Introduction: Studies suggest that up to 56% of node-negative patients have tumor deposits in their lymph nodes that are missed by routine pathological examination. However, few studies differentiate between isolated tumor cells and micrometastases using reproducible criteria, and their prognostic significance has not been established. **Methods**: We identified 119 patients who had undergone surgical resection for esophageal cancer between 1997 and 2007, and who were classified as node-negative. Relevant paraffin blocks were identified, and three additional levels, each 250-microns apart, were cut of all lymph nodes. Isolated tumor cells and micrometastases were defined according to size criteria but additional data and characteristics were recorded. Two slides were made at each level (one for hematoxylin and eosin, one for immunohistochemistry). Results were correlated with survival. **Results**: One patient was found to have a metastasis (>2mm), 8 patients (7%) had micrometastases, and 22 patients (18%) had isolated tumor cells. The 5-year survival rates were 60% for patients who remained node-negative, 33% for patients with isolated tumor cells, 40% for patients with micrometastases, and 0 for the patient with a metastasis (P=0.02). A significant difference was found between node-negative patients versus patients whose lymph nodes contained isolated tumor cells (P=0.014). Most tumor deposits (71%) were identified on the first additional section. **Conclusions**: Our results suggest that isolated tumor cells are as important as micrometastases in determining survival in patients with esophageal cancer. This has important implications in the retrieval and pathological analysis of lymph nodes.

4.3 INTRODUCTION

Tumor recurrence in patients with esophageal cancer who have seemingly had a complete resection of their tumor suggests that undetectable tumor deposits must be present at the time of operation. Lymph nodes are a frequent site of tumor recurrence indicating they contain occult disease. Recent studies indicate that up to 56% of pathologically node negative patients have nodal metastases that are missed by routine pathologic examination.[1,2]

Robust, sensitive immunohistochemical (IHC) techniques using antibodies to detect epithelial tumor cells in lymphatic tissue have been in use since the mid-1990s.[3] It is therefore surprising that no consensus exists regarding the prognostic significance of immunohistochemically-identified tumor cells.[4-19] The main reason for this is the lack of unequivocal results showing their prognostic significance in various solid tumors. In esophageal cancer, some studies[4-14] have shown a significant correlation between occult lymph node metastases and decreased survival in esophageal cancer, but others have not.[15-19] However, most of the studies suffer from small numbers of patients, limited analysis of existing paraffin blocks (i.e. one additional section only), and most importantly, varying definitions for both isolated tumor cells and micrometastases. This explains the reluctance of pathologists to introduce additional routine tests that require time and money, both at a premium in most laboratories.

This study examined the yield of occult nodal metastases using both serial sectioning and IHC staining in a large group of node negative patients with *standardized, reproducible* definitions. The importance of free-lying isolated tumor cells and micrometastases in node-negative esophageal cancer patients was also determined.

4.4 MATERIALS AND METHODS

4.4.1 Patient Selection

All patients who had undergone a surgical resection for squamous cell carcinoma or adenocarcinoma (including high-grade dysplasia) were identified from an Adelaide-wide Esophageal Cancer Surgery audit database, held at the Royal Adelaide Hospital in Adelaide, Australia. Since July 1997, prospective follow-up data has been collected and stored in this database. The study group consisted of 119 node negative (pN0) patients: 49 (41%) had undergone surgery alone and 70 (59%) received neoadjuvant therapy prior to resection. This consisted of 2 cycles of cisplatin (80 mg/m^2 on day 1) and 5-FU (800 mg/m^2 continuous infusion for 5 days), plus 15 to 25 fractions of radiation therapy (over 3 to 5 weeks) to a total of 40 to 50 Gray. Surgical resection occurred 5 to 6 weeks later, and was usually performed by a 2-surgeon synchronous Ivor-Lewis technique via a right antero-lateral thoracotomy and an upper midline laparotomy, as described previously.[20] A conservative lymph node dissection (removal of all nodes adjacent to the tumor) was performed in all patients, regardless of operative technique.[21]

4.4.2 Pathology

The study was approved by the Research Ethics Committee at the Royal Adelaide Hospital and by the Flinders Clinical Research Ethics Committee, Bedford Park, South Australia. In the first part of our study[22], we re-examined 240 esophageal cancer pathology specimens to determine which variables could improve the accuracy of the TNM staging system. During this project, we recorded all paraffin blocks for each specimen containing lymph node(s). Specimen identification numbers were obtained from our database, and the designated paraffin blocks were then retrieved from one of 4 pathology laboratories providing services to all hospitals in which radical esophageal surgery is performed in

South Australia: Institute for Medical and Veterinary Science, ClinPath Laboratories, South Path Laboratories, and Adelaide Pathology Partners.

Two consecutive sections (approximately 4 μm thick) were cut at three levels, separated by 250 μm. One slide of each level was stained with hematoxylin and eosin (H&E), and the other was stained with the monoclonal antibody against the epithelial marker AE1/AE3 (DAKO, Carpinteria, CA) for immunohistochemistry (IHC) (Figure 1). The monoclonal antibody AE1/AE3 is widely used because it recognizes a broad range of keratin subtypes expressed in epithelial tumors including esophageal carcinomas.[3] Immunostaining was performed using an automated Dako Autostainer as follows[23]: each section was deparaffinized, dehydrated, and incubated with AE1/AE3 diluted at 1:200 at room temperature overnight. After incubation with the primary antibody, the slices were washed with phosphate buffered saline (PBS), and then incubated with a rabbit anti-mouse secondary antibody (DAKO, Carpinteria, CA) for 30 min at room temperature. For immunohistologic labeling, the slices were incubated with streptavidin peroxidase for 60 min. The sections were then counterstained with Mayer's hematoxylin and mounted.

A single experienced pathologist reviewed all three sets of slides (A.R.R.), along with an upper gastrointestinal surgeon (S.K.T.). The greatest dimension of the largest tumor deposit was measured with the Nano-Zoomer C9600 series slide scanner. Micrometastases were defined as a metastasis >0.2mm and ≤2mm, while isolated tumor cells were defined as a single tumor cell or a cluster of tumor cells ≤0.2mm in size.[24,25] Strict, widely recognized and accepted criteria were used to determine the malignant nature of cell(s) in lymph node tissue. These included nuclear enlargement, pleomorphism, hyperchromasia, and increased nuclear:cytoplasmic ratio (Figure 2). Only cells that unequivocally fulfilled these criteria and demonstrated cytoplasmic expression of AE1/AE3 were accepted as

malignant, i.e. an isolated tumor cell or micrometastasis. Not infrequently, cells were identified with cytoplasmic brown staining on AE1/AE3 preparation but without cytological features of atypia (e.g. macrophages with hemosiderin pigment, or damaged cells with brown pigment particularly in lymph nodes treated with neoadjuvant therapy).[2,6,14] These cells did not qualify as malignant cells.

The location of the tumor cell(s) was classified as within nodal parenchyma, within a subcapsular sinus/space, or within an intramedullary sinus/space. Cells identified in an extranodal location, or those within an afferent lymphatic vessel were excluded as per the European Working Group Study (i.e. considered lymphatic invasion only).[26] Irrespective of size, we recorded whether the tumor cell(s) had elicited a stromal reaction, had made contact with a vessel or lymph sinus wall, had begun to proliferate, and/or had begun to invade and penetrate a vessel or lymph sinus wall, according to Hermanek *et al*'s Union Internationale Contre le Cancer (UICC) guidelines.[24] In patients with multiple lymph nodes containing metastatic tumor deposits, the data and characteristics of the largest tumor deposit were recorded. Micrometastases were further classified according to whether they exhibited glandular formation. Isolated tumor cells were further classified into either single tumor cells or clusters of tumor cells. A cluster was defined as two or more adjacent tumor cells. If both single cells and clusters were identified in a single lymph node, they were classified as the cluster type. Lymph node metastases that were >2mm were re-classified as overt lymph node metastases. Appropriate positive controls were used in each immunohistochemistry run, and a negative control (primary antibody omitted) was always included.

4.4.3 Statistical Analysis

The presence of occult lymph node metastasis (either isolated tumor cells or

micrometastases) was correlated with clinical outcome. Overall survival was calculated from the date of operation to July 31, 2008 (if alive) or to the date of death (as recorded from the South Australian Cancer Registry) according to the Kaplan-Meier method. Differences in survival between the tumor deposit groups were assessed using a log-rank test. Where an overall difference in survival between the groups was found, post-hoc log-rank tests comparing the tumor deposit groups two at a time were performed. The Tukey-Kramer adjustment was used to adjust the post-hoc tests for multiple comparisons. Multivariate analysis was performed by Cox regression for selected variables that were significant on univariate analysis (all variables were not included to avoid over-fitting and colinearity). Subset analyses were performed for patients with adenocarcinoma, and those who had received neoadjuvant therapy prior to surgical resection. Statistical significance was set at the 5% level. Calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA).

4.5 RESULTS

4.5.1 Patients

There were 128 patients who were designated as node-negative esophageal cancer between July 1997 and January 2007. Nine cases were excluded from our study: 7 had no lymph nodes identified along with the specimen, 1 did not have retrievable paraffin blocks, and 1 patient did not give consent for his/her slides to be reviewed. The mean age of the remaining 119 patients was 61.3 years (Std dev=9.3 years). There were 93 men (78%) and 26 women (22%). Seventy patients (59%) underwent neoadjuvant therapy. The median number of lymph nodes per patient was 5, and a total of 661 lymph nodes were analyzed.

Esophagectomy was performed using the Ivor-Lewis technique in 70% of patients, using a three-stage or McKeown technique in 26%, and using a transhiatal technique in 8%. While there were no 30-day mortalities in our pN0 subset of patients, we have previously reported our 30-d mortality rate of 5.8% for 240 pN0 and pN1 esophagectomies during this 10-year time period.[22] Complete follow-up was available for all 119 patients with an overall 5-year survival rate of 53%, and a median survival of 68.5 months.

4.5.2 Tumor Deposits

Eighty-eight patients (74%) remained node-negative after serial sectioning and immunohistochemistry of the lymph nodes. Twenty-two (18%) had isolated tumor cells or clusters, eight (7%) had micrometastases, and one (1%) was upgraded to a lymph node metastasis (pN1). Patient and tumor characteristics are listed in Table 1. No association was found between the presence of AE1/AE3 tumor cells and patient age, sex, neoadjuvant therapy, tumor length, circumferential resection margin, pT-stage, and perineural invasion (Fisher's exact tests, $P>0.5$). The prevalence of occult disease was significantly higher in

patients with an adenocarcinoma, lower or junctional tumors (compared to middle third tumors), poorly differentiated grade, and vascular invasion.

Table 2 and Figure 3 show 5-year survival rates according to each tumor deposit group (*P*=0.02). Table 3 demonstrates a significant survival advantage in the node-negative group compared to the isolated tumor cell group (*P*=0.014). These results were confirmed with a multivariate analysis (Table 4) using age, sex, histological type, vascular invasion, and the presence of occult tumor deposits. The mean number of lymph nodes resected in the node-negative group (n=88) and the isolated tumor cell/micrometastasis group (n=31) was 5.1 (SD=3.47) and 6.8 (SD=5.29), respectively (*P*=0.10). There was also no significant difference in the distribution of the total number of nodes removed between these two groups (Wilcoxon test, *P*=0.54).

Three deeper levels, each separated by 250-microns, were analyzed with both H&E and IHC. Twenty-two of 31 patients (71%) had AE1/AE3 tumor deposits present on the first additional level (95%CI: 51.96%, 85.78%), 29 of 31 patients (94%) on the second level (95%CI: 51.96%, 99.21%), and 31 of 31 (100%) on the third level. Eight of 22 patients (36%) with isolated tumor cells had single tumor cells only, while fourteen patients (64%) were sub-categorized into the "cluster" group. There was a large difference in 5-year survival rates between these 2 groups : 48% vs. 0% but it was not a significant difference (Figure 4) (*P*=0.053). Similarly, 19 patients (86%) had an isolated tumor cell < 0.1 mm in size, while 3 patients (14%) had tumor clusters between 0.1-0.2 mm in size. No significant difference was found in 5-year survival between these groups *(P*=0.06). Five patients (23%) had isolated tumor cells/clusters located in the lymph node parenchyma, and 17 patients (77%) had isolated tumor cells/clusters located in the subcapsular or intramedullary sinuses. Again, no significant survival difference was observed (*P*=0.26).

4.5.3 Subset Analyses

Eighty-eight patients (74%) had an adenocarcinoma. Fifty-nine (67%) remained node-negative, 20 (23%) had isolated tumor cells, eight (9%) had micrometastases, and one (1%) was upgraded to a metastatic node. Similar significant differences in 5-year survival between tumor deposit groups were found in this subset of patients (P=0.01). Patients with isolated tumor cells had a 5-year survival of 31%, compared to 67% for those without (P=0.005).

Analyses were repeated in the subset of patients who received neoadjuvant chemo-radiotherapy (n=70). Fifty-three (76%) remained node-negative, 12 (17%) had isolated tumor cells, four (6%) had micrometastases, and one (1%) had a metastasis. Although node-negative patients had a 5-year survival rate of 61%, and those with isolated tumor cells had a 5-year survival of 35%, this difference was not significant (P=0.17) (Figure 5).

4.6 DISCUSSION

Thirty-one of 119 esophageal cancer patients (26%), originally classified as node negative, were found to have occult tumor deposits in their nodes following three additional serial sections, and immunohistochemical staining with the monoclonal antibody AE1/AE3. While our prevalence rate is within the range reported in previous studies (8 to 56%), there are a few observations to be made. Studies (like ours) that insisted upon: 1) morphological abnormalities detected in positive cells to classify them as tumor deposits, and 2) more than one additional level, had prevalence rates ranging from 24 to 38%.[4-7,12] Prevalence rates were generally higher in those studies that counted any positive cell as a tumor deposit irrespective of its malignant features (32 to 56%).[13-15,19] Similarly, the two studies that included morphological features in their definition of a tumor cell, but examined only one additional level had lower prevalence rates (8 to 11%).[11,16]

This study used reproducible, carefully defined, standardized methodology to distinguish occult lymph node deposits as either isolated tumor cells or micrometastases. We found that patients with esophageal cancer who remained node negative had a 5-year survival of 60%. In contrast, patients with newly-identified tumor deposits had a significantly reduced 5-year survival of 33% and 40%, for isolated tumor cells and micrometastases respectively (P=0.02). A significant difference in survival was also found between node negative patients versus patients whose lymph nodes contained tumor deposits <0.2 mm in size (P=0.014), suggesting that the presence of isolated tumor cells flags the potential for disseminated occult metastatic disease.

Cserni *et al* have stated that “interpretation of the pathologic findings is probably the most ignored aspect of the differences between laboratories”.[27] Part of the problem is in

deciphering the guidelines set out by two leading bodies, the UICC and the American Joint Committee on Cancer (AJCC).[24,26] Criteria include but are not limited to the microanatomic location of tumor deposits, rules for measuring multiple tumor cells or clusters, and qualitative features of the tumor cells (i.e. mitotic activity, stromal reaction). Different wording between the two sets of guidelines complicate things further. Recent studies have shown low interobserver reproducibility by pathologists' distinguishing isolated tumor cells from micrometastases using these guidelines even after a consensus statement.[27,28] We have observed that isolated tumor cells/clusters < 0.2mm in size do not show signs of mitotic activity nor do they elicit a stromal reaction (i.e. features generally associated with malignant potential), suggesting that most qualitative criteria are unnecessary in distinguishing isolated tumor cells from micrometastases.

Several authors have therefore proposed much simpler and uniform definitions[25,29-30]: micrometastases are occult metastases >0.2mm and ≤2mm, while isolated tumor cells are a single cell or a cluster of cells, *with malignant features*, ≤0.2mm in size. Much higher interobserver reproducibility has been demonstrated using size-based diagnostic criteria.[30] However, critics of this latter approach argue that definitions based on size criteria alone may lead to the under-treatment of some patients, depending on whether the designation of an isolated tumor cell or a micrometastasis is allocated to a particular node (especially a sentinel lymph node).[27]

In esophageal cancer, we found that the distinction between an isolated tumor cell and a micrometastasis was not important. Patients with either of these in one or more lymph node(s) had significantly reduced overall survival compared to patients who remained node negative after serial sections and immunohistochemistry. De Mascarel *et al* state that "there is likely a prognostic continuum between isolated tumor cells, micrometastases, and

metastases sized more than 2 mm".[29] We suggest that there is a positive association between isolated tumor cells and micrometastases rather than a continuum.

The importance of isolated tumor cells in lymph nodes has been reported not only in esophageal cancer[6,9-12,14], but also in several studies of gastric cancer[31,32], melanoma[33], breast cancer[34-36], colorectal cancer[37-39], and non-small-cell lung cancer[40]. Overwhelmingly, studies that included phenotypic malignant features in their definition for an isolated tumor cell (as shown in Figure 2) were much more likely to find a significant association between these cells and survival and/or poor prognostic indicators (pT-stage, vascular invasion). And contrary to several authors' belief that sub-micrometastatic disease (<0.1mm) has no impact on overall survival (albeit in melanoma and breast cancer)[41,42], we found that the presence of a single isolated tumor cell (on average 0.01 mm in size) significantly reduced the likelihood of 5-year survival. Moreover, we did not find any evidence to support the assertion that isolated tumor cell location, whether in the sinuses or the parenchyma, was more important than size in predicting non-sentinel lymph node metastases.[36] In the absence of neoadjuvant therapy, an isolated tumor cell/cluster was always located in a subcapsular or an intramedullary sinus (sometimes confused with nodal parenchyma), while micrometastases were located in the parenchyma, usually at the periphery. Following neoadjuvant therapy however, we did encounter viable isolated tumor cells *within* the parenchyma of the lymph node, presumably in nodes that had once been overtly metastatic.

It is possible that the clear association between isolated tumor cells and survival seen in our study was because esophageal cancer patients (unlike melanoma or breast cancer) do not require long follow-up times to observe survival differences.[34,43] In addition, examination of the deeper lymph node tissue, including immunohistochemistry, was

performed using standardized methods in the same laboratory and assessed by a single gastrointestinal pathologist. Some investigators may argue that our patients were understaged from the outset because of the low number of resected lymph nodes (median, 5) in our series. We have made 2 observations over the past year while conducting this study. First, removal of each lymph node station from the specimen, and placement of each group of nodes into a separate pot (prior to sending it to the pathologist), has resulted in an increased median number of detected lymph nodes. This is without any change in our conservative lymphadenectomy approach. Second, the detection of occult nodal disease was not influenced by the total number of lymph nodes resected (i.e. it did not matter whether two or 20 lymph nodes were resected) (P=0.54). MacGuill *et al* also report no difference in the average number of nodes sampled per case between the AE1/AE3 negative and positive groups in their study[11], and this supports our view that it is the detailed analysis of lymph nodes, not the absolute number resected, which is most important in determining prognosis (and perhaps optimizing postoperative therapy).

The implications of our findings are important for both surgeons and pathologists.[11,26] For the surgeon, increased consideration should be given towards performing sentinel lymph node localization prior to esophagectomy. This might limit the number of lymph nodes which need to have additional sectioning and immunohistochemistry. This technique has been reported by a few centers, and preliminary results are promising.[44,45] On average, two or three sentinel lymph nodes were detected for each patient. For the pathologist, a modification of the current approach for examining harvested lymph nodes may be required with supplementation of conventional histopathology (single section with H&E) with additional levels and immunohistochemistry, especially in patients who are node negative.[11] All single isolated tumor cells (as opposed to clusters) in our study were detected by immunohistochemistry and not by deeper levels, suggesting that

"…immunohistochemistry is a helpful tool to refine risk stratification in several solid pathologies".[12]

In addition, 9 patients (29%) had occult nodal deposits detected on the second or third section only, which means that a single section fails to detect tumor cells in up to one third of patients. Although some studies have shown that isolated tumor cells can be detected using real-time PCR[46,47], it cannot currently replace immunohistochemical analysis (and therefore minimize the number of deeper levels required) because it is not yet sensitive enough to detect tumor deposits less than 0.8 mm in size (i.e. will miss many micrometastases and all isolated tumor cells).[42] Therefore, for now, serial sections seem necessary to increase the amount of nodal tissue examined under the microscope, and consequently, minimize the false negative rate.[48] We chose 3 additional levels, each 250-microns apart, because exhaustive studies by Turner and Viale (in breast cancer) have shown that 77 to 81% of all occult nodal deposits are found in the first 2 or 3 sections.[49,50] We recognize that much lymph node tissue will still not be analyzed, but a sampling protocol using the sentinel nodes is realistic within today's monetary constraints.

4.7 CONCLUSION

In esophageal cancer patients, lymph nodes containing isolated tumor cells should not be designated pN0(i+) as per the AJCC's breast cancer staging system. Whether these cells represent tumor cells in transit is uncertain, but they are associated with a worse prognosis compared to more likely true node negative (pN0) patients. These cells appear to have the same clinical implication as micrometastases and represent microscopic tumor cell dissemination. Surgeons should consider adopting the sentinel lymph node concept in esophageal cancer patients, and pathologists should consider additional sections and immunohistochemistry for evaluating negative lymph nodes following conventional histopathological analysis.

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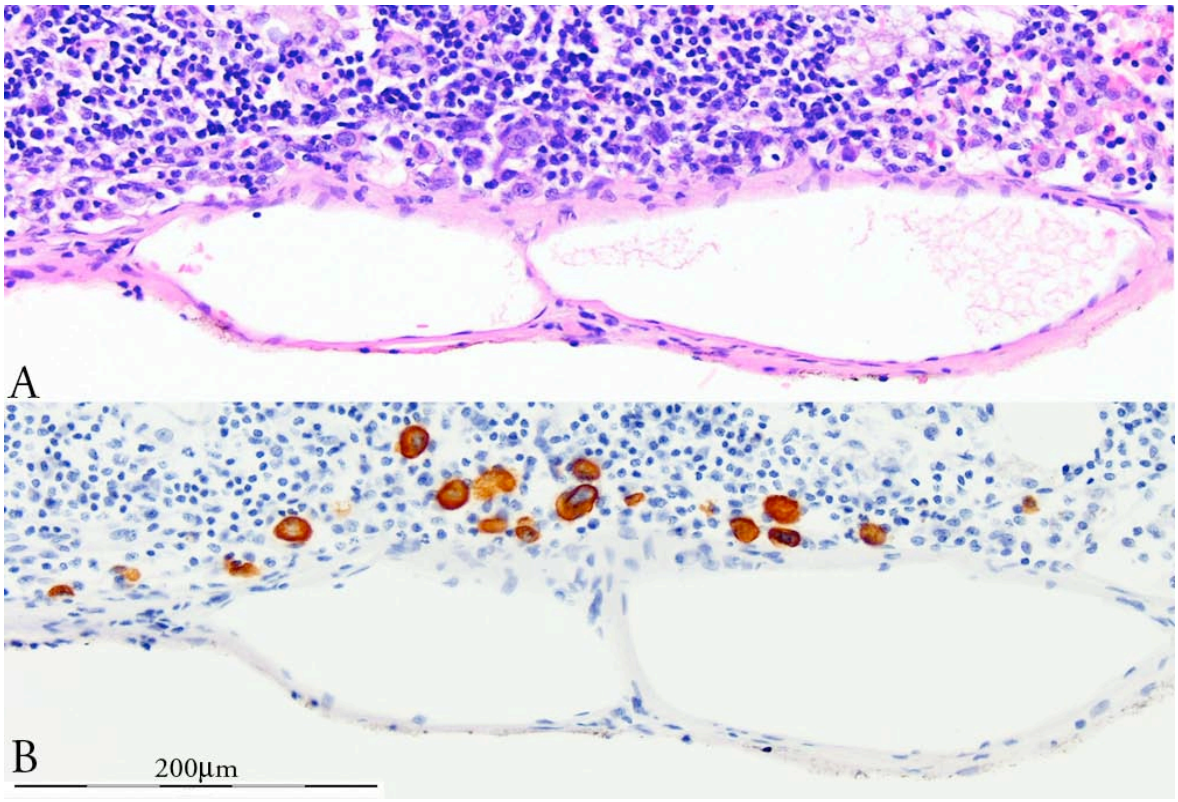


Figure 4.1. Lymph node section showing no overt metastatic cells (A, hematoxylin & eosin stain). Identical lymph node section demonstrating obvious isolated tumor cells (B, AE1/AE3 immunohistochemical stain).

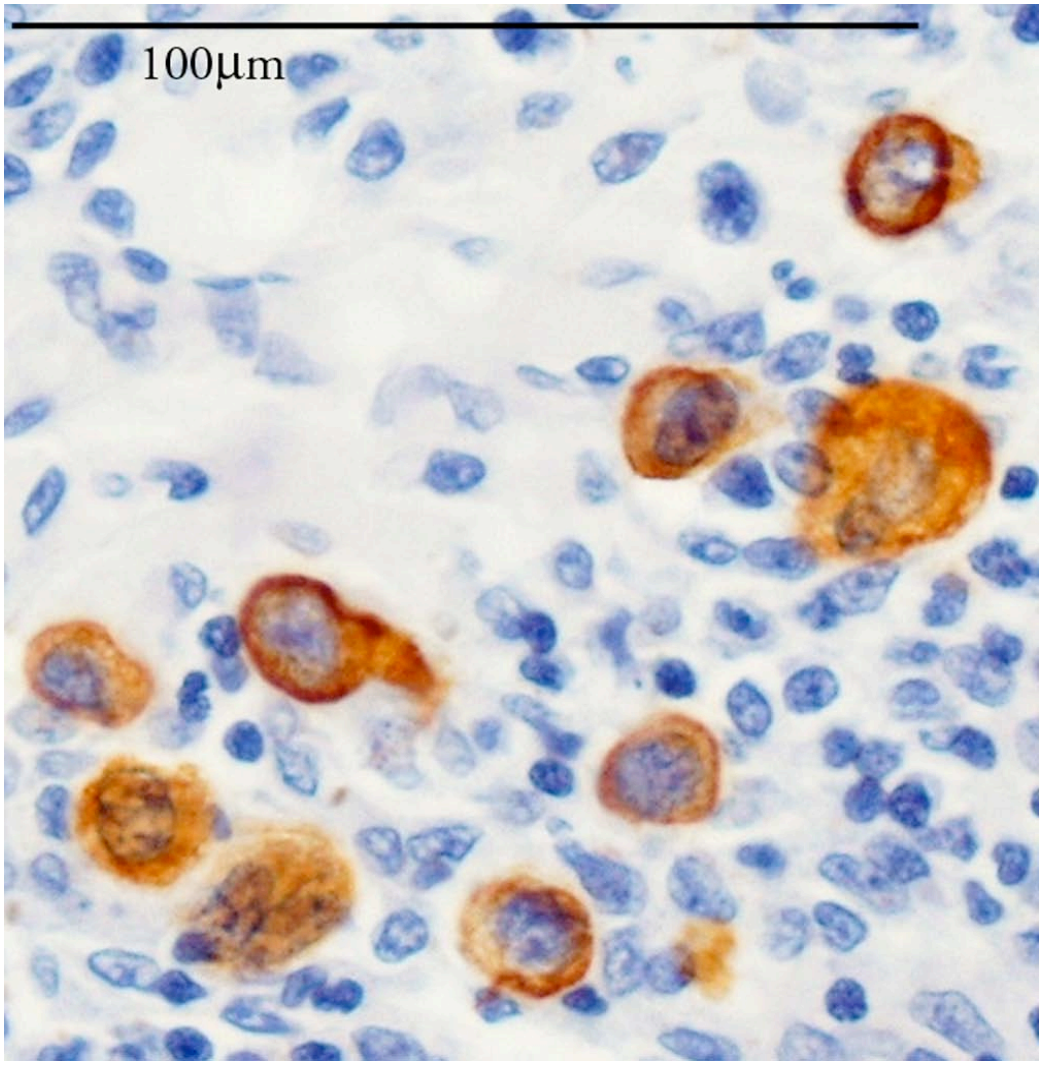
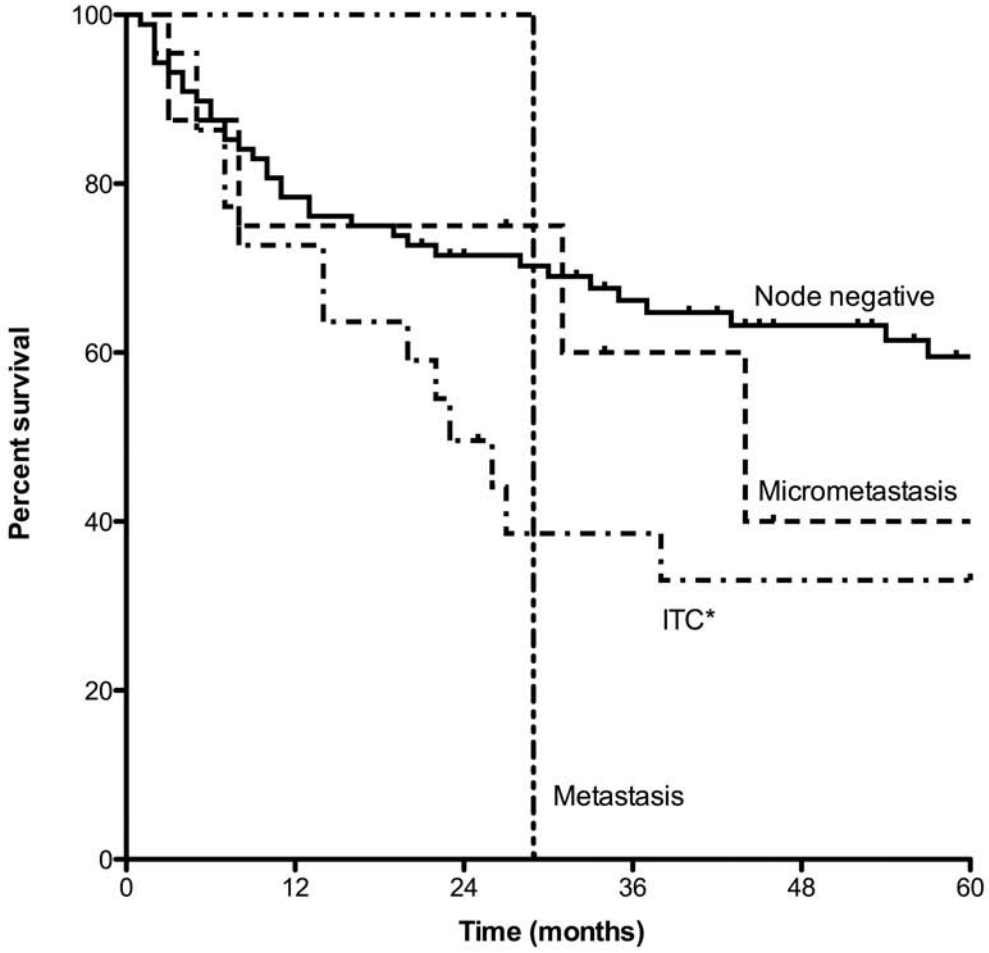


Figure 4.2. Isolated tumor cells in the subcapsular sinus of the lymph node, surrounded by normal lymphocytes. Note the cell's enlarged nucleus, hyperchromasia, and increased nuclear:cytoplasmic ratio (AE1/AE3 immunohistochemical stain).



No. at risk						
Node negative	88	68	63	57	55	52
ITC*	22	16	11	8	7	7
Micrometastasis	8	6	6	5	3	3
Metastasis	1	1	1	0	0	0

*Isolated tumor cell/cluster

Figure 4.3. Overall 5-year survival according to the presence or absence of isolated tumor cells or micrometastases for 119 esophageal cancer patients originally deemed node-negative after conventional histopathology. There was a significant difference in survival between the subgroups (*P*=0.02).

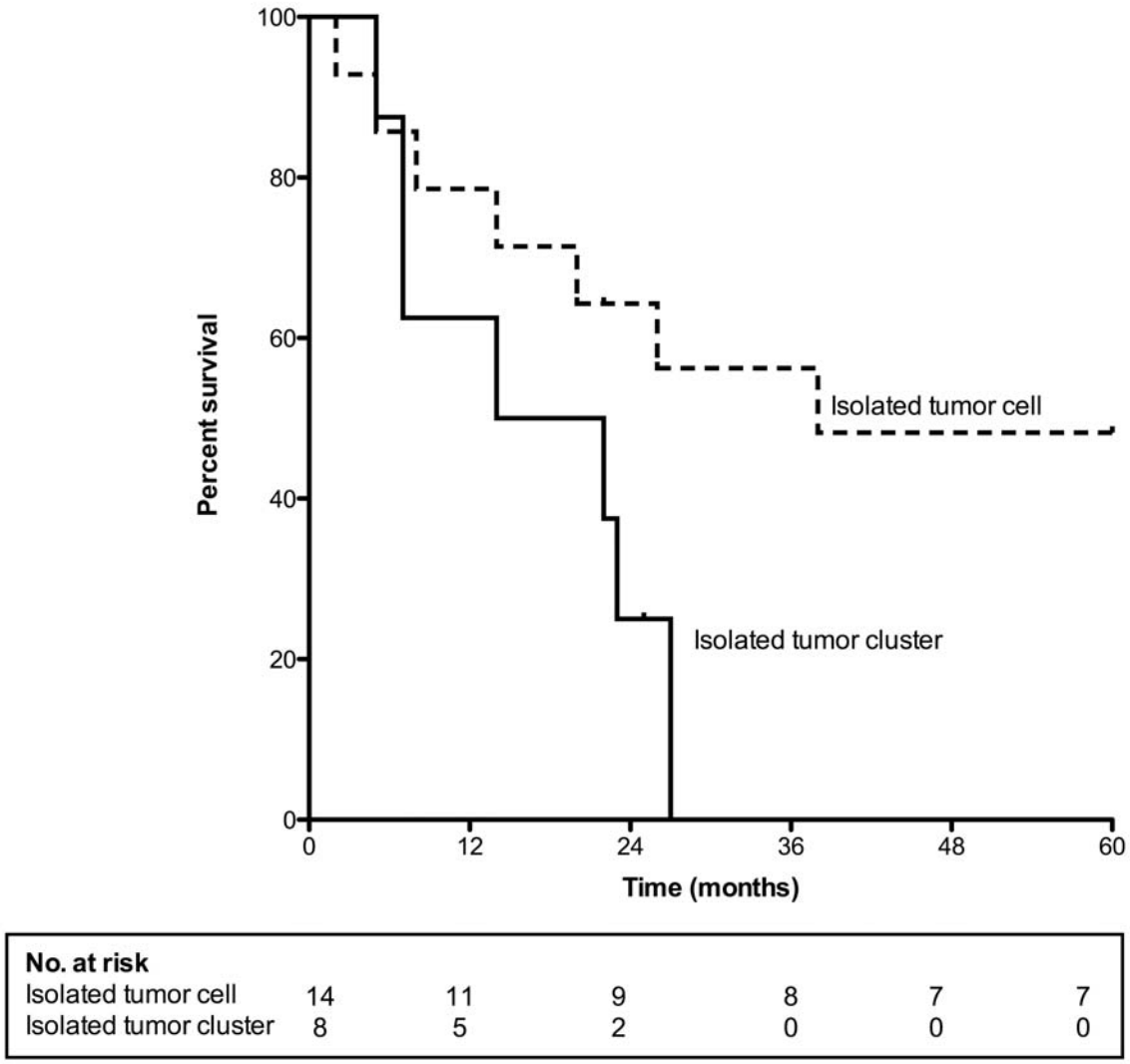
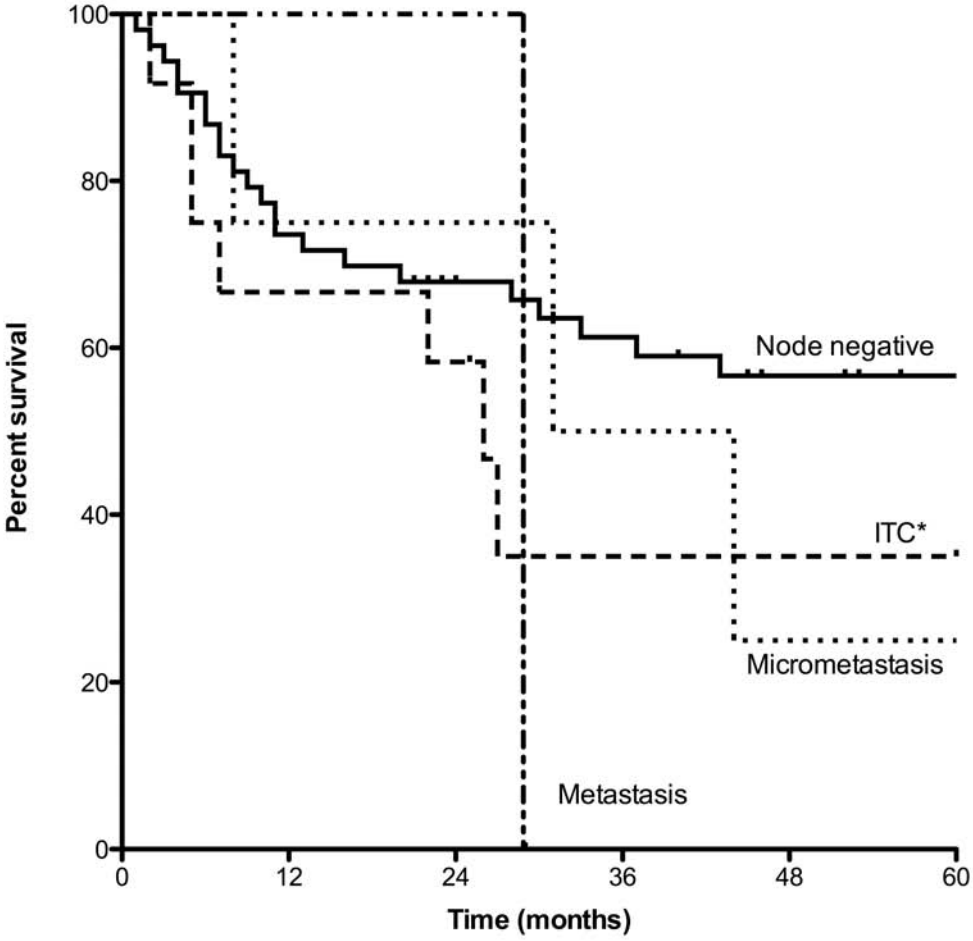


Figure 4.4. Overall 5-year survival for isolated tumor cells compared to isolated tumor clusters (< 0.2mm in size) for 22 esophageal cancer patients originally designated pN0 on the original pathology reports. There was a large difference in 5-year survival rates between these 2 groups : 48% vs. 0% but it was not a significant difference (*P*=0.053).



No. at risk						
Node negative	53	39	36	32	30	30
ITC*	12	8	7	4	4	4
Micrometastasis	4	3	3	2	1	1
Metastasis	1	1	1	0	0	0

*Isolated tumor cell/cluster

Figure 4.5. Overall 5-year survival according to the presence or absence of isolated tumor cells or micrometastases for 70 esophageal cancer patients treated with neoadjuvant therapy and originally designated pN0 after conventional histopathology. There was a difference in survival between patients without isolated tumor cells compared to those with tumor cells, but this was not significant (61% vs. 35%, respectively) (*P*=0.17).

Table 4.1. Correlation between patient and tumor characteristics and AE1/AE3 positivity (n = 119)

Variable	No. Patients	AE1/AE3 Positivity n (%)	*P* value
Age, y			0.55
< 70	102	28 (27)	
> 70	17	3 (18)	
Sex			0.21
Male	93	27 (29)	
Female	26	4 (15)	
Neoadjuvant therapy			0.67
No	49	14 (29)	
Yes	70	17 (24)	
Tumor length, cm			0.76
0-5	103	26 (25)	
> 5	16	5 (31)	
Tumor location			0.006*
Middle 1/3	19	1 (5)	
Lower 1/3	40	7 (18)	
GOJ[a]	60	23 (38)	
Grade of differentiation			0.01*
Well/moderate (G1 + G2)	53	8 (15)	
Poor/undifferentiated (G3 + G4)	58	22 (38)	
Histology			0.004*
Adenocarcinoma	88	29 (33)	
Squamous cell carcinoma	31	2 (6)	
Positive CRM[b]			0.80
No	93	25 (27)	
Yes	26	6 (23)	
pT-stage			0.10
T0[c]	22	5 (23)	
Tis	6	0 (0)	
T1	36	6[d] (17)	
T2	15	7 (47)	
T3	40	13 (33)	
Vascular invasion			0.03*
No	69	13 (19)	
Yes	41	16 (39)	
Perineural invasion			0.20
No	78	18 (23)	
Yes	15	6 (40)	

[a]GOJ = gastro-esophageal junction
[b]CRM = circumferential resection margin
[c]pT0 = no residual tumour in specimen
[d]All 6 patients were pT1b (submucosal tumors)
*$P < 0.05$

Table 4.2. Survival According to Presence of Isolated Tumor Cells/Clusters or Micrometastases in Lymph Nodes

Lymph node status	No. patients (n = 119)	Median Survival (mo.)	5-Yr Survival (%)	*P*
				0.02*
Negative (pN0)	88	Undefined[a]	60	
Isolated tumor cell(s)	22	23.5	33	
Micrometastasis	8	44.7	40	
Metastasis (pN1)	1	29.7	0	

*$P < 0.05$
[a]median survival > 125.7 months

Table 4.3. Differences in Survival across the 4 Tumor Deposit Groups using Post-Hoc Log Rank Tests (n = 119)

Strata Comparison		Chi-Square	Raw *P*	Tukey-Kramer *P*
ITC	M	6.6061	0.0102	0.049*
ITC	MIC	4.5776	0.0324	0.141
ITC	None	9.0516	0.0026	0.014*
M	MIC	0.0008	0.9779	1.000
M	None	8.2071	0.0042	0.022*
MIC	None	4.8367	0.0279	0.123

*$P < 0.05$

ITC, isolated tumor cell; M, metastasis; MIC, micrometastasis

Table 4.4. Results of multivariate survival analysis for node negative esophageal cancer patients

Variable	Hazard Ratio	95% CI	*P*
Age (< 70 y/> 70 y)	0.954	0.435 – 2.091	0.91
Sex	0.083	0.550 – 2.235	0.77
Vascular invasion (yes/no)	0.632	0.360 – 1.111	0.11
Histological type (adenocarcinoma/SCC[a])	0.720	0.360 – 1.440	0.35
AE1/AE3 positivity (yes/no)	0.512	0.283 – 0.927	0.02[*]

[*] $P < 0.05$
[a]SCC = squamous cell carcinoma

4.10 LETTER TO EDITOR AND AUTHOR REPLY

4.10.1 Letter to Editor

To the Editor: The very interesting article by Thompson et al. showed a prognostic significance of isolated tumor cells and micrometastases in patients with esophageal carcinoma[1]. However, as stated in the study, the median number of lymph nodes resected per patient was 5; therefore, in half of the cases fewer than 5 lymph nodes were analyzed. While some authors have suggested that at least "*18 nodes should be resected as the minimum necessary for accurate staging*"[2], the 7th edition of the American Joint Committee on Cancer/ International Union Against Cancer (AJCC/UICC) staging manual recommends resection of as many lymph nodes as possible (≥ 10 for T1, ≥ 20 for T2 and ≥30 for T3-T4, from the former minimum of 6 lymph nodes to be submitted to the pathologist for adequate staging). Therefore, it seems that about half of the patients in the series of Thompson et al. did not comply even with the former AJCC/UICC recommendations, and some of them were likely to be understaged as pN0[3]. Consequently, some patients deemed to be pN0 but having micrometastases and/or isolated tumor cells were probably pN1 (false negative) in which the positive lymph nodes were not retrieved. In this case, serial sections and immunochemistry seem to make up for the understaging, and it cannot be excluded that the true groups being compared were true pN0 patients (i.e. negative to both metastasis and micrometastasis) versus pN1 (i.e. patients in which the presence of micrometastases "*flags the potential for disseminated occult metastatic disease*", using Thompson words).

Additionally, in the "Results" section, no statistically significant difference was reported between the mean numbers of resected lymph nodes in the node-negative group (n=88) compared to the micrometastasis/isolated tumor cell group (n=31) (mean 5.1±3.47 and

6.8±5.29, respectively; P=0.10). However, from the median, mean and standard deviation as above indicated, it looks like most of patients had quite a limited number of lymph nodes resected, so the lack of a significant difference was probably due to the fact that the studied population included a rather homogeneous series of patients with a low number of dissected nodes. It is not clear whether the data analyzed might have enough statistical power to detect any significant difference (i.e. to avoid a Type II error). Therefore, in the "Discussion" section, the sentence "*the detection of occult nodal disease was not influenced by the total number of lymph nodes resected*" seems somewhat misleading.

Nevertheless, the article has valuable implications: a peculiar application of the concept of sentinel lymph node (SLN). In patients with breast cancer or melanoma, SLN biopsy is aimed to avoid unnecessary lymphadenectomy to reduce morbidity, whereas in esophageal cancer, given the multiple and scattered pattern of metastases, it seems to play a different role: 1) to guide a proper lymph node dissection; and 2) to provide with accurate staging by adding multiple sections and eventually immunochemistry in SLN in patients initially regarded as pN0 with hematoxylin and eosin single section conventional histopathology[4,5]. This appears to be even more important after the recently introduced esophageal staging system based upon the number of positive lymph nodes[3].

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4.10.2 Author Reply

We are pleased to comment on this letter by Dr. Incarbone. His comments regarding our low median number of lymph nodes are not surprising; there is of course much debate on the benefit of a radical versus conservative lymphadenectomy in esophageal cancer patients[1]. First, we would like to take this opportunity to correct the original wording in our aforementioned article. Rather than state that we had a "low median number of ***resected*** lymph nodes", we should have written that we had a "low median number of ***detected*** lymph nodes". This is an important distinction because, since the publication of our article, we continue to see a significant increase in the median number of lymph nodes in our esophageal cancer specimens. Since 2008, we have isolated each individual lymph node station on the back table prior to examination by the pathologist. We have subsequently noticed an increase in the median number of ***detected*** lymph nodes from 5 to 14, and this is without any change to our operative technique.

Second, while the 7th edition of the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) staging manual may suggest a minimum number of resected lymph nodes in order to achieve adequate staging, these numbers are arbitrary (i.e. there is no scientific evidence to support these recommendations). As described in our recent review paper[1], a conservative lymphadenectomy involves removal of lymph nodes in direct proximity to the tumor, esophagus, and upper stomach. This generally includes the paraesophageal nodes in the mediastinum, and the left gastric, right paracardial, and left paracardial nodes in the abdomen. We, along with others[2,3], have found that esophageal cancer (albeit, mostly adenocarcinoma) follows a predictable linear drainage pattern to one of these 'first tier' nodal stations if the patient is deemed node-positive. In fact, Van de Ven *et al* found that 90% of patients with node-positive esophageal adenocarcinoma had an involved node within 3 cm of the primary tumor[3]. Skip metastases are extremely rare in esophageal adenocarcinoma, noted in less than 5% of patients[4]. We therefore do not believe that many of our patients were understaged as pN0 despite our low median lymph node number. The reported low number of skip metastases may account for the lack of a clear survival benefit in patients undergoing a more radical lymphadenectomy.

Dr. Incarbone is correct that our article has valuable implications for esophageal cancer patients, and probably for all types of solid tumors. It is true that our study did not have a large number of patients. However, in a much larger study with a much lower possibility of a Type II error, De Boer *et al* found that isolated tumor cells or micrometastases in the sentinel nodes of women with early-stage breast cancer led to a comparable significant decrease in 5-year disease-free survival rates[5]. If we are to improve staging and ultimately survival in cancer patients, then we must not ignore the presence of isolated tumor cells in

lymph nodes. We agree with Dr. Incarbone that sentinel lymph node biopsy is the obvious answer and may become an essential component of surgical resection, not necessarily to avoid lymphadenectomy, but to better guide histopathological staging and adjuvant therapy.

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CHAPTER 5: FEASIBILITY STUDY OF SENTINEL LYMPH NODE BIOPSY IN ESOPHAGEAL CANCER WITH CONSERVATIVE LYMPHADENECTOMY

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NOTE:
Statements of authorship appear on pages 144-145 in the print copy of the thesis held in the University of Adelaide Library.

5.2 ABSTRACT

Introduction: Lymphoscintigraphy and sentinel node mapping is established in breast cancer and melanoma but not in esophageal cancer even though many centers have shown that occult tumor deposits in lymph nodes influence prognosis. We report our initial experience with lymphoscintigraphy and sentinel lymph node biopsy in patients undergoing resection for esophageal cancer. **Methods:** Sixteen of 17 consecutive patients underwent resection for invasive esophageal cancer along with sentinel lymph node retrieval (resection rate, 94%). Peritumoral injection of ^{99m}Tc antimony colloid was performed by upper endoscopy prior to the operation. A 2-surgeon synchronous approach via a right thoracotomy and laparotomy was performed with a conservative lymphadenectomy. Sentinel lymph nodes were identified with a gamma probe both *in* and *ex vivo*. Sentinel lymph nodes were sent off separately for serial sections and immunohistochemistry. **Results:** The median patient age was 60.4 years (range, 45-75 years). Fifteen were male, and thirteen had an adenocarcinoma. At least one sentinel lymph node (median, 2) was identified in 14 of 16 patients (success rate, 88%). Sentinel nodes were present in more than 1 nodal station in 5 patients (31%). In all 14 patients, the sentinel lymph node accurately predicted findings in non-sentinel nodes (accuracy, 100%). Three patients with positive sentinel lymph nodes had metastases identified in non-sentinel nodes (sensitivity, 100%). **Conclusions:** Sentinel lymph node biopsy is feasible in esophageal resections with conservative lymphadenectomy, and initial results suggest it is accurate in predicting overall nodal status. Further study is needed to assess the impact on patient management and prognosis.

5.3 INTRODUCTION

The sentinel lymph node concept describes the preferential lymphatic metastasis of a primary tumor to one or more regional lymph nodes [1]. This concept has gained acceptance in many solid organ tumors such as those located in the breast and colon [2,3]. However, its use in esophageal cancer remains controversial due to both the anatomy and the lymphatic flow of these tumors [4-8]. These two problems create certain technical challenges for the surgeon, and it is doubtful this technique will become standard of care without good evidence to support its use.

Minimally invasive surgery is one of the driving forces behind continued efforts to establish the sentinel node concept in upper gastrointestinal surgery. En bloc resections can be difficult both laparoscopically and thoracoscopically, so if it were possible to direct the surgeon to 1 or 2 sentinel lymph nodes, both the extent and morbidity of surgery might be reduced. Another important consideration is to direct the pathologist to a small number of lymph nodes upon which a detailed analysis can be performed. It is recognized that occult lymph node metastases (including both isolated tumor cells and micrometastases) have prognostic significance in esophageal cancer [9-15]. Isolated tumor cells (30 microns in diameter) will almost certainly be missed on routine pathological examination, requiring both serial sections and immunohistochemistry for detection. Thus, if the sentinel node concept can be applied to these tumors in the same way as it does to breast cancer, a closer examination can be confined to this lymph node group.

This study reports our initial experience with the sentinel lymph node concept in esophageal cancer. Aims included determining the feasibility of sentinel lymph node localization with a conservative lymphadenectomy, evaluating the accuracy of the sentinel

node in predicting the status of non-sentinel lymph nodes, and identifying potential technical problems in performing sentinel node biopsies in esophageal cancer.

5.4 MATERIALS AND METHODS

5.4.1 Patient Selection and Preparation for Surgery

Seventeen consecutive patients undergoing a surgical resection for invasive squamous cell carcinoma or adenocarcinoma of the esophagus were selected for the study. All operations were performed or closely supervised by one of 5 surgeons (S.K.T., P.G.D., G.G.J., Philip Game, and Andrew Lord). The study was approved by the Research Ethics Committee at the Royal Adelaide Hospital, Adelaide, South Australia.

Preoperative clinical staging included upper gastrointestinal endoscopy, computed tomography scans (chest, abdomen, pelvis), positron emission tomography (PET) scans, endoscopic ultrasonography (if minimal stricturing), and diagnostic laparoscopy (for gastroesophageal junction tumors). Selected patients (T2 or greater) were treated with neoadjuvant therapy according to protocol [16]. Eight patients (47%) received neoadjuvant therapy prior to resection. This consisted of 2 cycles of cisplatin (80 mg/m^2 on day 1) and 5-FU (800 mg/m^2 continuous infusion for 5 days) during weeks 1 and 5 of radiotherapy, plus 25 fractions of radiotherapy (over 5 weeks) to a total of 45 Gray. Patients underwent surgical resection 5 to 6 weeks after completion of neoadjuvant therapy.

5.4.2 Lymphoscintigraphy and Surgery

Peritumoral injection of four, 1-ml aliquots of 40 MBq ^{99m}Tc antimony colloid (Lymphflo) were undertaken once the patient was under general anesthetic immediately before surgery. At endoscopy, the peritumoral injections were performed into the submucosal layer at both the proximal and distal margins of the tumor [17,18]. If the tumor was circumferential and passage of the endoscope was not possible, the tracer was injected

proximal to the tumor only. Directed by the Ethics Review Board, a licensed nuclear medicine physician (D.B.) transported and injected the radioactive tracer.

Esophagectomy was usually performed by a 2-surgeon synchronous Ivor-Lewis technique via a right antero-lateral thoracotomy and an upper midline laparotomy, as described previously [19]. In 1 patient with a mid-esophageal tumor, a 3-stage (cervico-thoraco-abdominal) esophagectomy was performed. A gamma probe (gammasonics MK2) was used to identify any sentinel lymph node(s) in both the upper abdomen and thorax after mobilization of the esophagus and stomach. Readings were taken with the probe tip directed away from the tumor to minimize background interference. A sentinel node was defined as any node with an activity twice that of surrounding tissue [1,17]. Readings were also taken after esophageal and gastric resection to identify any residual sentinel node(s) because it is our practice to perform a conservative lymph node dissection (removal of all nodes adjacent to the tumor) rather than a two-field radical lymphadenectomy [20]. Continuity of the gastrointestinal tract was restored by either a handsewn or stapled end-to-side esophago-gastrostomy, depending on surgeon preference.

5.4.3 Specimen Handling and Pathology

Each specimen was dissected on the back table in the operating room by the surgeon. Lymph node stations were removed sequentially from the specimen. A lymph node was confirmed as the sentinel node by a background ratio of 10:1 *ex vivo*. In addition, other hot nodes containing more than 10% of the activity in the hottest node in the lymphatic basin were classified as sentinel nodes [1]. Each lymph node station and sentinel node was sent separately for pathological analysis.

Non-sentinel lymph nodes were bisected once, fixed in formalin, embedded in paraffin,

and stained with hematoxylin and eosin (H&E) according to standard procedures. Sentinel lymph nodes were bisected along their longitudinal axis, or cut into 2- or 3-mm slices if thicker than 5 mm. On the first section, one slide was stained with H&E, and the other with the monoclonal epithelial antibody AE1/AE3 (DAKO, Carpinteria, CA) for immunohistochemistry (IHC). The monoclonal antibody AE1/AE3 is widely used because it recognizes a broad range of keratin subtypes expressed in esophageal carcinomas [21]. Sections of primary tumors were used as positive controls with each run, and a negative control (primary antibody omitted) was also included.

Sentinel lymph nodes that remained tumor-free by both H&E and IHC on the first section had a minimum of two further serial step sections performed [22-24]. Lymph node metastases were defined as a metastasis >2mm in size. Micrometastases were defined as a metastasis >0.2mm and ≤2mm, while isolated tumor cells were defined as a single tumor cell or a cluster of tumor cells ≤0.2mm in size [25,26]. Strict criteria were used to designate a positive cell(s) as an isolated tumor cell(s). Cells had to demonstrate cytologic and microanatomic features of a tumor cell, including increased cell size, enlarged nuclear size, and increased nuclear: cytoplasmic ratio [26].

5.4.4 Statistical Analysis

Data were collected prospectively. Calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). The Chi-square test was used to compare groups, if applicable. The sensitivity, specificity, and accuracy of sentinel lymph node biopsy were calculated by the standard definitions [18]. Statistical significance was set at the 5% level.

5.5 RESULTS

5.5.1 Patient and Tumor Characteristics

The median patient age was 60.4 years (range, 45-75 years), and 15 of 17 patients were male. Thirteen patients had an adenocarcinoma. Nine of these were lower esophageal tumors (Siewert type I), and four were located at the gastroesophageal junction (Siewert type II). Two of the four squamous cell carcinomas were located in the mid-esophagus, with the remaining two in the lower esophagus. Eight patients (47%) underwent neoadjuvant therapy. One patient who had undergone neoadjuvant therapy was deemed unresectable at the time of operation because his tumor was invading the right atrium (resection rate, 94%). The remaining 16 patients had pT-stage tumors as follows: four (25%) were pT1a (intramucosal), 4 (25%) were pT1b (submucosal), 1 (6%) was pT2, 4 (25%) were pT3, and 3 (19%) were pT0 after a complete response (pCR) following neoadjuvant therapy.

5.5.2 Lymphoscintigraphy

The sentinel lymph node detection rate using lymphoscintigraphy was 88% (14 of 16 patients). One of the two patients (both Siewert type I) in whom we could not identify a sentinel lymph node had had extensive prior upper gastrointestinal surgery. The second patient was morbidly obese with a body mass index of 42. In the remaining 14 patients, there were 37 sentinel lymph nodes, with a median of 2 lymph nodes per patient (range, 2-5 lymph nodes). A total of 239 lymph nodes were resected (as identified by the pathologist) with a median of 16 per patient (range, 4-30 lymph nodes).

Sentinel node characteristics are listed in Table 1. In patients with a Siewert type I tumor, the sentinel lymph nodes were mostly located in the peri-esophageal tissue (60%). In

Siewert type II tumors, the sentinel nodes were located more often in the peri-gastric tissue (82%). Five patients had sentinel nodes present in more than one lymph node station. Three of 14 patients had sentinel lymph nodes identified in the tumor basin once the esophageal cancer and adjacent lymph nodes had been removed (1 peri-esophageal lymph node, and 2 celiac artery lymph nodes). These were all negative for metastasis.

5.5.3 Accuracy of Sentinel Lymph Node(s)

Three patients (21%) had overt metastases in the sentinel lymph node(s), one of whom had an occult tumor deposit (micrometastasis) in a second sentinel node, and one patient (7%) had isolated tumor cells in a sentinel node. The remaining 10 patients had negative sentinel nodes. Three patients were categorized as pN1, and these were the same patients with a positive sentinel node (sensitivity, 100%). One patient was categorized as a pN0(i+) and his non-sentinel nodes were all negative. No patient had positive non-sentinel lymph nodes and negative sentinel nodes. This corresponds to an accuracy of 100% for the sentinel lymph node procedure in our study.

5.6 DISCUSSION

The sentinel lymph node concept has been shown by others to be feasible in esophageal cancer, and we have confirmed this in our study with successful localization of the sentinel node in 14 of 16 patients (88%). This success rate is equivalent to other studies using radioactive tracer (range, 85 to 100%) [17,18,27-29]. This study is unique because we performed sentinel lymph node biopsy in conjunction with a *conservative* lymphadenectomy. While this approach contributed to our initial difficulties with the procedure (*vide infra*), it also enabled detection of hot nodes which would have otherwise not been resected, in 3 of 14 patients. We therefore agree with Kitagawa [4] that this method has great potential, especially with the development of increasingly minimally invasive surgery where a more radical lymph node dissection may not be feasible.

A median of 2 sentinel nodes per patient were removed, with a median number of 16 lymph nodes identified. This is an increase from our usual number of resected lymph nodes (median, 7) [30] without a change in our conservative lymphadenectomy approach. This was achieved by submitting lymph node stations separately from the specimen, and probably represents increased identification of lymph nodes in the resected specimen rather than a more radical procedure. Veeramachaneni *et al* [31] observed a significant increase in the number of lymph nodes resected in patients with esophageal cancer depending on whether they had submitted named packets of nodal stations (16 ± 9 nodes/patient) or the entire un-dissected specimen (10 ± 8 nodes/patient). A recent study in gastric cancer looking at factors influencing lymph node recovery from the operative specimen also suggests that dissecting the specimen prior to fixation may increase lymph node yield [32].

Sentinel lymph nodes in Siewert type I tumors were mostly located in the peri-esophageal tissue (78%), whilst Siewert type II tumors were found more often in the peri-gastric tissue (75%). Lymphatic mapping studies support these findings with lower esophageal cancers and junctional tumors disseminating in a longitudinal fashion (rather than segmental) to lower mediastinal and abdominal lymph nodes [7,8,33,34]. While sentinel nodes were found in more than one nodal station per patient in 31% of cases, no skip metastases were found in 14 patients (i.e. neither hot celiac artery node was positive). It is possible that 2 of 16 patients had skip metastases, contributing to unsuccessful localization of sentinel nodes.

In our study, the sentinel node was extremely accurate (100%) in predicting the status of non-sentinel lymph nodes. Other studies have shown similar findings with the use of radiocolloid tracer: 91-96% [17,18,29]. Grotenhuis *et al* [35] performed sentinel node biopsy in 40 esophageal adenocarcinomas with blue dye only. While they were successful in identifying a sentinel lymph node in 98%, they had an unacceptably high false negative rate of 15%. Perhaps the high number of pT3 tumors (65%) in their study contributed to their low accuracy rate, but most studies in esophageal cancer support radiocolloid tracer as superior to the dye method [4,17]. Lamb et al [17] used Patent Blue V in addition to radioactive colloid in 20 of 57 patients. The addition of blue dye failed to identify any additional sentinel nodes. When injecting blue dye endoscopically prior to the procedure (along with radioactive tracer), they found that too much time elapsed from time of injection to sentinel node identification. When injecting blue dye intraoperatively through the esophageal wall, they found extensive staining of adjacent tissues that obscured the surgical field. It is for these reasons that we chose not to inject blue dye along with radiocolloid tracer.

With the use of serial sections and immunohistochemistry on the sentinel lymph nodes, one patient was up-staged to 3 positive lymph nodes (rather than the original 2 on conventional H&E staining). Many believe this has important prognostic significance [30,36,37] and, in our patient, the number of positive nodes influenced the decision for further postoperative chemoradiotherapy. A second patient had isolated tumor cells in their sentinel lymph node while all other nodes were negative on conventional staining. We have recently shown that the presence of isolated tumor cells significantly decreases 5-year survival, compared to patients who remain node-negative following additional analysis of their lymph nodes [15].

Writing about the feasibility of the sentinel node concept to esophageal surgery, Udugawa [6] predicted that: "The theory is elegant, but there are many questions to be answered and technical hurdles to overcome before its application is widely accepted." True to this comment, we encountered several technical problems in adopting this approach (Table 2).

5.6.1 Choice of Radioactive Tracer

Colloid particles between 4 to 100 nm in size are necessary to translocate from the interstitial injection site to lymphatic channels, and to be retained within the first lymph node(s) encountered along such pathways [38,39]. However, the type of radiocolloid available for clinical use is strongly dependent on that particular country's legislation [38]. Filtered ^{99m}Tc-sulphur colloid is used routinely in North America, ^{99m}Tc-albumin nanocolloid in Europe, ^{99m}Tc-tin fluoride colloid in Japan, and ^{99m}Tc-antimony trisulfide colloid in Australia [1,40]. This has important implications for surgical planning. ^{99m}Tc-tin colloid with a particle size of 100 nm results in a long period of tracer deposition in the lymph which allows the surgeon to perform a lymphoscintigraphy 24 hours prior to surgical resection [5]. The remaining 3 radiocolloids have smaller particle sizes, with a

median transit time of 10 minutes to sentinel nodes, and a half-time for washout of activity in the node(s) between 4-8 hours [1,38]. As a consequence, surgery must be planned shortly after peritumoral injection (or in the case of antimony colloid, a much higher dose must be given). This leaves little time for lymphoscintigraphy, and in a health care system (such as Australia's) with limited resources, it may be more cost-effective to undertake peritumoral injection immediately prior to esophagectomy.

5.6.2. Preoperative Endoscopy & Peritumoral Injection

Similar to Lamb *et al*'s landmark study [17], we opted to perform upper endoscopy following insertion of the double-lumen endotracheal tube. We encountered difficulty inserting the endoscope in one patient with a short wide (bull) neck. In all other patients, insertion of the endoscope was straight-forward with the assistance of the anesthetist (who performed a jaw thrust). Peritumoral injection was best situated 5-10 mm away from the proximal and distal tumour margins (or post-treatment scar) in order to target normal submucosal lymphatics and to avoid tracer spillage [38]. In the setting of a tight stricture, radioactive tracer was injected only above the proximal margin. In patients who had received neoadjuvant therapy, or in patients with multicentric malignant degeneration within a Barrett's segment, identification of the tumor was at times difficult [27]. It is our policy to endoscope all patients prior to multi-disciplinary treatment planning, so we relied heavily on the pre-treatment endoscopy reports in these cases.

5.6.3. In vivo Identification of Sentinel Lymph Node(s)

Shine-through phenomenon (a strong overlapping signal from the primary tumor) is a problem in esophageal and gastric cancer because the tumor and sentinel nodes are often adjacent to each other [42]. It is important to angle the gamma probe away from the tumor

at all times. If there has been inadvertent spillage of tracer into the esophageal or gastric lumen, identification of sentinel nodes can be difficult. In this situation, it is usually best to abandon *in vivo* localization, and resort to *ex vivo* sentinel node identification.

Attempts to identify a hot node intra-operatively were hampered by extensive adhesions in one patient, and morbid obesity in another. Nakahara *et al* found that an increase in body mass index from 22 to 24 was significantly associated with unsuccessful sentinel lymph node localization in gastric cancer [43]. It should be noted that the average BMI in our patient population was 28, with two patients above 40. Our surgical unit is considering whether we should put morbidly obese patients on a very low calorie diet (1,000 kcal/day) for 3 to 4 weeks prior to surgical resection.

5.6.4. Definition of Sentinel Lymph Node

There is no standard criteria for defining hot nodes or sentinel lymph nodes [44,45]. The EANM-EORTC guidelines for sentinel node diagnostics in melanoma, published in 2009 [1], state that a sentinel node is the hottest node plus any other hot nodes containing more than 10% of the activity in the hottest node in the lymphatic basin. Other investigators have defined a sentinel node as any node with an activity more than twice that of surrounding tissue, and those with an *ex vivo* gamma probe reading of more than 10 times background activity [17,38]. Yasuda *et al* [44] measured the sensitivity of the gamma probe in a laboratory study, and they found that 51% of esophageal cancer hot nodes had activity levels below the detection sensitivity of the probe. They attribute this to spillage of radiocolloid during endoscopic injection. Spillage was not a factor in the two patients in whom we were unable to locate sentinel lymph nodes. In our study, we found that all sentinel nodes contained 20% or more of the activity of the hottest node.

5.6.5. Ex-vivo Identification of Sentinel Lymph Node(s)

Increased vigilance must be taken during the intra-operative search for sentinel nodes in the setting of a conservative lymphadenectomy. If no sentinel nodes are identified *ex vivo*, one must assume that the sentinel node remains in the patient (via an unexpected drainage pattern) or that there was a technical error with radiocolloid injection [4]. Lymph node stations were separated from the main specimen, and a thorough search for sentinel nodes was undertaken. Although our lymph node number was slightly decreased following neoadjuvant therapy (13 vs 15), we did not experience any difficulty in sentinel lymph node identification. In fact, all four patients with overt or occult tumor in their sentinel nodes had had neoadjuvant therapy. We also did not experience increased difficulty in identifying sentinel nodes in patients with more advanced tumors. Several authors have found a significant correlation between a higher metastatic area and lower radioisotope counts [38,45]. However, these studies have used the 100 nm ^{99m}Tc-tin colloid particles. It is possible that smaller particles such as those used in this study are still able to penetrate metastatic lymph nodes.

5.6.6. Pathological Analysis

Analysis of the circumferential margin cannot be performed by the pathologist once the lymph node stations have been removed by the surgeon in the operating room. Designated sentinel lymph nodes should be analyzed with a minimum of three serial sections and immunohistochemisty if negative on initial analysis [22-24]. In the absence of an internationally-recognized sentinel lymph node analysis protocol, the number of sections and choice of cytokeratin agent is dependent upon both the laboratory and the pathologist.

5.7 CONCLUSION

Sentinel lymph node biopsy is feasible in esophageal resections with conservative lymphadenectomy and, when successful, initial results suggest it is very accurate in predicting overall nodal status. Further work is needed to optimize radiocolloid type, refine the technique, standardize sentinel lymph node definitions, and develop a quick and accurate way to determine sentinel lymph node status intra-operatively. Sentinel lymph node biopsy may become standard of care in esophageal cancer in the upcoming decade, especially in the setting of minimally invasive surgery.

5.8 ACKNOWLEDGMENTS

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Table 5.1. Sentinel lymph node characteristics in 16 patients

Patient	Tumor type	Neoadj therapy	BMI	Tumor Location	pT	pN	No. SLN	Location of the SLN	SLN status[a]	Non-SLN status[b]
1	SCC	N	25	Middle	1b	0	3	middle paraesophageal; perigastric	−	−
2	ACA	N	25	Lower	1a	0	2	lower paraesophageal	−	−
3	ACA	N	27	Lower	1b	0	2	lower paraesophageal	−	−
4	ACA	N	26	GOJ	3	0	2	lower paraesophageal	−	−
5	ACA	Y	33	Lower	0	0	2	lower paraesophageal	−	−
6	ACA	Y	34	Lower	3	1	3	lower paraesophageal; perigastric	M/MIC	+
7	ACA	N	32	GOJ	1a	0	4	perigastric	−	−
8	ACA	N	25	Lower	1a	0	2	lower paraesophageal; celiac artery	−	−
9	ACA	N	26	Lower	1a	0	0	NA	NA	−
10	ACA	N	42	Lower	1b	0	0	NA	NA	−
11	SCC	N	41	Lower	1b	0	5	perigastric	−	−
12	SCC	Y	22	Middle	0	0	3	lower paraesophageal	−	−
13	ACA	Y	23	GOJ	3	1	2	perigastric	M	+
14	ACA	Y	23	Lower	3	1	2	lower paraesophageal	M	+
15	ACA	Y	33	GOJ	0	0	3	perigastric	ITC	−
16	SCC	Y	16	Lower	2	0	2	lower paraesophageal; celiac artery	−	−

BMI: body mass index (kg/m^2); SLN = sentinel lymph node; SCC: squamous cell carcinoma; ACA: adenocarcinoma; GOJ: gastro-esophageal junction; pT0: no residual tumour in specimen; NA: not applicable

[a]Sentinel lymph node metastases were defined as pathologically negative (−), with isolated tumor cells (ITC), with micrometastases (MIC), or positive (M)

[b]Non-sentinel lymph node metastases were defined as pathologically negative (−) or positive (+)

Table 5.2. Technical considerations for sentinel lymph node biopsy and same-day esophagectomy.

Problem	Solution
Insertion of endoscope	Experienced endoscopist; jaw thrust by anesthesiologist
Narrow stricture	Injection above proximal margin only
No tumor visible	Injection based on tumor description on pre-treatment endoscopy report
Radiocolloid spillage	Peritumoral injection at least 5-10 mm away from tumor; if junctional tumor, avoid injection in the retroflexed position
Shine-through phenomenon	Angle gamma probe away from primary tumor; if spillage of tracer has occurred, may need to remove specimen and perform *ex vivo* identification of sentinel nodes
Obesity	Consider low-calorie diet prior to surgical resection (if BMI >35)
Ex vivo identification of sentinel nodes	Designate any node(s) with >20% the activity of the hottest node as a SLN; if a section of tissue is hot but no lymph node is palpable, submit section of tissue separately as SLN (nodes <3 mm may not be palpable)

BMI: body mass index (kg/m^2); SLN = sentinel lymph node

5.10 PUBLISHED QUESTIONS & ANSWERS

5.10.1 Discussant

Blair Jobe: Doctor Thompson, this is a fantastic paper, and the way you just presented that, I learned a lot for how I want to structure my talks in the future because I thought it was phenomenally presented. I think that your manuscript was very well written and very organized and I got a lot out of it and I learned a lot from it, and I think as I was reflecting, I think perhaps the most important potential application of sentinel lymph node assessment would be in the staging of lymph node status when considering endoluminal resection of T1 with a goal of esophageal preservation; because right now we use tumor depth as a proxy for lymph node status, and it would be really nice to have a minimally invasive approach, either through a transthoracic approach or maybe even a node approach to sample the sentinel node in patients who have a superficial cancer so we can sleep at night and know that they do not have some acute disease there.

I just have a couple of quick questions; first, please address the potential differences in the patterns of lymphatic spread; so we know that squamous cell carcinoma has a tendency to skip nodal basins versus adenocarcinoma which tends to spread in a linear fashion away from the tumor.

Second, we know that an increasing number of lymph nodes harvested has recently been demonstrated to be an independent predictor for improved prognosis in patients with esophageal cancer and you suggest that the sentinel node assessment could be used to tailor the degree of lymphadenectomy; will you discuss how your technique may be employed in this context? So in other words, we are all talking about the more nodes you can get out the better the patient is going to do, and is that just because we do not have the resolution

of really cutting up a lymph node and looking at it with IHC or what you are doing? So it sounds like the sentinel nodes, you are really going over them with a fine-tooth comb, and it sounds like you are maybe increasing the resolution a little bit.

And then finally, what do you think the impact of radiation therapy may be on the sensitivity and/or accuracy of sentinel node sampling? Will lymphatic sclerosis caused by induction therapy lead to a misinterpretation of your lymph node basin status?

So once again I congratulate you, I think this is important work and I plan on trying to apply this to more superficial cancers and looking at ways we can do this in a minimally invasive approach.

5.10.2 Author Reply

Sarah Thompson: Thanks for those great questions. In response to your first question, it is important to differentiate between multiple levels and skip metastases. Just over 30% of patients in our small series had sentinel nodes in more than one lymph node station, sometimes on either side of the diaphragm. We do not consider these to be skip metastases, rather simultaneous lymphatic drainage of the primary tumor to two or more lymph nodes; and I think this is how esophageal cancer is unique compared to other cancers using this concept. You are right in that skip metastases are described commonly in squamous cell cancer and there is a high incidence of nodal spread to the cervical nodes in both middle and lower third esophageal squamous cell tumors. In adenocarcinoma however, Lamb et al have done the largest series of SLN biopsy in this group of patients and they found that close to 80% of sentinel nodes were within 3 cm of the primary tumor. So I think preoperative lymphoscintigraphy is probably more important in squamous cell cancer and certainly if you are thinking about doing an endoluminal approach. In

adenocarcinoma, linear lymphatic spread seems to be the norm and this is highlighted in our study because we had a success rate of 88% when performing a conservative lymphadenectomy.

In answering your second question, we are aware of these studies and our overall survival rates are around 36%, which is comparable to centers that perform a radical lymphadenectomy. There are several reasons why studies, which relate numbers of lymph nodes, resected and survival may be drawing the wrong conclusion. Suffice it to say in the context of our study, we believe the most practical outcome of sentinel node studies will be in directing the pathologist to the most important lymph nodes for additional analyses for better detection of isolated tumor cells and micrometastases. Whether it will ever play a role in tailoring lymphadenectomies remains to be demonstrated.

Finally, half of our patients had received neo-adjuvant therapy and in our center this includes 45 Gray of radiotherapy. We did not have any increased difficulty in identifying sentinel lymph nodes in these patients, and all three patients with positive nodes had received neo-adjuvant therapy. As well, the two failures were in patients who had not received neo-adjuvant therapy.

CHAPTER 6: SENTINEL LYMPH NODE BIOPSY IN ESOPHAGEAL CANCER: SHOULD IT BE STANDARD OF CARE?

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NOTE:
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6.2 ABSTRACT

Introduction: Sentinel node mapping is established in some superficial cancers but remains controversial in harder-to-access solid tumors. There are an increasing number of recent studies suggesting that isolated tumor cells have prognostic significance in predicting poor survival, in breast cancer, esophageal cancer, and others. It is for this reason that we have persevered with the sentinel lymph node concept in our esophagectomy cancer patients, and we report our results since 2008. **Methods:** Thirty-one of 32 consecutive patients underwent resection for invasive esophageal cancer along with sentinel lymph node retrieval (resection rate, 97%). Peritumoral injection of ^{99m}Tc antimony colloid was performed by upper endoscopy prior to the operation. A 2-surgeon synchronous approach via a right thoracotomy and laparotomy was performed with a conservative lymphadenectomy. Sentinel lymph nodes were identified with a gamma probe both *in* and *ex vivo*, and sent off separately for 3 serial sections and immunohistochemistry with AE1/AE3. **Results:** The median patient age was 63.4 years (range, 45-75 years). Most patients (81%) had an adenocarcinoma, and 61% had received neoadjuvant therapy. At least one sentinel lymph node (median, 3) was identified in 29 of 31 patients (success rate, 94%). Sentinel nodes were present in more than 1 nodal station in 16 patients (55%). One false negative case led to a sensitivity of 90%. In 28 of 29 patients, the sentinel lymph node accurately predicted findings in non-sentinel nodes (accuracy, 96%). **Conclusions:** Sentinel lymph node biopsy is both feasible and accurate in esophageal resections with conservative lymphadenectomy. It allows targeted serial sectioning and immunohistochemical studies of those nodes and should become standard of care in patients undergoing esophagectomy for esophageal cancer.

6.3 INTRODUCTION

The sentinel lymph node (SLN) concept describes the preferential lymphatic drainage of a primary tumor to a regional lymph node(s)[1]. Since its inception by Morton in 1992, sentinel lymph node biopsy has become the gold standard for patients with melanoma and breast cancer. However, its use in other solid tumors has been more controversial with continued debate regarding its role, if any, in staging and treatment algorithms[2-4].

Perhaps recent studies have strengthened the case for the routine use of sentinel lymph node biopsy in the treatment of esophageal cancer patients. First, we (and others) have recently shown that occult tumor deposits in lymph nodes have prognostic significance for decreased survival[5,6]. These results have been replicated in larger studies in other solid tumor types such as breast cancer[7]. The smallest of the occult tumor deposits, isolated tumor cells, are on average 10 to 30 microns in size (0.01-0.03 mm), making their detection virtually impossible without the use of serial sections and immunohistochemistry. Sentinel lymph node biopsy is the only practical method in today's economic climate to identify the most important lymph nodes for more detailed histopathological analysis.

The second reason to establish this technique in esophageal cancer is to promote the introduction of improved sentinel lymph node tracers that may lead to better diagnostic and staging investigations. We do not agree that other imaging techniques "may be as accurate (as SLN biopsy) in detecting esophageal cancer metastases", as written by Zhang and colleagues in 2010[8]. Positron emission tomography/computed tomography (PET/CT) cannot distinguish positive lymph nodes in close proximity to the primary tumor due to the shine-through effect (a strong overlapping signal from the tumor)[9], nor can it detect

positive lymph nodes less than 7 to 8 mm in size. It most certainly does not have the sensitivity required to detect lymph nodes containing only micrometastatic disease[10]. Similarly, endoscopic ultrasound is not able to identify occult tumor deposits within a lymph node from a fine needle aspirate.

We recently published our initial experience with sentinel lymph node (SLN) biopsy with conservative lymphadenectomy in esophageal cancer and we showed that it was feasible to identify the SLN in 88% of cases, and it was accurate 92% of the time[11]. We have persevered with this approach because we do not believe the current pathological analysis for non-sentinel lymph nodes is sufficient. In this prospective study, our aims included evaluating the accuracy of the sentinel node in predicting the status of non-sentinel lymph nodes with a larger sample size, and determining the frequency of skip metastases in esophageal cancer.

6.4 MATERIALS AND METHODS

6.4.1 Patient Selection and Preparation for Surgery

Thirty-two consecutive patients undergoing a surgical resection for invasive squamous cell carcinoma or adenocarcinoma of the esophagus were selected for the study. These patients were recruited between June 2008 and March 2011, and include 17 patients from our prior publication[11]. All operations were performed or closely supervised by one of 5 surgeons who are involved with our unit. The study was approved by the Research Ethics Committee at the Royal Adelaide Hospital, Adelaide, South Australia.

Preoperative clinical staging included upper gastrointestinal endoscopy, computed tomography scans (chest, abdomen, pelvis), PET/CT scans, endoscopic ultrasonography (if minimal stricturing), and diagnostic laparoscopy (for gastroesophageal junction tumors). Selected patients (T2 or greater) were treated with neoadjuvant therapy according to protocol[12]. This consisted of 2 cycles of cisplatin (80 mg/m^2 on day 1) and 5-FU (800 mg/m^2 continuous infusion for 5 days) during weeks 1 and 5 of radiotherapy, plus 25 fractions of radiotherapy (over 5 weeks) to a total of 45 Gray. Patients underwent surgical resection 5 to 6 weeks after completion of neoadjuvant therapy.

6.4.2 Lymphoscintigraphy and Surgery

As previously described, peritumoral injection of four, 1-ml aliquots of 10 MBq ^{99m}Tc antimony colloid (Lymphflo), maximum 40 MBq, were undertaken once the patient was under general anesthetic immediately before surgery. At endoscopy, injections were performed into the submucosal layer at both the proximal and distal margins (if possible) of the tumor[13]. In accordance with our Ethics Review Board, a licensed nuclear medicine physician (D.B.) transported and injected the radioactive tracer.

Esophagectomy was usually performed by a 2-surgeon synchronous Ivor-Lewis technique via a right antero-lateral thoracotomy and an upper midline laparotomy, as described previously[14]. A gamma probe (gammasonics MK2) was used to identify any sentinel lymph node(s) in both the upper abdomen and thorax after mobilization of the esophagus and stomach. Readings were taken with the probe tip directed away from the tumor to minimize background interference. A sentinel node was defined *in vivo* as any node with an activity twice that of surrounding tissue[1,13]. Readings were also taken after esophageal and gastric resection to identify any residual sentinel node(s) because it is our practice to perform a conservative lymph node dissection (removal of all nodes adjacent to the tumor) rather than a two-field radical lymphadenectomy[15]. Continuity of the gastrointestinal tract was restored by either a handsewn or stapled end-to-side esophago-gastrostomy, depending on surgeon preference.

6.4.3 Specimen Handling and Pathology

Each specimen was dissected on the back table in the operating room by S.K.T. Lymph node stations were removed sequentially from the specimen. Using the EANM-EORTC guidelines for sentinel node diagnosis in melanoma, a sentinel node was defined *ex vivo* as the hottest node plus any other hot nodes containing more than 10% of the activity in the hottest node in the lymphatic basin[1]. In our feasibility study, we had found that all sentinel nodes contained 20% or more of the activity of the hottest node[11]. Each lymph node station and sentinel node was sent separately for pathological analysis.

Non-sentinel lymph nodes were bisected once, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) according to standard procedures. Sentinel lymph nodes were bisected along their longitudinal axis, or cut into 2- or 3-mm slices if

thicker than 5 mm. On the first section, one slide was stained with H&E, and the other with the monoclonal epithelial antibody AE1/AE3 (DAKO, Carpinteria, CA) for immunohistochemistry (IHC)[16]. Sections of primary tumors were used as positive controls with each run, and a negative control (primary antibody omitted) was also included.

Sentinel lymph nodes that remained tumor-free by both H&E and IHC on the first section had a minimum of two further serial step sections performed[17-19]. A lymph node metastasis was defined as a metastasis >2mm in size (pN1). A micrometastasis was defined as a metastasis >0.2mm and ≤2mm [pN1mi(sn)], while isolated tumor cells were defined as single tumor cell(s) or cluster(s) of tumor cells ≤0.2mm in size [pN0(i+)(sn)][20-22]. Strict criteria were used to designate a positive cell(s) as an isolated tumor cell(s), including increased cell size, enlarged nuclear size, and increased nuclear: cytoplasmic ratio[21].

6.4.4 Statistical Analysis

Data were collected prospectively. Calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). The Chi-square test was used to compare groups, if applicable. The sensitivity, specificity, and accuracy of sentinel lymph node biopsy were calculated by the standard definitions[23]. Statistical significance was set at the 5% level.

6.5 RESULTS

6.5.1 Patient and Tumor Characteristics

One patient who had undergone neoadjuvant therapy was deemed unresectable at the time of operation because his tumor was invading the right atrium (resection rate, 97%). The median patient age of the remaining patients was 63.4 years (range, 45-75 years), and 28 of 31 patients were male. The average body mass index (BMI) in our patient population was 27.6 kg/m^2, with 8 patients above 30 kg/m^2 and two above 40 kg/m^2. Tumor characteristics are listed in Table 1. Twenty-five of 31 patients (81%) had an adenocarcinoma, and the majority of these (64%) were lower esophageal tumors (Siewert type I). Nineteen patients (61%) underwent neoadjuvant therapy. Of these, six (32%) had a complete pathological response with no residual viable tumour cells on final conventional pathology (i.e. without taking into account the results of immunohistochemistry).

6.5.2 Sentinel Node Identification

The sentinel lymph node detection rate using lymphoscintigraphy was 94% (29 of 31 patients). One of the two patients (both Siewert type I adenocarcinomas) in whom we could not identify a sentinel lymph node had had extensive prior upper gastrointestinal surgery. The second patient was morbidly obese with a body mass index of 42. In the remaining 29 patients, there were 92 sentinel lymph nodes, with a median of 3 lymph nodes per patient (range, 1-8 lymph nodes). A total of 438 lymph nodes were resected (as identified by the pathologist) with a median of 14 per patient (range, 4-31 lymph nodes).

The majority of sentinel lymph nodes were located in one of the following lymph node stations (in conjunction with a conservative lymphadenectomy): lower para-esophageal,

left paracardial, and left gastric (Fig. 1). In patients with a Siewert type I tumor, the sentinel lymph nodes were mostly located in the para-esophageal tissue (75%) although in 31% of patients, sentinel nodes were found on both sides of the diaphragm. In Siewert type II tumors, the sentinel nodes were located more often in the para-gastric tissue (83%). Sixteen patients (55%) had sentinel nodes present in more than one lymph node station. Nine of 29 patients (31%) had sentinel lymph nodes identified in the tumor basin once the esophageal cancer and adjacent lymph nodes had been removed (in the para-esophageal, celiac artery, and carinal lymph node locations). These were all negative for metastasis except for one celiac artery sentinel node.

6.5.3 Accuracy of Sentinel Lymph Node(s)

Overall, sentinel lymph nodes were significantly more likely to contain tumour than non-sentinel nodes: 13 of 92 (14%) positive sentinel nodes *versus* 11 of 346 (3%) positive non-sentinel nodes ($P<0.001$). A total of 13 sentinel lymph nodes were positive in 9 patients (9/29, 31%). Eight of these nodes contained overt metastases, three had micrometastatic disease, and two had isolated tumour cells.

The accuracy of the sentinel lymph node procedure in predicting the status of non-sentinel nodes is shown in Table 2. Six patients (21%) had overt metastases in the sentinel lymph node(s), and four of these had corresponding positive non-sentinel nodes on routine H&E staining. Three patients had positive sentinel nodes on IHC staining, two of whom had micrometastatic deposits, and one with isolated tumour cells only. The non-sentinel nodes for all three of these patients were negative on routine lymph node analysis. We had one false-negative result in our series. This particular patient had an advanced long 10-cm oesophageal tumour with overt metastases in four non-sentinel nodes, but no metastatic deposits in two identified sentinel nodes. The sensitivity of sentinel lymph node biopsy in

our series was therefore 90% (9/10). The overall accuracy of sentinel lymph node biopsy was 96% (28/29) using immunohistochemistry and a minimum of 3 serial sections for all sentinel lymph nodes.

6.6 DISCUSSION

Sentinel lymph node biopsy was performed successfully in 29 of 31 (94%) consecutive esophageal cancer patients. A median of 3 sentinel nodes per patient were removed, and the diagnostic accuracy based on SLN status was 96%. SLN mapping was successful even with a *conservative* lymphadenectomy, an average body mass index (BMI) of 28, and the addition of neoadjuvant therapy in 61% of patients.

Four studies (with a sample size of at least 20 patients) using a radio-guided approach to find sentinel lymph nodes in esophageal cancer have reported success rates of 85 to 100%, and accuracy rates of 88 to 96%[13,24-26]. These results are superior to the two existing studies in the literature which used the blue dye method in esophageal cancer patients[27,28]. Grotenhuis *et al*[27] identified a sentinel lymph node in 98% of patients, but they had an unacceptably high false negative rate of 15% and an overall accuracy rate of only 85%. Similarly, Bhat *et al* detected a SLN in 81% of patients with an accuracy rate of only 75%[28]. Both studies had a high number of pT3 tumors (65% and 72%, respectively) but radiocolloid tracer is uniformly regarded as superior to the dye method for SLN biopsy in most solid tumor types[4,13,29].

There is no doubt that obesity contributed to increased difficulty in our patients with surgical resection and identification of sentinel lymph nodes. It is also noteworthy that, despite some reports to the contrary, the addition of neoadjuvant therapy prior to surgical resection did not affect our results. In fact, all 9 patients with overt or occult tumor in their sentinel nodes had undergone neoadjuvant therapy. Several authors have found a significant correlation between a higher metastatic area within the node, and lower radioisotope counts[30,31]. However, these studies have used the 100 nm ^{99m}Tc-tin colloid

particles. We believe smaller particles, such as 10±3 nm ^{99m}Tc-antimony trisulfide colloid, are able to penetrate metastatic lymph nodes, contributing to our high accuracy rate in the setting of advanced esophageal cancer.

With the use of 3 serial sections and immunohistochemistry (IHC) on negative sentinel lymph nodes, 14% (3/22) of patients were up-staged: two from pN0 to pN1mi(sn), and one from pN0 to pN0(i+)(sn). Lamb *et al* also found that 12% (3/25) of pN0 patients were upstaged following IHC analysis in their landmark study[13]. We recently published results showing that node-negative patients with either isolated tumor cells or micrometastases detected by IHC have a significantly decreased 5-year survival compared to those who remain node-negative following additional analysis of their lymph nodes (33% and 40% *versus* 60%, respectively)[5]. These patients may benefit from adjuvant therapy. A further patient in our series was up-graded from pN1 (2 positive lymph nodes) to pN2 (3 or more positive lymph nodes) with the identification of a micrometastasis within a sentinel lymph node. This patient went on to receive adjuvant chemoradiotherapy and is currently well with no evidence of tumor recurrence 21 months later.

Much of the lack of enthusiasm surrounding the routine use of sentinel lymph node biopsy in esophageal cancer is because, at present, it cannot alter or limit the extent of lymphadenectomy in the same way as is seen in breast cancer and melanoma. Most hospitals, like ours, do not have a dedicated pathologist who is willing to perform *intraoperative* rapid immunohistochemical analysis on the sentinel nodes. And in esophageal cancer, preoperative access to sentinel nodes may be as invasive, and as morbid, as the operation itself. But, if one agrees that isolated tumor cells have prognostic significance in esophageal cancer and, as shown above, are detected in 12-14% of node-negative patients using serial sections and immunohistochemistry, then the sentinel lymph

node concept becomes the only practical method of improving pathological staging. So, although sentinel node biopsy has not yet been shown to minimize the extent of lymphadenectomy, it may influence postoperative therapy for a significant number of patients.

Another criticism in the literature regarding sentinel lymph node biopsy in esophageal cancer is the reported high incidence of skip metastases, although most of these findings have been in patients with squamous cell carcinomas. It is well-known that lower esophageal cancers and junctional tumors (albeit, mostly adenocarcinomas) disseminate in a longitudinal fashion (rather than segmental) to lower mediastinal and abdominal lymph nodes[32-34]. And, sentinel lymph nodes in esophageal cancer are often multiple and found in more than 1 nodal station (range: 21 to 55%)[13,27]. However, it is important not to confuse multiple sentinel nodes with true "skip metastases". Tumor cells in esophageal cancer follow a predictable linear drainage pattern to 'first tier' nodal stations, and over 90% of them seem to be within 3 cm of the primary tumor[35]. Similar to Lamb's study[13], every one of our 29 patients had a sentinel node in one of the 'first tier' lymph node groups: lower para-oesophageal, right or left paracardial, or left gastric. One patient in our study was found to have a positive celiac lymph node in conjunction with a negative left gastric sentinel node. But, as celiac lymph nodes are now considered regional nodes according to the 7th edition of the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) staging manual[22], not even this can be called a skip metastasis.

Probably the biggest limitation with sentinel lymph node biopsy in esophageal cancer is the variable type of sentinel lymph node tracer legislated for clinical use in each country[30]. The vastly different particle sizes hinder wide application of the concept and creation of a uniform protocol. For example, Japan's ^{99m}Tc-tin colloid (100 nm in size) allows for

lymphoscintigraphy 24 hours prior to surgical resection[26], while other smaller radiocolloids (like Australia's ^{99m}Tc-antimony trisulfide colloid) have much shorter transit times in the sentinel nodes[1,30]. Facilitating preoperative lymphoscintigraphy in between endoscopic peritumoral injection and same-day surgery is often not practical. Future efforts should be made to design better sentinel lymph node tracers with dual imaging capabilities and, ultimately, the ability to differentiate a positive node (containing only micrometastatic tumor deposits) from a negative one prior to the initiation of any treatment.

6.7 CONCLUSION

Sentinel lymph node biopsy is both feasible and accurate in esophageal resections with conservative lymphadenectomy. There is no doubt that SLN biopsy improves pathological staging and may then influence further treatment decisions. Further work is needed to optimize sentinel node tracer type particularly with recent advances in imaging technology, but it is our opinion that SLN biopsy should become standard of care in patients with esophageal cancer. Whether it will ever be useful as a tool for tailoring a lymphadenectomy is a question for the future.

6.8 ACKNOWLEDGMENTS

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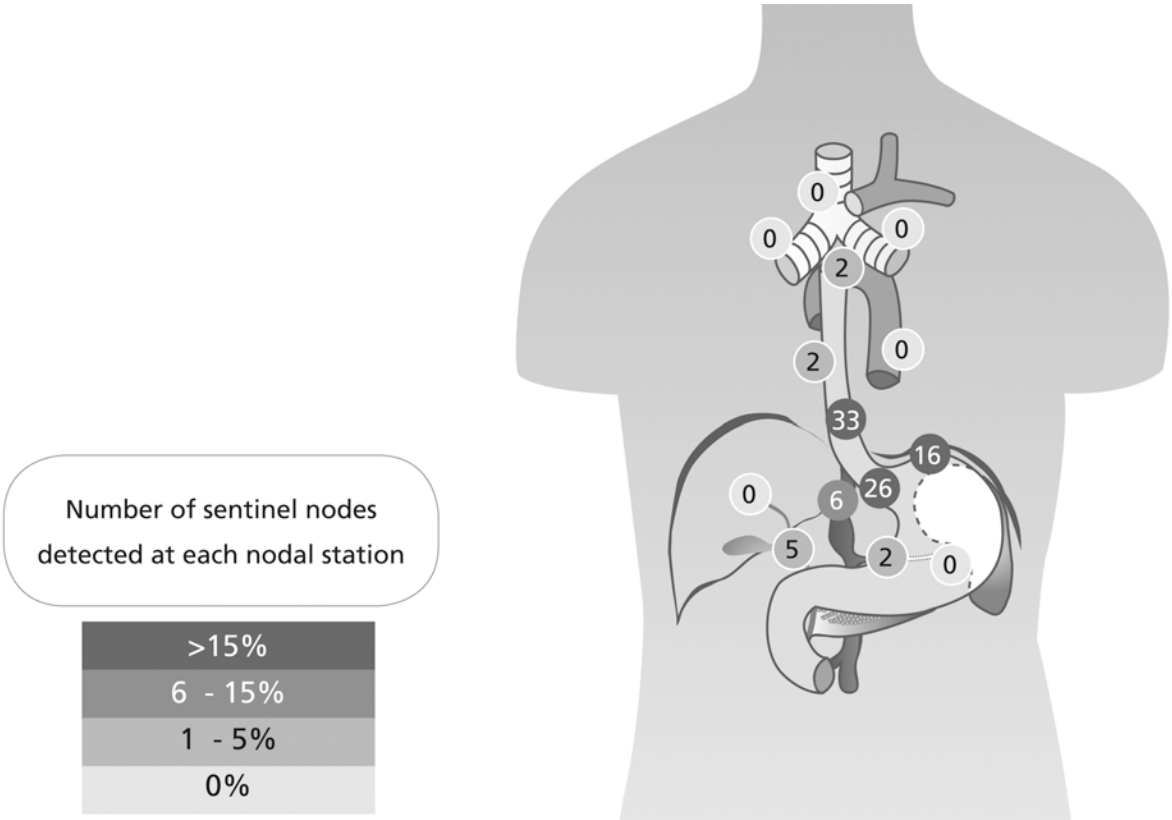

Figure 6.1. Graphical depiction of 92 sentinel lymph nodes in 29 esophageal cancer patients. Sentinel nodes were most commonly located in the lower para-esophageal, left paracardial, and left gastric lymph node stations.

Table 6.1. Patient and tumor characteristics (n= 31).

Variable	No. patients (%)
Histology	
Adenocarcinoma	25 (81)
Squamous cell carcinoma	6 (19)
Neoadjuvant therapy	
No	12 (39)
Yes	19 (61)
Tumor location	
Middle 1/3 esophagus	3 (10)
Lower 1/3 esophagus	22 (71)
GOJ[a]	6 (19)
Grade of differentiation	
Well/moderate (G1 + G2)	15 (48)
Poor/undifferentiated (G3 + G4)	14 (45)
Not assessable	2 (7)
pT-stage	
T0[b]	6 (19)
T1a	6 (19)
T1b	6 (19)
T2	4 (13)
T3	9 (30)
pN-stage	
N0	23 (77)
N1	4 (13)
N2	3 (10)
Vascular invasion	
No	25 (81)
Yes	6 (19)
Perineural invasion	
No	24 (77)
Yes	3 (10)
Not reported	4 (13)
Barrett's oesophagus	
No	9 (29)
Yes	22 (71)

[a]GOJ = gastro-esophageal junction
[b]T0 = no residual viable tumor cells

Table 6.2. Accuracy of the sentinel node in predicting the status of non-sentinel nodes (n=29).

	Overall Nodal Pathology	
	H&E[a] positive	Negative
Sentinel lymph node		
H&E positive	4	2
IHC[b] positive		3
Negative	1	19

[a]H&E = hematoxylin & eosin stain (routine pathology)
[b]IHC = immunohistochemisty (with epithelial antibody AE1/AE3)

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

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7.1 CONCLUSIONS

7.1.1 Aim #1

To examine the prognostic value of the following variables following oesophagectomy for oesophageal cancer on overall survival:

a. ***Sub-division of T1 lesions into T1a (intramucosal) and T1b (submucosal) lesions***
b. ***Refinement of lymph node status into N1a (< 3 metastatic lymph nodes) and N1b (≥ 3 metastatic lymph nodes)***
c. ***Presence or absence of lymph node invasion (extra-capsular tumour extension)***
d. ***Presence or absence of a positive circumferential resection margin***
e. ***Degree of tumour response to neoadjuvant therapy (if applicable)***

Two-hundred and forty oesophageal cancer specimens were re-analyzed to determine whether the accuracy of the current TNM staging system could be improved. In keeping with the literature, although many independent prognostic factors were found that could predict improved 5-year survival, most did not retain their significance after multivariate analysis. Only two factors remained significant: grade of differentiation and number of positive lymph nodes (0, 1-2, >2 nodes). Further, these results were applicable to patients receiving neoadjuvant therapy.

This study: 1) adds further weight to the necessity of a refinement in nodal staging, 2) discounts the ability of many independent prognostic factors to increase the accuracy of the current TNM model, and 3) is applicable to patients who received neoadjuvant therapy and those who were treated with surgery alone. We therefore concluded that a revised TNM staging system should incorporate grade of differentiation and number of positive lymph nodes and furthermore, this should be irrespective of neoadjuvant therapy.

Since this paper's publication in 2008, a revised TNM staging system has been published (7th edition of the AJCC/IUCC *Cancer Staging Manual*)[19,83]. This new edition includes many of the conclusions of the work that follows (in particular Chapter 2) regarding the importance histopathologic cell type and histologic grade, as well as the number of positive lymph nodes (a new pN classification). The impetus for these changes was in part due to the growing body of literature concerning the inadequacies of the (then) current staging system.

7.1.2 Aim #2

To determine the prognostic value of HER2/neu gene amplification and overexpression in oesophageal adenocarcinoma.

Eighty-nine oesophageal adenocarcinoma patients were identified and their respective paraffin blocks retrieved. Using silver-enhanced *in situ* hybridization (SISH), we found an incidence of HER2 gene amplification in 16% of patients, and a corresponding incidence of HER2 protein overexpression in 13.5% of patients [correlation coefficient between SISH and immunohistochemistry = 0.636 (moderate/strong), $P<0.0001$].

The presence of HER2 gene amplification did not correlate with any clinicopathological factors nor did it predict decreased survival. In fact, patients with HER2 amplification had a 5-year survival of 57% compared to only 32% for those without. However, this survival difference was not significant ($P=0.37$).

This publication concluded that although a subset of oesophageal adenocarcinoma patients may meet the criteria for anti-HER2 monoclonal antibody therapy, it is too early to suggest

that such therapy may increase long-term survival. As well, further research into targeted molecular therapies should not exclude patients who have undergone neoadjuvant therapy, nor those with metastatic disease.

7.1.3 Aim #3

In resection specimens classified as lymph node-negative on conventional histological analysis, to determine the incidence and prognostic value of:

a. immunohistochemically (IHC)-identified micrometastases and

b. isolated tumour cells

in the histologically negative nodes.

Thirty-one of 119 oesophageal cancer patients (26%), originally classified as node negative, were found to have occult tumour deposits in their lymph nodes following three additional serial sections, and immunohistochemical staining with the monoclonal antibody AE1/AE3. Twenty-two (18%) had isolated tumor cells or clusters, eight (7%) had micrometastases, and one (1%) was upgraded to a lymph node metastasis (pN1).

This study used reproducible, carefully defined, standardised methodology to distinguish occult lymph node deposits as either isolated tumour cells or micrometastases. We found that patients with oesophageal cancer who remained node negative had a 5-year survival of 60%. In contrast, patients with newly-identified tumour deposits had a significantly reduced 5-year survival of 33% and 40%, for isolated tumour cells and micrometastases, respectively (P=0.02).

We, like many others, did not think isolated tumour cells would significantly decrease survival in patients with oesophageal cancer. However, our findings confirm that these

tiny cells are probably not just tumour cells in transit, but microscopic tumour cell dissemination. Nearer to the time this paper was published in the *Annals of Surgery*, de Boer *et al* published a much larger trial showing that isolated tumour cells in the sentinel nodes of women with early-stage breast cancer led to a comparable significant decrease in 5-year disease-free survival rates[84]. This reduces the possibility that our results were due to a type II statistical error.

If we are to improve staging and ultimately survival in oesophageal cancer patients, then we must not ignore the presence of isolated tumour cells in lymph nodes. Surgeons should consider adopting the sentinel lymph node concept in oesophageal cancer patients. In today's economic climate, sentinel node biopsy may become an essential component of surgical resection, not necessarily to avoid lymphadenectomy, but to better guide histopathological staging and adjuvant therapy.

7.1.4 Aim #4

To validate the described technique for identification of sentinel lymph nodes in patients with cancer of the oesophagus and gastro-oesophageal junction, and confirm its accuracy in predicting nodal involvement of non-sentinel lymph nodes.

Sentinel lymph node (SLN) biopsy was performed successfully in 29 of 31 (94%) consecutive oesophageal cancer patients, although the majority of sentinel nodes were identified *ex vivo* (on the back table) due to interference from the primary tumour *in vivo*. A median of 3 sentinel nodes per patient were removed, and the diagnostic accuracy based on SLN status was 96%. Eighty-one percent of our patients had an oesophageal adenocarcinoma, 61% had undergone neoadjuvant chemoradiotherapy, and all had had a *conservative* lymphadenectomy.

With the use of 3 serial sections and immunohistochemistry (IHC) on negative sentinel lymph nodes, 14% of patients were up-staged: two with micrometastases, and one with isolated tumour cells. As shown in the 3rd part of this thesis, these patients may benefit from adjuvant therapy. In addition, another patient was up-graded from pN1 (2 positive lymph nodes) to pN2 (3 or more positive lymph nodes) with the identification of a micrometastasis within a sentinel lymph node. This patient went on to receive adjuvant chemoradiotherapy and is currently well with no evidence of tumour recurrence 21 months later.

Our study is one of only 5 studies (with a sample size greater than 20) examining the role of radio-guided SLN mapping in patients with oesophageal cancer. These all have similar results with success rates between 85 to 100%, and accuracy rates between 88 and 96%. It is curious then why the technique has not gained more acceptance in oesophageal cancer. There is no doubt that the procedure is easier to perform with an open oesophagectomy and, in our case, a synchronous approach whereby both the chest and abdomen are open at the same time. However, laparoscopic gamma probes do exist and are not difficult to use. Probably the main reason is that, unlike breast cancer and melanoma, SLN biopsy in oesophageal cancer does not offer an immediate trade-off by tailoring the extent of lymphadenectomy.

We concluded this work by suggesting that sentinel lymph node biopsy become standard of care in the treatment of patients with oesophageal cancer. Future efforts should be made to design better sentinel lymph node tracers with dual imaging capabilities and, ultimately, the ability to differentiate a positive node (containing only micrometastatic tumour deposits) from a negative one prior to the initiation of any treatment.

7.2 FUTURE DIRECTIONS

7.2.1 Limitations of Current Sentinel Lymph Node Tracers

As much of the previous work has shown, accurate staging of oesophageal cancer is critical to 1) predict overall prognosis, 2) decide upon the appropriate treatment(s), and 3) evaluate the tumour's response to those treatments. The current staging system has 2 major limitations[83].

First, there are no preoperative investigations that can predict lymph node involvement with satisfactory accuracy. Positron emission tomography (PET) with ^{18}FDG cannot currently distinguish a positive node from a negative one unless it is greater than 7-8mm in size, thereby limiting its application to smaller lymph nodes with or without micrometastatic disease. Endoscopic ultrasound has a sensitivity of 85% for accurately diagnosing positive regional lymph nodes, but only those, which are completely replaced by tumour cells (i.e. metastatic lymph nodes, not micrometastic disease). Precision in the preoperative detection of lymph node metastases is of great importance as the trend towards endoscopic management and minimally invasive surgery gathers momentum.

Second, we have shown that almost 30% of pathologically node negative (pN0) patients had isolated tumour cells in their lymph nodes, which were missed by routine pathologic examination. Importantly, these patients had a significantly reduced 5-year survival rate compared to those patients who remained node-negative and they may have benefited from adjuvant therapy. It is very important that we identify patients with occult tumour cells in their lymph nodes so that we can improve overall survival rates with additional treatment(s).

The final part of this thesis examined the success and accuracy of applying the *sentinel lymph node (SLN) concept* to patients with oesophageal cancer. With a total of 30 cases, we found a sentinel node in 94% of patients, and the sentinel nodes were accurate 96% of the time. And, importantly, three of 22 patients were upstaged (14%) from node negative with additional sectioning and staining of their sentinel lymph nodes. But, in order to increase the ease with which sentinel lymph nodes are found in oesophageal cancer, we need novel lymph node tracers. This is because, unlike many other cancers, approximately 90% of sentinel nodes lie within 3 cm of the tumour in oesophageal cancer[43]. Therefore the proximity of a tumour can limit accurate localisation of SLNs by lymphoscintigraphy. Furthermore, blue dye does not improve SLN localisation in oesophageal cancer because too much time elapses between injection and localisation of SLNs. Novel tracers are needed which are visible by multiple radiological signals to accurately locate SLNs prior to surgery.

7.2.2 Nanotechnology

Taking advantage of advances in fundamental physics and chemistry, a wealth of novel nanomaterials have been proposed in recent years for medical applications[85]. Although more studies are required to fully ascertain the safety profile of these novel nanoparticulate contrast agents, they are rapidly progressing towards clinical medicine. Iron oxide nanoparticles have already received approval for clinical use and they have shown promising results as lymphotropic MRI contrast agents[86]. MRI provides high spatial resolution and enables dynamic imaging of contrast agents with very high sensitivity, two features critical towards the successful implementation of nanoparticles for lymph node staging in oesophageal cancer. Although the behavior of nanoparticles within the lymphatics is directly correlated to their size and surface chemistry, the structure-activity relationship remains unclear. Using state-of-the-art synchrotron based imaging

technology, we aim to bridge that knowledge gap and provide an improved understanding of lymphatic anatomy in oesophageal cancer.

7.2.3 Proposed Study Design

7.2.3.1 Aims

i) To determine the structure-activity relationship of nanoparticles in the lymphatics

ii) To create a novel sentinel lymph node tracer capable of dual visibility: i) currently accepted radioactivity to facilitate detection by gamma probe intraoperatively, and ii) magnetic resonance imaging (MRI) contrast agent to allow detection on preoperative MRI scans for precise anatomical localization

iii) To validate the novel multimodal tracer in a large animal model

7.2.3.2 Hypotheses

i) Accurate staging is critical in understanding the biology of oesophageal adenocarcinoma, and in the evaluation of response predictors for these patients.

ii) Advanced nanoparticulate tracers with tailored functionalities can provide the required accurate staging in oesophageal adenocarcinoma.

7.2.3.3 Methods

Gold nanoparticles with different sizes (from 12 to 200 nm) and surface chemistry (anionic, cationic, and hydrophilic) will be prepared following procedures developed in Thierry's lab and used as models[87]. The unique capabilities of synchrotron X-Ray imaging will enable us to achieve hitherto unattainable real-time, high resolution, and quantitative X-ray imaging of nanoparticles within the lymphatics. Access to the X-ray imaging facility at the National Synchrotron Radiation Research Center (Hsinchu, Taiwan) has already

been granted. Preliminary studies will be performed at the SA ANFF facility (Ian Wark) using the flagship micro-CT imaging system (MicroXCT-400, Xradia). Anaesthetised rats will be injected intradermally at the top of the foot and the migration pattern of the nanoparticles will be imaged.

Building on the outcome of Aim #1 and on the team expertise in the design of nuclear imaging tracers, we will equip MRI contrast agents with technetium-99m (^{99m}Tc) as radiotracer. Iron oxide and gadolinium oxide nanoparticles will be used as T2 and T1 MRI contrast agents, respectively. Importantly gadolinium oxide also provides strong contrast in CT imaging, thereby enabling dual CT/MRI imaging. The lymphatic pattern of these multimodal imaging agents will be determined in rats as described above using nuclear imaging (RAH), microCT imaging (Ian Wark) and the 16.4T micro-imaging MRI system available through the National Imaging Facility (University of Queensland). The novel lymphotropic agents will be validated against ^{99m}Tc-antimony trisulfide.

Swine (40 kg) will be anaesthetised intramuscularly and subsequently intubated. 100 uL of the best multimodal tracer from Aim #2 will be injected into the submucosal layer of the lower oesophagus (n= 6). The migration of the lymphotropic nanoparticles from the injection site will be imaged in real time using the MRI system in the Large Animal Research & Imaging Facility (IMVS Veterinary Division, Gilles Plains). Identified lymph nodes will be harvested post-mortem using a gamma probe, and the concentration of nanoparticulate tracer will be measured. Other swine organs will also be harvested and analysed to determine the physiological distribution of nanoparticulate tracer. Ethical approval will be sought from the Royal Adelaide Hospital Animal Ethics Committee.

7.2.4 Proposed Research Team

The multi-institutional research team will be composed of Dr S Thompson (PhD candidate) and Prof G Jamieson (Surgery/University of Adelaide), Dr B Thierry and Prof T Nann (Ian Wark Research Institute/UniSA), and Dr C Tsopelas and Dr D Bartholomeusz (Nuclear Medicine/Royal Adelaide Hospital). All members of this collaboration have proven track records. This is a translational study with great potential for a subsequent clinical study with wider application (to other solid tumours). Following completion of the above project, the applicants plan to apply for a national project grant and, depending on the results, validation in a human oesophagus model will begin.

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