Predictive Factors in Esophageal Carcinoma

MARTIN DREILICH
Abstract

Esophageal carcinoma is a malignancy with a poor prognosis and is the sixth cause of cancer related death worldwide. In Sweden approximately 400 new cases are diagnosed every year. The aim of this present thesis was to investigate predictive factors for esophageal carcinoma patients. 126 esophageal carcinoma patients admitted to the department of Oncology at the University Hospital in Uppsala between 1990-2000 were investigated with focus on known and potential prognostic factors. Performance status and stage of the disease were the only independent prognostic factors (p-values <0.001).

Angiogenic factors VEGF and bFGF were correlated to platelet and leukocyte counts and VEGF was correlated to tumor volume (p=0.04) whereas bFGF was not (p=0.08) in pre-treatment serum samples from 42 esophageal carcinoma patients. The use of the angiogenic factors as prognostic factors, prior to therapy in patients with esophageal carcinoma, according to the results from the present study, seems limited.

HER-2 overexpression was seen in 17% of 97 investigated esophageal tumor samples. In squamous cell carcinoma patients, HER-2 overexpression correlated with poorer survival (p=0.035), whereas in adenocarcinoma patients, HER-2 status did not. HER-2 overexpression seems to be associated with poorer survival in esophageal carcinomas, especially in patients with squamous cell esophageal carcinoma.

Telomerase activity was detected in all esophageal cell lines, with a broad range of activity levels. No correlation was found between telomerase activity levels and sensitivity to investigated cytotoxic drugs. We therefore conclude that basal telomerase activity level is not a key determinant of sensitivity to standard cytotoxic drugs in esophageal carcinoma cell lines.

The virus HPV-16 was detected in 16% of the patients; no other type HPV was detected. HPV-16 infection had no significant effect on survival (p=0.72). Our results did not show that HPV-16 increases survival or improve therapy response in patients with esophageal carcinoma.

Keywords: Esophageal carcinoma, Survival, Prognosis, Angiogenesis, HER-2, Telomerase, FMCA, HPV

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Pessimisten klagar över blåsten

Optimisten räknar med att vinden ska vända

Realisten skotar seglen
List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


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## Abbreviations

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<tr>
<td>5-FU</td>
<td>5-Flourouracil (cytotoxic drug)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosintriophosphate</td>
</tr>
<tr>
<td>BFGF</td>
<td>basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>Cag-A</td>
<td>Cytotoxin associated gene A</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRT</td>
<td>Chemoradiotherapy</td>
</tr>
<tr>
<td>CT</td>
<td>Computer Tomography</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Validation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DPD</td>
<td>Dihydropyrimidine Dehydrogenase</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EUS</td>
<td>Endoscopic Ultrasound</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein Diacetate</td>
</tr>
<tr>
<td>FDG</td>
<td>18-Fluoro-Deoxy-Glucose</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast Growth Factor Receptor</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric Microculture Cytotoxic Assay</td>
</tr>
<tr>
<td>GERD</td>
<td>Gastro Esophageal Reflux Disease</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray (Unit for absorbed radiation dose)</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human Epidermal growth factor Receptor 2</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat Induced Epitope Retrieval</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>hTERT</td>
<td>human Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>HTR</td>
<td>human Telomerase RNA</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>KDa</td>
<td>kiloDalton (unit for molecular weight)</td>
</tr>
<tr>
<td>MAP-K</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi Drug Resistance</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metallo Proteinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>MRCOC</td>
<td>Medical Research Council Oesophageal Cancer working group</td>
</tr>
<tr>
<td>MRT</td>
<td>Magnetic Resonance Tomography</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidyl-Inositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced Folate Carrier</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RTOG</td>
<td>Radiation Oncology Group</td>
</tr>
<tr>
<td>SI</td>
<td>Survival Index</td>
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<tr>
<td>TNM</td>
<td>Tumor Node Metastasis</td>
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<tr>
<td>TRAP</td>
<td>Telomeric Repeat Amplification Protocol</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate Synthetase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
Introduction

Epidemiology
Esophageal carcinoma is a malignancy with a poor prognosis. It is the sixth cause of cancer-related death worldwide (2002) [1]. In the year 2002, 462,000 new cases were diagnosed globally, accounting for 4.2% of all cancer diagnoses [1]. The incidence of esophageal carcinoma is characterized by a large geographical variation [2]. Esophageal carcinoma is on average seven times more common in men than in women. The incidence rate is close to the prevalence rate, indicating a short overall survival time [3]. During the past three decades, the incidence of adenocarcinoma has increased in the United States and Europe as well as in Scandinavia [4]; while in the 1970s approximately 85% of all esophageal tumors were squamous cell carcinomas [5], today adenocarcinoma accounts for approximately 2/3 of all cases in the United States [5]. This histological shift is most evident among white men in the Western countries (especially in the United States); it is not seen amongst women. While squamous cell carcinoma is still the dominant histology in Sweden (and in Asia [6]), adenocarcinoma incidence has increased and now accounts for over 25% of all cases [7]. Improved detection methods for separating tumors originating in the gastro-esophageal junction into gastric tumors and esophageal tumors do not seem sufficient to explain the observed histological shift.

The Western world is considered to be a low incidence area for esophageal carcinoma; mortality rates seldom rise above 10/100,000 [2]. The highest mortality rates, exceeding 100/100,000, are seen in specific areas in China, South-East Asia, Persia, South Africa, and Chile [2]. In Sweden, approximately 400 new cases of esophageal carcinoma are diagnosed every year, with around three times as many cases among men as among women; the incidence rate is 7.3/100,000 for men and 2.0/100,000 for women [3]. Median age at time of diagnosis in Sweden is 72 years [3].

Pathology
The growth pattern of esophageal tumors is characterized by projection into the lumen, small ulcerating lesions, infiltrating tumors with growth in the esophageal wall, or a combination of all these features. Tumors projecting
into the esophageal lumen are more likely to cause obstruction whereas ulcerative tumors tend to bleed [8].

Adenocarcinoma and squamous cell carcinoma are the dominant histologies for esophageal carcinoma patients [9]. Small cell carcinoma is seen in approximately 1% of cases [10] and mukoepidermoid carcinoma in less than 1% [11]. Other uncommon histologies, seen only in rare cases, include carcinosarcoma, adenocystic carcinoma, carcinoid, malignant melanoma, oat cell carcinoma, spindle cell sarcoma, malignant lymphoma, and Kaposi's sarcoma [12].

Histological classification is based on morphological considerations and is ultimately determined by the microscopic appearance of the tumor cells. Resemblance to the tissue of origin determines whether a tumor is classified as adenocarcinoma or squamous cell carcinoma; glandular-like tumors are classified as adenocarcinoma whereas epithelial-like tumors are classified as squamous cell carcinoma. The tumor grade is a measure of the resemblance between the tumor and the original tissue and is based on several factors such as the shape of the cells and their nuclei, and the number of mitoses observed in the tumor. Tumors are graded as well-differentiated, moderately differentiated, poorly differentiated, or undifferentiated.

Barrett's esophagus is an acquired condition which often acts as a pre-malignant stage for esophageal adenocarcinoma [13]. It affects the lower third of the esophagus, and comprises a columnar metaplasia (replacement) of the normal squamous epithelium, often as a result of chronic gastro-esophageal reflux. This metaplasia is characterized by a glandular mucosa in the gastro-esophageal junction with either a circumferential growth pattern, or tongs of growth extending up into the esophagus, or both [14]. Three histological types of metaplasia can be distinguished: the junctional type, the fundic type, and the specialized intestinal-like mucosa. The histology seen in the junctional type is similar to that of the cardia whereas the fundic type resembles the histology found in the fundus.

It is only the specialized intestinal-like mucosa with typically goblet cells that has been associated with an increased risk of adenocarcinoma. The development into adenocarcinoma is evidenced by a series of morphological changes which are characterized by an increasing grade of cell dysplasia [15]. This dysplasia is divided into low-grade and high-grade dysplasia, depending on morphological criteria, and adenocarcinoma is defined as tumor cell invasion of the lamina propria. The vast majority of esophageal adenocarcinomas arise from Barrett’s esophagus, although some cases develop from submucosal glands or ectopic gastric epithelium [16].
No similar pre-malignant conditions have been identified for esophageal squamous cell carcinoma. Squamous cell carcinoma is believed to develop from the esophageal epithelial cells but is currently poorly understood, although knowledge regarding its molecular pathogenesis is accumulating [17].

Risk factors

Smoking and alcohol consumption are the dominant risk factors for squamous cell carcinoma of the esophagus, since they both cause chronic irritation and inflammation of the esophageal mucosa [9]. In a case-control study of current smokers, the risk of developing esophageal carcinoma increased with the number of cigarettes smoked. However, the risk of developing adenocarcinoma was significantly lower than that of developing squamous cell carcinoma [18].

Excess alcohol consumption is a major risk factor for developing squamous cell carcinoma but not an established risk factor for developing adenocarcinoma [18,19]. When heavy alcohol drinking is combined with tobacco smoking, the risk of esophageal cancer increases exponentially [20].

The increasing incidence of adenocarcinoma over the last three decades suggests a new etiologic factor for esophageal adenocarcinoma. It has been conjectured that the current epidemic of esophageal adenocarcinoma is best explained by the parallel epidemic of obesity [5]. Conversely, an obese person is at lower risk of developing squamous cell carcinoma. In a nationwide population based case-control study in Sweden, the body mass index (BMI) was used to determine the odds ratio (OR) and estimate the relative risk for developing esophageal adenocarcinoma. The authors reported a strong association between adenocarcinoma and BMI among subjects with BMI>22 (OR=7.6). The association became even stronger when only obese subjects (BMI>30) were included (OR=16.2) [21].

Long-standing gastro-esophageal reflux disease (GERD) is an important risk factor for Barrett’s esophagus [22]. Anti-cholinergic medication and other medication that lowers the distal esophageal sphincter tonus may increase the risk of GERD and esophageal adenocarcinoma [23]. Several risk factors for esophageal adenocarcinoma, such as obesity and smoking, are also true risk factors for gastro-esophageal reflux. Investigations into the causal connection between obesity and reflux symptoms have not demonstrated any relationship [21,24,25]. Further, the acid juice from the stomach has been shown to be an risk factor for Barrett’s metaplasia [26] whereas infection with Helicobacter pylori has been postulated to reduce the risk for developing esophageal adenocarcinoma [27]. It has also been suggested that certain bacterial strains carrying the specific cytotoxin-associated gene A (Cag-A) have a protective role against adenocarcinoma [28].
Low socioeconomic status is a concept that covers factors such as smoking and alcohol consumption but also includes nutritional condition, oral health, and general sanitation. Low socioeconomic status has been identified as a risk factor in high incidence areas of esophageal carcinoma. In some of the high incidence areas of squamous cell carcinoma where smoking or alcohol consumption is less common, dietary factors as well as oral health and certain virus infections have been suggested as underlying causes for esophageal carcinoma. The histological shift towards increased incidence of adenocarcinoma is not seen in these high incidence areas. In case-control studies from China, a high intake of vegetables and fruit was associated with lower risk while broiled meat was associated with an increased risk for esophageal carcinoma [29,30]. Results from a case-control adenocarcinoma study performed in a low incidence area showed the same protective pattern as in high incidence areas concerning nutrients from plant-based food and an increased risk from animal-derived food, particularly animal fat [31]. Although several dietary risk factors were identified in the Chinese studies, the protective role of plant-derived food seems to have a larger penetrating effect than in similar studies performed in low incidence countries [32,33].

The role of human papilloma virus (HPV) infection as a risk factor for developing esophageal tumors is another area under investigation. High-risk HPV type infections are present in approximately 15% of patients with esophageal carcinomas [2]; however, the incidence among esophageal carcinoma patients varies between different geographical areas. It has been postulated that areas with high incidence of esophageal carcinoma also have high detection rates of HPV, but these data are also to some extent in conjunction with socioeconomic status [2,34].

Diagnosis

Symptoms

At the time of esophageal carcinoma diagnosis, approximately 70% of patients experience dysphagia and 20% feel pain when swallowing food or beverages. Besides swallowing problems, weight loss is frequently seen in patients with esophageal carcinoma [9]. Although long-standing gastro-esophageal reflux is often seen in this group of patients, this is a nonspecific sign of esophageal carcinoma [35].

Since the esophagus and the trachea are located closely together in the mediastinal compartment, tumors infiltrating into the trachea might cause fistulas to the airways. Cough can thus be a symptom of an infiltrating esophageal tumor, and fistulas between the esophagus and the trachea might cause dyspnea and aspiration pneumonia [36].
Diagnostic investigations

When an esophageal tumor is suspected, the initial investigation comprises either an esophagoscopy with contingency to take biopsy excisions for pathological examination, or a barium swallow aimed at detecting either strictures or ulcerations of the esophagus, or both. Further pre-treatment evaluation is generally performed with a computer tomography (CT) scan, magnetic resonance tomography (MRT), or endoscopic ultrasound (EUS) to determine tumor extension [37]. The presence of distant metastasis in the abdomen is determined by a CT scan or ultrasound or both [37]. However, standard imaging methods cannot predict the presence of local lymph node involvement with sufficient accuracy.

Pathological examinations of the tumor biopsy confirm the histology of the tumor and determine the grade of differentiation.

As well as assessment of the tumor extension and pathology, an evaluation of the patient’s performance status and general medical condition is performed.

Staging

Esophageal carcinoma is classified according to the tumor-node-metastasis (TNM) system of the 2002 American Joint Committee on Cancer, a classification system for characterizing the primary tumor, regional nodal status, and distant metastasis [38]. The definitions of the TNM classification system and the different pathological and clinical criteria for esophageal carcinoma are shown in Table 1a.

Tis is defined as in situ tumor growth in the esophageal wall. T1 tumors invade the mucosa, T2 tumors invade the muscularis propria, T3 tumors grow into the adventitia, and T4 tumors invade adjacent organs. Table 1b provides further grouping of esophageal carcinomas in terms of tumor size, nodal involvement, and distant metastases.
Table 1a. TNM classification.

<table>
<thead>
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<th>Primary Tumor</th>
<th>Pathology</th>
<th>CT/MR</th>
<th>EUS</th>
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<tbody>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
<td>Unable to differentiate</td>
<td>Unable to differentiate</td>
</tr>
<tr>
<td>T1</td>
<td>Invades submucosa</td>
<td>Wall thickness &gt;5-10 mm</td>
<td>Invasion into the first three layers</td>
</tr>
<tr>
<td>T2</td>
<td>Invades muscularis propria</td>
<td>Unable to differentiate</td>
<td>Invasion into the fourth layer</td>
</tr>
<tr>
<td>T3</td>
<td>Invades adventitia</td>
<td>Wall thickness &gt;10 mm</td>
<td>Invasion into the fifth layer</td>
</tr>
<tr>
<td>T4</td>
<td>Invades adjacent structures</td>
<td>Invasion into adjacent organs</td>
<td>Invasion into adjacent organs</td>
</tr>
</tbody>
</table>

Regional Lymph node

| N0 | No lymph node involvement | - | - |
| N1 | Regional lymph node involvement | Regional lymph node >10 mm | Regional lymph node >10 mm |

Distant Metastasis

| M0 | None present | - | - |
| M1a| Cervical or celiac lymph node involvement | Celiac lymph node >5 mm | Unable to differentiate |
| M1b| Distant metastasis | Metastasis in other organs | |

Table 1b. Stages of disease.

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>I</td>
<td>T1, T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>II a</td>
<td>T1, T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>II b</td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>III</td>
<td>T4</td>
<td>N</td>
<td>M0</td>
</tr>
<tr>
<td>IV a</td>
<td>T</td>
<td>N</td>
<td>M1a</td>
</tr>
<tr>
<td>IV b</td>
<td>T</td>
<td>N</td>
<td>M1b</td>
</tr>
</tbody>
</table>
T stage

T stage (tumor depth or size) can be determined to an accuracy of 25–30% with barium swallow or endoscope [39]. Barium swallowing has the advantage over other imaging methods of also revealing the functional status of the swallowing act. Endoscopic ultrasound (EUS) has been demonstrated as perhaps the most accurate non invasive technique for preoperative loco-regional staging of esophageal tumors [40]. EUS enables determination of the five anatomical layers of esophagus [41]. The reported accuracy of EUS for determination of T stage is approximately 90%, and even higher for T3 disease [42]. The limitations of EUS are due firstly to the size of the transducer (30% of all examinations are precluded by narrow conditions) and secondly to the fact that the investigation is dependent on the skill of the operator [43].

Computer tomography and magnetic resonance imaging are comparable imaging methods for T stage determination in esophageal carcinoma [44]. They have better accuracy than barium swallowing for T stage determination, but lower accuracy than EUS [43]. The technologies behind MRI and CT are undergoing rapid improvement, resulting in increased resolution, and it is possible that in the near future both CT and MRI will be capable of determining T stage as accurately as EUS does today [45,46].

Lymph nodes

Nodes larger than 1 cm detected with MRI, CT, or EUS are considered to be malignant [47,48]. In one study, only 3% of nodes with a size less than 5 mm and 8% of nodes sized between 5 and 10 mm were found to be malignant [49]. EUS seems to be better than CT for determining local node involvement; EUS reaches accuracy in about 80% of cases, while the sensitivity of CT for local node involvement is 60% [42,47]. MRI as a method for determining nodal status in esophageal carcinoma has been poorly investigated, but reports indicate a similar sensitivity to that of CT imaging [50-52].

Celiac lymph nodes are one of the most common sites for distant metastases from esophageal carcinoma. Metastasis to celiac nodes (M1a) can be investigated with CT, MRI, EUS, or positron emission tomography (PET). PET is based on differences in cellular metabolism and not anatomical abnormalities as in conventional image diagnostic technology. Administered radio-labeled 18-F-fluoro-deoxy-D-glucose (FDG) is preferentially taken up by cells with a high glucose use, such as tumor cells, and is then trapped inside the tumor cells due to limitations in glucose metabolism. Normal cells do not have this deficient metabolism; FDG is trapped only in tumor cells and can then be detected by positron emission [53]. Metastasis to other locations (M1b) can be detected by the same radiological methods as for other malignancies, i.e., CT, MRI, ultrasonography, or PET. Reliability tests using
CT and EUS for M1a show similar results, with a sensitivity rate of about 65%. Concerning distant metastasis, FDG-PET has been found to have superior accuracy compared to the combination of CT and EUS in diagnosing stage IV disease [54]. Further, PET scanning has proved capable of detecting additional metastatic spreading in 15% of patients diagnosed with CT as having only localized disease, [55].

Treatment

Surgery

Management of esophageal carcinoma is based on tumor extent according to the TNM classification and is divided into curative and palliative treatment. Patients with loco-regional disease (Stage I-II), in good medical condition, are often offered curative treatment. Surgery still remains the first choice for patients with early stage disease, and is the standard with which all other treatment regimes are compared. Commonly used techniques for resection of localized esophageal carcinoma are the transthoracic and right transthoracic approaches [56]. There are no significant differences in survival or operative mortality between the two types of surgery [57]. Although surgical techniques and postoperative care have improved, reported operative mortality rates are still as high as 4–10% [9].

In surgical series of esophageal carcinoma patients, presence of lymph nodes are detected by the pathologist in approximately half of all cases [58]. In a study by von Rahden et al., lymphatic vessel invasion was found in 11.6% of T1 tumors and in over half of all T2 and T3 tumors [59]. This might explain the high incidence of relapse for this patient category.

The number of malignant lymph nodes involved has also been subject to prognostic investigation [60]. Xiao et al. reported that three or more malignant lymph nodes were associated with worse survival in comparison with patients with no malignant lymph nodes. However, the same authors also reported that there were no significant differences in terms of survival between patients with no malignant lymph nodes and those with one [61].

Esophageal carcinoma with malignant lymph node involvement has very low survival rates but extensive lymph node resection does not seem to add any survival benefit. The incidence of perioperative complications such as infections, anastomosis leakage, and pulmonary complications is considerable for both of the main surgical methods; the rate of such complications has been reported as 26–41% [9]. These complications might explain the dismal results of extensive lymph node resection.
In the hope of improving survival rates, combined treatment modalities including radiotherapy and chemotherapy in different settings together with surgery have been investigated in several randomized clinical trials.

Table 2a presents a selection of randomized clinical trials comparing survival following pre-operative radiation therapy versus surgery alone. In the studies using pre-operative radiotherapy, radiation treatment was delivered with doses ranging from 20-90 Gy. No statistically-significant survival differences were detected in either of the studies. Further, a meta-analysis of five randomized trials investigating pre-operative versus surgery alone has concluded that there might be a small benefit for the pre-operative treatment arm [62]. Regarding pre-operative versus post-operative radiotherapy, one randomized study has been performed without finding any survival differences.
**Table 2a. Randomized clinical trials of preoperative radiation and surgery versus surgery alone.**

<table>
<thead>
<tr>
<th>No of patients</th>
<th>Intervention</th>
<th>Median survival (months)</th>
<th>5-year survival (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Launois et al. 1981 [63]</td>
<td>67 Pre-op radiation 64-90 Gy + esophagectomy</td>
<td>4.5</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>57 Esophagectomy</td>
<td>8.2</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gignoux et al. 1987 [64]</td>
<td>115 Pre-op radiation 33 Gy + esophagectomy</td>
<td>12.3</td>
<td>10</td>
<td>p=0.94</td>
</tr>
<tr>
<td>114 Esophagectomy</td>
<td>12</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang et al. 1989 [65]</td>
<td>104 Pre-op radiation 40 Gy + esophagectomy</td>
<td>ND</td>
<td>35</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>102 Esophagectomy</td>
<td>ND</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nygaard et al. 1992** [66]</td>
<td>58 Pre-op radiation 35 Gy + esophagectomy</td>
<td>10</td>
<td>21*</td>
<td>p=0.08</td>
</tr>
<tr>
<td>50 Esophagectomy</td>
<td>7</td>
<td>9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arnott et al. 1992 [67]</td>
<td>90 Pre-op radiation 20 Gy + esophagectomy</td>
<td>8</td>
<td>9</td>
<td>p=0.40</td>
</tr>
<tr>
<td>86 Esophagectomy</td>
<td>8</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fok et al. 1994** [68]</td>
<td>40 Pre-op radiation 24-53 Gy + esophagectomy</td>
<td>11</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>39 Esophagectomy</td>
<td>22</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Three-year survival; ** = Randomized in four groups, data from radiation and surgery versus surgery; ND = not determined
Three meta-analyses have been performed on pooled data from trials evaluating pre-operative chemotherapy versus surgery alone. None of these could find any survival differences over the first three years. However, in a study including 5-year survival in the analysis, a survival benefit (p=0.02) was detected for the pre-operative chemotherapy group [69]. Table 2b presents survival rates for a compilation of randomized phase II trials concerning pre-operative chemotherapy versus surgery alone.

Data from a study evaluating pre-operative with additional post-operative chemotherapy versus surgery alone could not detect survival advantages for either group (p=0.34) [70]. None of the studies evaluating post-operative cisplatin-based chemotherapy versus surgery alone could identify survival improvement for patients with complete resection [71].

A multicenter phase III randomized trial performed by Kelsen et al. reported no difference in survival rates between patients receiving pre-operative chemotherapy compared to surgery alone [72]. However, a phase III trial performed by the Medical Research Council Oesophageal Cancer working party (MRCOC) to compare pre-operative chemotherapy with surgery alone, with 400 patients included in each treatment arm, reported better survival rates for the pre-operative chemotherapy arm than the arm treated only with surgery [73]. These conflicting results have not been fully explained, but it should be noted that the chemotherapy regimes differed in dose and treatment time.
Table 2b. Randomized clinical trials of preoperative chemotherapy versus surgery alone.

<table>
<thead>
<tr>
<th>No of patients</th>
<th>Intervention</th>
<th>Median survival (months)</th>
<th>5-year survival (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nygaard et al. 1992** [66]</td>
<td>56 Cisplatin + Bleomycin + esophagectomy</td>
<td>7</td>
<td>3*</td>
<td>ND</td>
</tr>
<tr>
<td>50 Esophagectomy</td>
<td></td>
<td>7</td>
<td>9*</td>
<td></td>
</tr>
<tr>
<td>Schlag et al. 1992 [74]</td>
<td>22 Cisplatin + 5-FU + esophagectomy</td>
<td>7.5</td>
<td>ND</td>
<td>p=0.91</td>
</tr>
<tr>
<td>24 Esophagectomy</td>
<td></td>
<td>5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Maipang et al. 1994 [75]</td>
<td>24 Cisplatin + Bleomycin + Vinblastine + esophagectomy</td>
<td>17</td>
<td>31*</td>
<td>p=0.186</td>
</tr>
<tr>
<td>22 Esophagectomy</td>
<td></td>
<td>17</td>
<td>36*</td>
<td></td>
</tr>
<tr>
<td>Law et al. 1997 [76]</td>
<td>74 Cisplatin + 5-FU + esophagectomy</td>
<td>16.8</td>
<td>38*</td>
<td>p=0.17</td>
</tr>
<tr>
<td>73 Esophagectomy</td>
<td></td>
<td>13</td>
<td>14*</td>
<td></td>
</tr>
<tr>
<td>Kok et al. 1997 [77]</td>
<td>74 Cisplatin + Etoposide + esophagectomy</td>
<td>18.5</td>
<td>ND</td>
<td>p=0.002</td>
</tr>
<tr>
<td>74 Esophagectomy</td>
<td></td>
<td>11</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Ancona et al. 2001 [78]</td>
<td>47 Cisplatin + 5-FU + esophagectomy</td>
<td>25</td>
<td>34</td>
<td>ND</td>
</tr>
<tr>
<td>47 Esophagectomy</td>
<td></td>
<td>24</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

* = Three-year survival; ND = not determined
Several randomized trials have compared pre-operative treatment including both chemotherapy and radiation therapy with surgery alone. One study of 58 patients performed by Walsh et al. reported improved survival for patients treated with pre-operative chemo-radiation therapy [79]. **Table 2c** summarizes some results from these randomized trials of pre-operative chemo-radiation.

**Table 2c. Randomized clinical trials of preoperative chemo-radiation (CRT) and surgery versus surgery alone.**

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Intervention</th>
<th>Median survival (months)</th>
<th>5-year survival (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nygaard et al. 1992** [66]</td>
<td>53 Cisplatin + Bleomycin + 35 Gy</td>
<td>7</td>
<td>17*</td>
<td>p=0.30</td>
</tr>
<tr>
<td></td>
<td>50 Esophagectomy</td>
<td>7</td>
<td>9*</td>
<td></td>
</tr>
<tr>
<td>Le Prise et al. 1994 [80]</td>
<td>41 Cisplatin + 5-FU + 20 Gy</td>
<td>11</td>
<td>19*</td>
<td>p=0.56</td>
</tr>
<tr>
<td></td>
<td>45 Esophagectomy</td>
<td>11</td>
<td>14*</td>
<td></td>
</tr>
<tr>
<td>Apinop et al. 1994 [81]</td>
<td>35 Cisplatin + 5-FU + 40 Gy</td>
<td>9.7</td>
<td>24</td>
<td>p=0.40</td>
</tr>
<tr>
<td></td>
<td>34 Esophagectomy</td>
<td>7.4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Walsh et al. 1996 [79]</td>
<td>58 Cisplatin + 5-FU + 40 Gy</td>
<td>16</td>
<td>32*</td>
<td>p=0.001</td>
</tr>
<tr>
<td></td>
<td>55 Esophagectomy</td>
<td>11</td>
<td>6*</td>
<td></td>
</tr>
<tr>
<td>Bosset et al. 1997 [82]</td>
<td>143 Cisplatin + 37 Gy</td>
<td>18.6</td>
<td>33</td>
<td>p=0.78</td>
</tr>
<tr>
<td></td>
<td>139 Esophagectomy</td>
<td>18.6</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Urba et al. 2001 [83]</td>
<td>50 Cisplatin + Vinblastine + 5-FU + 45 Gy</td>
<td>17.6</td>
<td>30*</td>
<td>p=0.15</td>
</tr>
<tr>
<td></td>
<td>50 Esophagectomy</td>
<td>16.9</td>
<td>13*</td>
<td></td>
</tr>
<tr>
<td>Burmeister et al. 2002 [84]</td>
<td>128 Cisplatin + 5-FU + 35 Gy</td>
<td>22</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>128 Esophagectomy</td>
<td>19</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Lee et al. 2004 [85]</td>
<td>52 Cisplatin + 5-FU + 45.6 Gy</td>
<td>28.2</td>
<td>ND</td>
<td>p=0.67</td>
</tr>
<tr>
<td></td>
<td>50 Esophagectomy</td>
<td>27.3</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* = Three-year survival; ND = not determined
Radiation

Curatively-intended radiation therapy can be performed as conventional external radiotherapy, as intra-luminal brachytherapy, or in combination. External radiation is usually given in fractions of 1.8–2.0 Gy five times a week until the final dose of 40–70 Gy is reached. Radiotherapy is given with a 5 cm margin both distal and proximal of the tumor; a 3–5 cm margin is used in the transversal plane depending on nodal involvement. The dose-limiting structures surrounding the esophagus are the spinal cord (45 Gy), the pericardium (40 Gy), and the lungs (20 Gy) [86]. Studies have shown a correlation between the total radiation dose and the median survival time [87].

Brachytherapy has the advantage of delivering the radiation dose to a limited area, thus protecting the dose-limiting structures as well as delivering a much higher total dose of radiation to the tumor (>100 Gy). Two studies have investigated overall survival rates following brachytherapy in combination with external radiation or chemoradiation therapy, but found no survival advantage for patients receiving treatment according to the experimental arm [88,89]. However, a study by Okawa et al. of patients at T-stage T1 and T2 demonstrated a trend (p=0.088) in the brachytherapy group toward better survival; and in tumors less than 5 cm, the survival benefit was significant (p=0.025) [89]. Further, in a study including only patients with superficial esophageal tumors, a significantly improved survival was reported for the group that received a boost of intraluminal brachytherapy instead of a continued external radiation schedule [90].

In a study comparing a conventional fraction schedule (1.7–2.0 Gy/day, five times/week) with a hyperfractionated scheme comprising 2.0 Gy and 1.2 Gy (field-in-field), or 1.5 Gy and 1.5 Gy at five to six hour intervals, a trend (p=0.07) towards better survival was observed for the hyperfractionation schedules [91].

Only one randomized trial has investigated radiation alone versus surgery in patients with localized tumors, and comparable survival rates were reported [92]. Earlam et al. began a prospective randomized trial to investigate survival rates between surgery and radiotherapy alone in patients with resectable squamous cell carcinoma, but the study was discontinued after 18 months due to poor recruitment [93].

Chemotherapy

Cisplatin in combination with 5-fluorouracil (5-FU) is considered the standard cytotoxic drug combination for esophageal carcinoma. The response rate for cisplatin as a single agent is approximately 20% [94,95]. The combination of cisplatin and continuous-infusion 5-FU has shown a response rate ranging from 35% to 65% [96,97]. In a phase II trial, the combination of
Paclitaxel and Cisplatin has been reported to have an response rate of 40%, and only 10% of the study subjects required hospitalization due to treatment related complications [98]. Illson et al. reported a 36% response rate to irinotecan and cisplatin; the major toxicity was grade 3/4 neutopenia, which was reported in 22% of subjects [99,100].

Despite these dismal survival rates for patients with esophageal carcinoma, there are no phase III studies regarding chemotherapy treatment. In a phase I study of esophageal carcinoma, trastuzumab was combined with paclitaxel, cisplatin, and radiation to explore the toxicity profile; however, so far, no randomized clinical trial has investigated whether trastuzumab is effective in HER-2 overexpressing esophageal tumors [101]. Although studies of new combinations of chemotherapeutics have reported higher response rates, the survival rates for patients with advanced disease remain unchanged.

Chemoradiotherapy

Reasons for not operating on patients with loco-regional disease include patient refusal, poor medical condition, and technically-inoperable tumors. Instead of surgery, such patients are offered treatment with chemotherapy and concurrent radiotherapy, with curative intent. One of the studies behind this treatment approach was published in 1992 by Herskovic et al. [102]. 121 patients were randomized between radiation treatment alone (a total dose of 64 Gy) and two courses of concomitant chemotherapy (with a total radiation dose of 50 Gy) followed by additional two courses of chemotherapy. The chemotherapy consisted of cisplatin and 5-FU and was administrated on days 1 and 4 of a four-week schedule, concomitant with radiotherapy, and then every three weeks after radiation. In the experimental arm, the median survival was 14.2 months and the five-year survival 27%. When compared with the radiation treatment alone (where no patients were alive after five years) a highly statistically-significant survival advantage was found for the chemoradiotherapy arm (p<0.0001) [102]. However, toxicity was considerably higher in the combined treatment arm, with severe side effects reported in 44% of the patients compared with 25% for patients that received radiotherapy alone.

This issue of concurrent chemoradiotherapy has been further studied in a meta-analysis by Wong, in which the author concluded that concurrent chemoradiotherapy for localized esophageal carcinoma produces better survival rates than for radiotherapy alone, but that there is a significant increase in adverse effects including life-threatening toxicities [103]. Zhang et al. report that esophageal carcinoma patients receiving concurrent chemoradiotherapy in a total dose of 54–64.8 Gy had a significantly better loco-regional tumor control and prolonged survival compared to patients receiving less than 51 Gy [87]. Abitbol et al. investigated paclitaxel in combination
with 5-FU and cisplatin, concomitant with radiation treatment, and reported a 70% complete response; although toxicity was considerable, and eight patients were hospitalized for acute complications, 81% of the patients had restored swallowing function one year after treatment [104].

In a randomized trial comparing chemoradiation alone with pre-operative chemoradiation in patients with advanced loco-regional squamous cell carcinoma, patients responding to chemoradiation did not benefit from additional surgery [105]. Further, treatment-related mortality was significantly higher for pre-operative treatment group than in the chemoradiation alone group. In another study, by Bedenne et al., patients with major response from chemoradiotherapy treatment were randomized to surgery versus no surgery and again no survival benefit was found for patients that underwent additional surgery [106].

**Palliative treatment**

The goals for palliative treatment in esophageal carcinoma are relief of dysphagia, maintenance of nutrition, and — as for other terminal cancer patients — acceptable quality of life. The majority of patients diagnosed with advanced disease have an expected survival time of less than one year. Management methods for advanced disease include radiotherapy, brachytherapy, chemotherapeutic drugs, and dilatation techniques for maintaining the esophageal lumen.

Patients with malignant dysphagia are often treated with endoscopic palliative treatment modalities aimed at improving swallowing function, such as balloon dilation, expandable metallic stents, and laser treatment [107]. The usage of dilation tools as balloons has been shown to temporarily restore the esophageal lumen, and is often used as a prelude to the application of a stent [108]. Metallic stents have been reported to be more effective than external radiotherapy and chemotherapy in terms of an immediate effect for patients with malignant dysphagia [109]. Adverse effects associated with stents are tumor overgrowth and migration of the stent, and are reported in approximately 15% of cases [110].

Radiotherapy as palliative treatment is used for relieving dysphagia [111]. Swallowing ability is improved in the majority of patients treated with radiotherapy; the improvement has been reported to have a duration of approximately 10 months [112]. Intraluminal brachytherapy is reported to give relief from dysphagia to the same extent as externally delivered radiation [113]. However, intraluminal brachytherapy delivered as a booster following external radiotherapy has not been shown to result in improved symptom relief [114,115]. While stent placement provides faster dysphagia relief than brachytherapy, it is less effective in the long term [116]. Further, well-being and swallowing function seem to be better for patients receiving brachytherapy [117].
Patients with advanced disease are in most cases older, and have attenuated performance status that limits their tolerance for cytotoxic drugs. The use of single agent chemotherapy is generally only considered in the palliative setting to reduce unfavorable side effects and toxicity. Response rates for single agent chemotherapy are poor, with a brief clinical effect lasting only a few months; however, quality of life improvements have been demonstrated for patients in this setting [96]. Chemotherapeutic drugs administered as palliative therapy for metastatic disease include cisplatin, vindesine, and paclitaxel [118].
Prognosis and Prediction

Prognostic factors are those that predict the survival time and/or the prognosis of the disease. Predictive factors are defined as any factor that can predict the outcome of an intervention or a certain event.

There are several approaches to identifying prognostic or predictive factors, and they can be divided into clinical markers and biological markers. Clinical markers include the patient’s performance status, weight loss, and stage of disease. Worsened performance status and weight loss (>10% of body mass index) have been reported as indicators of poor prognosis [119].

Biological markers can be further subclassified depending on whether they can be detected in the tumor tissue or in the blood. Table 3 presents a compilation of recently published studies concerning prognostic factors for patients with esophageal carcinoma, divided into two parts: (3a) serological prognostic factors and (3b) prognostic factors comprising protein expression in tumor tissue.
<table>
<thead>
<tr>
<th>Serological factor</th>
<th>No of patients in study</th>
<th>Reference</th>
<th>p-value</th>
<th>Relation to prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>67 [120]</td>
<td>p=0.061</td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td></td>
<td>356 [121]</td>
<td>p=0.0285</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 [122]</td>
<td>p=0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>258 [123]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>262 [124]</td>
<td>p=0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>330 [125]</td>
<td>p=0.0061</td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 [126]</td>
<td>p&lt;0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>309 [127]</td>
<td>p=0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEA</td>
<td>330 [125]</td>
<td></td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 [126]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>215 [127]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>74 [128]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>90 [129]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53-abs</td>
<td>258 [123]</td>
<td></td>
<td>Associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td></td>
<td>105 [130]</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>34 [131]</td>
<td>p=0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>42 [132]</td>
<td>p=0.66</td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>IL-12, -18</td>
<td>15 [133]</td>
<td></td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>IL-2 receptor alpha</td>
<td>125 [134]</td>
<td>p=0.0209</td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>80 [135]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trombocytes</td>
<td>374 [136]</td>
<td>p=0.009</td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td>Microsatellite DNA</td>
<td>28 [137]</td>
<td></td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>Midkine</td>
<td>93 [138]</td>
<td></td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td>IAP</td>
<td>115 [139]</td>
<td></td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
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<tr>
<td>E-selectin</td>
<td>135 [140]</td>
<td>p=0.065</td>
<td>Elevated levels showed a trend towards poorer survival</td>
<td></td>
</tr>
<tr>
<td>PDGF</td>
<td>153 [141]</td>
<td></td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>ICTP</td>
<td>50 [142]</td>
<td></td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>96 [143]</td>
<td>p=0.001</td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>[144]</td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
<td>-------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td>BFGF</td>
<td>42</td>
<td>[144]</td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>Cystatin C</td>
<td>42</td>
<td>[144]</td>
<td>No relationship with survival</td>
<td></td>
</tr>
<tr>
<td>CYFRA 21-1</td>
<td>50</td>
<td>[145]</td>
<td>Elevated levels associated with poorer survival</td>
<td></td>
</tr>
</tbody>
</table>
### Table 3b.

<table>
<thead>
<tr>
<th>Apoptosis-related Proteins</th>
<th>No of patients in study</th>
<th>Reference</th>
<th>p-value</th>
<th>Relation to prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 [146]</td>
<td></td>
<td></td>
<td>No relationship with survival</td>
</tr>
<tr>
<td></td>
<td>76 [147]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>53 [148]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 [149]</td>
<td>p&lt;0.03</td>
<td></td>
<td>Expression associated with worse survival</td>
</tr>
<tr>
<td>Bax</td>
<td></td>
<td></td>
<td></td>
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Angiogenesis

The development of new blood vessels from the existing vascular bed is known as angiogenesis. Although angiogenesis is normally under tight regulatory control [179], the balance between pro-angiogenic and anti-angiogenic growth factors can be altered by pathological stimuli such as hypoxia, hypoglycemia, and genetic alterations, or as a response to secreted proteins in the microenvironment [180]. Physiological angiogenesis occurs during fetal development as well as during the female reproductive cycle [181].

Pathological angiogenesis is the result of excessive neo-vascularization. It has been postulated that tumors depend on angiogenesis to be able to grow beyond 2 mm³ in size [182]. The process of angiogenesis is dependent on a process called the angiogenic switch, an event divided into two phases: the pre-vascular phase and the vascular phase [179]. During the pre-vascular phase, tumor cells undergo phenotypic changes which then induce phenotypic changes in the endothelial cells, making them more susceptible to pro-angiogenic factors. During the vascular phase, the production of pro-angiogenic factors stimulates the endothelial cells into further and continuous neo-vascularization [179]. Figure 1 shows a simplified illustration of the events which occur during the angiogenic process. (Adopted after an illustration provided by Roche.)

Figure 1
VEGF

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the most thoroughly-studied pro-angiogenic growth factors, while well-known anti-angiogenic factors include interferon alpha, beta, and gamma, and angiostatin [183].

VEGF is expressed in tumor cells, T cells, and macrophages as well as in astrocytes and keratinocytes [184]. The VEGF gene is located on the short arm of chromosome 6 and its coding region is composed of eight exons [185,186]. VEGF regulates vascular permeability and proliferation and is also believed to interfere with apoptosis, thus providing anti-apoptosis protection to endothelial cells [187]. VEGF also activates cell surface receptor tyrosine kinases. The VEGF receptor family consists of VEGFR-1 and VEGFR-2, which mediate growth factor signals for blood vascular endothelial cells, and VEGFR-3, which mainly regulates lymphatic endothelial cells. Expression of VEGFR-1 and VEGFR-2 is mainly restricted to the vascular endothelium [188].

bFGF

Fibroblast growth factors (FGF) are one of the largest families of growth and differentiation factors for cells with mesodermal and neuroectodermal origin [189,190]. Fibroblast growth factors have been shown to be mitogenic for many different cell types, being involved in inflammatory processes, hematopoiesis, and wound healing, as well as angiogenesis [191]. The FGF family is basically comprised of two prototypic members, basic fibroblast growth factor and acidic fibroblast growth factor, but another 21 related polypeptide growth factors have also been identified [191]. These isoforms mediate their signals through four distinct tyrosine kinase receptors, FGFR1-4 [192]. The bFGF gene is localized on the short arm of chromosome 4 and has gained most attention for its angiogenic potential [193].

Cystatin C

Cystatin C is a 13 kDa basic protein. It belongs to the cystatin super-family of cystein protease inhibitors [194]. Cystatin C is synthesized at a constant rate by all nucleated cells and is mainly found in extra-cellular fluids such as blood. It is freely filtered in the glomeruli and has been shown to be an accurate marker for glomerular filtration rate [195].

Protease inhibitors are believed to play a physiological role in the regulation of proteases that are secreted or leaked from lysosomes during cell death processes such as apoptosis or necrosis; this feature links cystatin C to carcinogenesis and tumor progression [196]. Protease degradation of the extra-
cellular matrix is believed to facilitate tumor growth and invasion into the surrounding tissue and vasculature [197].

HER-2

The human epidermal growth factor receptor (HER) family is comprised of four distinct monomer transmembrane receptors: HER-1/EGFR, HER-2, HER-3, and HER-4 [198]. These receptors have an extracellular ligand binding domain, a transmembrane region, and, except for HER-3, an intracellular domain with tyrosine kinase activity [199]. The HER-2 gene is located on chromosome 17q and is involved in physiological events during normal conditions as well as in malignant progression in several human cancers [199].

Several endogenous ligands specific to HER have been discovered; they are divided into subgroups according to their binding specificity [200]. During activation of HER receptors, defined ligands bind to the extracellular domain, which prompts the inactive monomers to either homodimerize or heterodimerize with an HER family member [200]. Activation of the intracellular tyrosine kinase occurs after dimerization of the receptors and leads to phosphorylation, which activates intracellular residues [200]. Intracellular signal molecules are recruited and activated by the intracellular residue on the HER receptor [201]. The phosphorylated HER receptor recruits signal molecules and starts downstream signaling including the MAP-K and PI-3K/AKT pathways. Activation of MAP-K and the PI-3K/AKT pathways eventually leads to proliferation, angiogenesis, altered cell-cell interactions, increased cell motility, metastases, and resistance to apoptosis in the malignant cell [202]. Figure 2 provides a simplified illustration of the HER-2 signaling pathways.

Several molecular mechanisms have been described to explain how HER receptors are constitutively activated in cancer cells, including overexpression of the receptor, mutations, and the induction of autocrine loops [203]. HER-2 overexpression has been shown to be necessary for maintenance of cellular proliferation of tumor cells [204]. The aggressive malignant cell proliferation seen in HER-2 overexpressing tumors is believed to depend on increased cyclin D1 overcoming the cell cycle G1 block [205].

Targeting the HER-2 receptor in malignant cells has resulted in the inhibition of its downstream signaling pathways. Blocking the HER-2 receptor decreases the mitogenic signaling and also Myc activation in the PI-3K/Akt pathway that eventually leads to downregulation of cyclin D and G1 arrest [206]. Further, HER-2 blocking also leads to inhibition of PKB in the PI-3K pathway, and enables redistribution of p27Kip from the cytoplasm into the nucleus where it forms a complex with cyclin E/cdk2 and a block to the G1 phase [206].
Telomerase

The ends of chromosomes are formed from highly specialized structures known as telomeres. Telomeres are repetitive, non-coding DNA sequences (5'-TTAGGG-3') ranging in size from 5 to 20 kb. They protect the chromosomes against damage and degradation by ligases and exonucleases [207].

Telomeres in somatic cells become progressively shorter at each cell division due to the inability of the DNA polymerase complex to replicate the extreme end of the DNA strand. The telomere length may function as a mitotic clock, “counting” each cell division, and a telomere becoming critically short may be a signal for an exit out of the cell cycle into senescence [208]. In human cells, the predominant check on telomere loss is telomerase, which is normally only present in germ cells, certain stem cells, and resting lymphocytes [209].

Telomerase is a large ribonucleoprotein complex that maintains telomere length and compensates for telomere sequences lost during replication by using an intrinsic RNA component as a template for polymerization [210]. Telomerase activation inhibits telomere shortening, thereby preventing the cell cycle arrest and apoptosis that would otherwise be activated by shortened telomeres or chromosomal rearrangement [209]. Telomerase contains two catalytic subunits, human telomerase reverse transcriptase (hTERT) and...
telomerase RNA template (hTR) [211]. The interaction between telomerase and telomeres is still not fully understood but is known to include several recruiting and regulating factors to facilitate elongation of the telomere.

Telomerase is activated in most immortal cell lines and in the vast majority of malignant tumors, but not in normal somatic cells. Limitless potential for replication has been postulated as essential for malignant growth; and telomerase activation allows for such ability in cancer progression. However, despite intensified research, suppression of telomerase activity in somatic cells and molecular mechanisms during neoplastic processes is still poorly understood.

Investigation of gene expression of hTERT, hTR and other genes in the telomerase complex has shown that hTERT is a limiting factor in telomerase activity [211]. The hTERT gene is suppressed in normal somatic cells and up regulated in immortal cell lines and cancer cells. This alteration of gene expression is not seen among the other genes related to the telomerase ribonucleoprotein complex [207]. Recent studies of hTERT gene expression have revealed that the promoter site contains binding sites for several transcriptional factors, indicating a multi-regulatory mechanism.

Telomerase activity has been shown to distinguish Barrett’s metaplasia from adenocarcinoma [212] and precancerous lesions from gastric adenocarcinoma [213,214].

Human papilloma virus and carcinogenesis

There are accumulating data suggesting that human papilloma virus (HPV) infection may play a role in the development of esophageal squamous cell carcinoma [2]. More than 100 types of HPV have been described; they are divided into high-risk and low-risk types according to their oncogenic ability [215]. HPV is a double stranded DNA virus. The virus genome is divided into a non-coding region, an early region, and a late region [216]. The non-coding region contains a promoter and an enhancer region for DNA replication. The two proteins encoded by the late region (L1-L2) are structural proteins of the virus capsid [216]. There are seven early proteins (E1–E7) and two of them (E6 and E7) are of special interest for the oncogenic ability seen in high-risk HPV types [217]. The E6 and E7 proteins are both classified as independent oncogenes, although they are not sufficient either alone or together to cause a malignant cell phenotype. E6 interferes with the p53 protein and E7 with the retinoblastoma (Rb) protein [217]. The virus protein inactivates the tumor suppressor genes p53 and Rb, resulting in a diminished cell cycle control which ultimately might cause carcinogenesis [217].

There is extensive ongoing research aimed at a full elucidation of the role of HPV in carcinogenesis. The virus has an intrigue way of interfering with our immune system i.e. avoiding to be detected by antigen presenting mole-
cules. This makes a persistent HPV infection more likely and is probably necessary for further host-cell modifications [218].

Cytotoxic drug sensitivity

Cytotoxic drug resistance limits the effectiveness of chemotherapy in cancer treatment. Tumors can either be intrinsically resistant to cytotoxic drugs before treatment is given or acquire a resistant phenotype that evolves during chemotherapy treatment [219].

Increased drug efflux, drug inactivation, alterations in drug targets, and evasion of apoptosis are examples of mechanisms that can lead to cytotoxic drug resistance [219]. The mechanism by which many cytotoxic drugs are delivered into the tumor cell is unknown but some drug carriers have been identified and have been shown to be associated with cytotoxic drug resistance. The reduced folate carrier (RFC) is associated with cytotoxic drug resistance for anti-folate drugs such as methotrexate and tomudex [220]. Another transport mechanism concerning cytotoxic drug resistance is the active efflux transport out of the tumor cell facilitated by ATP transporter proteins such as multi-drug resistance protein (MDR) [221]. Hydrophobic substances such as anthracyclines, taxanes, and vinca alkaloids have been shown to be particularly susceptible to the active efflux transport mediated by MDR [222].

Further, an increase in catabolizing enzymes (DPD) may lead to lower amounts of free drug (5-FU) and lowered drug efficacy, as has been observed in patients with colorectal carcinoma [223]. For platinum drugs such as cisplatin, oxilaplatin, and carboplatin, a conjugation with glutathione (GSH) is seen which leads to inactivation of the cytotoxic drugs [224]. The conjugate is then removed from the tumor cells by the active ATP efflux transport proteins. High levels of GSH are seen in tumor cells with resistance to platinum compounds [224].

Genetic phenotypes and changes in the phenotype are also mechanisms for cytotoxic drug resistance [219]. Inhibition of thymidylate synthetase (TS) is believed to be the main anti-tumor effect of the antimitabolite 5-FU. TS expression has been shown to be a key factor for 5-FU resistance in tumor cells [225]. Similar patterns of downregulation of drug target enzymes or related proteins are seen for topoisomerase inhibitors as well as for drugs interfering with microtubule formation [226,227].

Another aspect of cytotoxic drug resistance is the cells’ response and survival mechanisms triggered when cytotoxic drugs cause severe cell and DNA damage. The cell has two principal choices for making sure that incorrect DNA information is not inherited by a daughter cell; either repair, or cell death. The DNA repair machinery consists of several machineries such as nucleotide excision repair (NER) and mismatch repair (MMR). When
DNA damage occurs, repair is activated by signaling pathways and is paralleled by a cell cycle arrest. Cell death is expected for cells that have extensive damage or if the repair machinery fails. Cytotoxic drug sensitivity regarding cell damage can be altered either by the cell’s DNA repair system or by the cell’s survival ability.

One rate-limiting protein, ECC1 in the NER DNA repair machinery has been shown to be a determinant for platinum compounds cytotoxic sensitivity in tumor cell lines. Deficient MMR has been observed to cause resistance to topoisomerase II drugs.

The primary goal of cytotoxic drug therapy is cell death, which can be achieved by inducing apoptosis. Apoptosis is a programmed cell death with a morphological pattern of cellular events including membrane blebbing, DNA fragmentation, and cell shrinkage. It occurs in both pathological and physiological situations [228]. The determinants of whether a cell will turn on apoptosis or choose to repair its DNA are poorly understood, but, regardless, the cell’s choice has an effect on drug sensitivity.

There are two principal activation methods for programmed cell death, both involving the apoptotic effector molecules known as caspases. The death ligand (FasL) pathway activates caspases directly, while the mitochondria-dependent pathway activates caspases via the release of cytochrome c. Mitochondrial cytochrome c release is dependent on the activation of several up-stream signaling molecules. This network of apoptotic signaling is summarized (and simplified) in Figure 3, along with the FasL pathway.
Figure 3
Aims of this thesis

I. To characterize esophageal carcinoma patients admitted to the department of oncology in Uppsala between 1990 and 2000, and to investigate clinical predictive factors.

II. To investigate the correlation of the circulating angiogenic molecules VEGF and bFGF with cystatin C, and their relationship with survival in esophageal carcinoma serum samples.

III. To determine the prognostic value of HER-2 overexpression and gene amplification in esophageal carcinoma tissue samples.

IV. To investigate the association between telomerase activity and cytotoxic drug activity in esophageal carcinoma cell lines.

V. To investigate the presence of and determine the viral load of high-risk human papilloma virus types in esophageal carcinoma tissue samples.
Patients and methods

Patient characteristics

Between 1990 and 2000, 126 patients were recorded as having received treatment for esophageal carcinoma at the Department of Oncology, Uppsala University Hospital, Sweden.

The following parameters were studied: gender, age, weight loss, smoking and alcohol habits, ECOG performance status at first admittance, and difficulty in swallowing (recorded according to the WHO classification). Other parameters were operation, localization, tumor differentiation, tumor involvement at first admittance (localized or metastatic disease), recurrence location, and time to recurrence. Tumor localization was grouped into the upper part (15–24 cm), the middle part (25–34 cm), and the lower part (35–46 cm) of the esophagus. Blood parameters measured at first admittance were hemoglobin, platelets, leukocytes, serum lactate dehydrogenase, and serum albumin. Tumor volume estimates were re-evaluated with CT scans. The majority of CT scans were obtained within one week after collection of the serum sample, but scans performed within 45 days of serum sampling were included.

Treatment strategy was recorded as:
1. Preoperative radiation treatment with a total dose of 40 Gy in 2 Gy fractions. This irradiation was given concomitant with chemotherapy delivered as cisplatin at 100 mg/m² and 5-FU at 750 mg/m² in three cycles (two of which were given concomitantly) with an interval of three weeks.
2. Planned curatively-intended radiation treatment alone to a total dose of 60-64 Gy.
3. Palliative treatment including radiation treatment with a total dose of 36 Gy in 3 Gy fractions. Some of these patients also received palliative chemotherapy and brachytherapy.
4. Palliative treatment only, including chemotherapy.

Patients were followed until 2003-03-12.
Cell lines

10 cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany, established by Shimada et al. [229]. The cells were cultured in RPMI 1640 with 5% fetal calf serum (FCS), 5% new-born calf serum (NCS), and 1% glutamine at 37°C in 95% oxygen / 5% carbon dioxide. To maintain exponential growth the cell lines were cultivated every third day. Reference cell lines KTC-1[230], HTh 7 [231], LP-1[232], Karpas 707 [233], and human diploid neonatal foreskin fibroblasts AG-1523 were purchased from the Human Genetic Mutant Cell Repository, Camden, NJ.

The fluorometric microculture cytotoxic assay (FMCA)

The cell lines were tested against a total of 20 different standard cytotoxic agents, mostly obtained from commercial sources. The drugs used were topoisomerase inhibitors (n=9), alkylating agents (n=4), tubulin-active agents (n=5), and antimetabolites (n=2). The compounds were dissolved in phosphate-buffered saline (PBS) and sterile water according to the manufacturers’ instructions. 800 μL of 1mg/ml solution was dispensed in 24 5-ml tubes. Two 96-well microtiter plates were prepared to obtain serial dilution of each drug with a concentration ranging from 0.001-10 μg/ml and 0.001-10 μM of CHS. These plates were used as templates for the preparation of 384-well microtiter plates (Nunc, Roskilde, Denmark) using an automated Biomek 2000 robotic system. 5 ml of each drug was dispensed into each well of the 384-well plate. Compounds from two template plates were transferred to one 384-well plate. The plates were sealed and kept frozen at –70 ºC until further use.

The FMCA method is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes [234]. Higher fluorescence signals a higher Survival Index (SI), or number of surviving cells. 45 μL of cell suspension at a density between 2.5 × 10³ and 5 × 10⁵ cells per well was seeded into the drug-prepared plates using an automated Precision 2000 robot. In each plate, two columns with cells but without drugs served as control, and two columns containing only culture medium served as blank. The plates were incubated at 37°C in 5% CO₂ for 72 hours without change of medium. At the end of the incubation period, the medium was aspirated and the cells washed in PBS. 25 μL of solution containing 0.1% of FDA was added to each well using the multidrop. The plates were then incubated for 40 minutes at 37°C in 5% CO₂ and the fluorescence in each well was measured in the Fluostar (wavelength of 520 and 590 nm). Computer software was connected to the Fluostar and the fluorescence in each well was measured and stored in an Excel spread-
sheet. 50% cell kill was used as the cut-off selection criterion for the cytotoxic drugs. For the dose response study, cell survival was presented as Survival Index (SI), defined as the fluorescence in experimental wells as a percentage of that in control wells, with blank values subtracted.

Quality criteria for a successful assay are (i) a fluorescence signal in the control wells of more than ten times the mean blank values, and (ii) a coefficient of variation (CV) in the control wells and blank wells of less than 30%.

**Telomerase assay**

Telomerase activity in KYSE cell lines was analyzed using the TRAPeze® Telomerase Detection Kit (Chemicon International, a division of Serologicals® Corporation, Temecula, CA, a former Intergen Company), which is an extension of the original TRAP (telomeric repeat amplification protocol) method described by Kim et al. [209]. 2x10^5 cells were collected and washed once in PBS. Cell extracts were prepared according to the manufacturers’ instructions. Protein content was determined using the BCA™ Protein Assay Reagent kit (Pierce Chemical Company, Rockford, IL) according to the manufacturers’ instructions. Cell extracts were assayed for telomerase activity in 50µL reactions provided with the TRAPeze® Telomerase Detection Kit with the exception of AmpliTaq® DNA polymerase (Perkin-Elmer LifeScience Inc., Boston, MA). A combined elongation and amplification reaction was performed in a PTC-225 Peltier Thermal Cycler. After 30 minutes of incubation at 30°C the reaction mixture was heated to 94°C for 5 minutes and then subjected to 33 cycles of 94°C for 30 seconds and 55°C for 30 seconds. The reaction mixtures were size-fractionated by electrophoresis in a 10% non-denaturating polyacrylamide gel and stained with SYBR® Green 1 dye (BioWhittaker Molecular Applications, Rockland, ME) according to the manufacturers’ instructions. The gels were photographed using the GelFotosystem GFS1000 (Techtum, Umeå, Sweden) and analyzed with the Gel-Pro™ Analyzer software (MediaCybernetics). In order to remain in the linear range in the polymerase chain reaction (PCR), dilution series were made for each cell line to determine the correct amount of protein to be used in each assay. The relative telomerase activity of each extract was determined from the ratio of the entire telomerase ladder to that of the internal control. To allow comparison of telomerase activity between different cell lines, relative telomerase activity was correlated to the total amount of protein run in each assay. Telomerase activity is shown as mean ± standard error of the mean. Statistical analysis was performed using the StatView program.
Measurement of serological factors

VEGF and bFGF

For detection of circulating VEGF and bFGF in sera, assays from R&D Systems were used (Quantikine™ human VEGF and Quantikine™ HS human FGF basic, R&D Systems, Minneapolis, MN, USA). Cut-off values were defined by the manufacturer as follows: 500 pg/mL for VEGF and 7.25 pg/mL for bFGF.

Cystatin C

Plasma cystatin C measurements were performed using latex-enhanced reagent (N Latex Cystatin C, Dade Behring, Deerfield, IL, USA) and a Behring BN ProSpec analyzer (Dade Behring). The total analytical imprecision of the method was 4.8% at 0.56 mg/L and 3.7% at 2.85 mg/L.

Methods involving tumor material

HER-2 immunohistochemical (IHC) staining

HER-2 immunohistochemical staining was performed in 97 archival paraffin-embedded tumor samples. Tumor samples were cut into 3.5 µm sections and placed onto Superfrost/plus® slides (Mentzel, Germany) and dried overnight. The samples were deparaffinized in xylene and rehydrated in graded alcohols. HIER retrieval was performed with TRIS-EDTA buffer pH 9.1 and rinsed in dH2O and TRIS-buffer. Sections were incubated with the primary antibody (A0485, DAKO) for 30 minutes at room temperature, and then incubated with EnVision® (Dual Link), DAKO, for 30 minutes at room temperature. Chromogen development was performed with DAB (K3466) DAKO. The slides were manually counterstained in Mayers hematoxylin (Histolab, Gothenburg, Sweden). Finally, the slides were dehydrated through graded alcohols to xylene and mounted in organic mounting medium (Per- tex, Histolab, Gothenburg, Sweden). The scored overexpression of HER-2 is equal to HercepTest® ® (+3), characterized as strong positive staining of the whole membrane in at least 10% of the neoplastic cell population. According to the HercepTest® protocol, all tumors scored as 0, +1, or +2 were classified as negative, and all tumors classified as +3 were considered overexpressed. Samples with known expression of HER-2 (breast carcinoma) were used as positive control.
HER-2 Chromogenic in situ hybridization (CISH)

Tumor samples were cut into 4.5 μm sections, placed onto Superfrost/plus® slides (Mentzel, Germany), and dried over night at 65°C. The samples were then deparaffinized in fresh xylene, absolute alcohol, and air-dried. HIER retrieval was performed with Tris-EDTA buffer, pH 7.0 (pretreatment kit, Zymed Laboratories Inc.), for 20 minutes at 92°C, and rinsed in dH2O before enzymatic digestion with 0.25% pepsin for 10 minutes at 37°C (WVR 1.07185.0100). The samples were thoroughly rinsed in dH2O. The slides were then dehydrated in fresh graded alcohols and air-dried. After applying the probe (Her2, 84.0146, Zymed), each slide was sealed with a cover slip, rubber cemented, and placed into a hybridizer. The slides were denaturated at 94°C for 5 min. and left to hybridize overnight at 37°C.

Stringency wash was in 0.5× SCC at 75°C for 5 minutes. Rinsing took place in dH2O, for 3 × 2 minutes.

Enzymatic digestion was in 3% H2O2 in absolute methanol for 10 minutes, and rinse was PBS-Tween for 2 × 3 minutes. Detection kits (84-9243, Zymed Laboratories Inc.) were used for blocking, FITC-anti-DIG antibody, HRP-anti-FITC, and development with DAB. The slides were manually counterstained in Mayers hematoxylin (Histolab, Gothenburg, Sweden). Finally, the slides were dehydrated through graded alcohols to xylene and mounted in organic mounting medium (Pertex, Histolab, Gothenburg, Sweden). Amplification of HER-2 was defined when a large gene copy cluster in 50% of carcinoma cells or numerous (10) separate gene copies were seen.

HPV investigation

HPV plasmids:
Plasmids containing HPV 16, 18, 31, 33, 39, 45, 52, 58 and 67 were supplied by T. Matsukura (National Institute of Health, Japan), A. Lörrincz (Digene Corporation) or G. Orth (Institut Pasteur, Paris) or prepared by cloning from PCR products of clinical samples. The plasmids were used both as positive controls and to estimate the sensitivity of the assay.

Deparaffination procedure:
The biopsies were fixated by treatment in buffered formalin followed by paraffin embedding. Paraffin blocks from the primary tumor were cut in 10-μm sections and 4 sections / patient were collected in the same microcentrifuge tube. Samples were de-waxed in 1 ml xylene for 5 min. in room temperature and centrifuged at 14,000 rpm for 5 min. The supernatant was removed. This step was then repeated 5 times. Samples were gently vortexed between each step. 1 ml 95% ethanol was then added for 5 min. in room temperature and this was repeated with 70 % ethanol. The samples were centrifuged 14.000 rpm for 5 min. between the ethanol changes. The samples
were then dried in a 37°C heated block with open lids for 20 min. to remove residual ethanol.

**Proteinase K digestion:**

200-400 µl of dilution buffer was added to each tube (0.2 M Tris-HCL pH 8.0, 1% sodium dodecyl sulfate, 10 mM EDTA, 1 mg/ml proteinase K). The volume of the dilution buffer was dependent of the amount of tissue available. Samples were subsequently incubated at 50°C overnight and thereafter heated to 94°C for 10 min. to inactivate the proteinase K.

**Protein precipitation:**

50 µl of saturated NaCl solution (approximately 6 M) was added to each tube and the samples gently vortexed for 5 min. The tubes were then centrifuged at 14,000 rpm at room temperature for 5 min. After centrifugation, white pellets were visible in the bottom of the tubes and supernatants were transferred to a new tube.

**DNA precipitation:**

DNA was precipitated with 2.5 volumes of 95 % ethanol in -20°C for 1 hour and then pelleted by centrifugation at 14,000 rpm in room temperature for 30 min. The pellet was then washed with 70 % ethanol followed by centrifugation for 5 min. DNA pellets were air dried and finally re-suspended in 100 µl TE buffer (100 mM Tris-HCL, pH8.0, 1 mM EDTA).

**Real-time PCR:**

The real-time PCR assay detects and quantifies HPV 16, 18, 31, 33, 35, 39, 45, 52, 58 and 67. The assay is based on three parallel real-time PCRs from each patient sample: a) Reaction 1 detects and quantifies HPV types 16, 31, 18 and 45 (HPV 18 and 45 detected and quantified together) using three different fluorophores, b) Reaction 2 detects and quantifies HPV types 33, 35, 39, 52, 58 and 67 (HPV 33, 52, 58 and 67 detected and quantified together), again using three different fluorophores, and c) Reaction 3 detects and quantifies the amount of a human single copy gene (HMBS, Homo sapiens hydroxymethylbilane synthase, GenBank accession number M95623.1). Reaction 1 includes a total of seven PCR primers and three probes, Reaction 2 a total of seven PCR primers and three probes and Reaction 3, two PCR primers and a single probe. By relating the HPV copy number to the number of nuclear gene equivalents (from Reaction 3) a measure of HPV load is obtained.

The system has a dynamic range from $10^2$ to $10^7$ HPV copies per assay and is applicable to both fresh clinical samples and DNA extracted from archival samples. Reconstitution experiments, made to mimic infections with several HPV types, shows that individual HPV types can be detected in a mixture as long as they represent 1-10 percent of the main type. The system
has been evaluated with respect to technical specificity and sensitivity, reproducibility, reagents stability, sample preparation protocol and applied to the analysis of clinical samples.

The PCR amplification was performed in a 25 µl volume containing 1x PCR buffer gold (Applied Biosystems, Foster City, CA, USA), 300 µM 6-carboxy-X-rodamine (Molecular Probes inc, Eugene OR, USA), 3.5 mM MgCl₂, 200 nM each of dATP, dCTP, dGTP and 400 nM dUTP (Pharmacia Biotech, Uppsala, Sweden), 0.625U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 3µg BSA (Sigma Chemical Co., St. Louis, MO, USA) and 200 nM of each primer and probe, and 3 µl DNA extract. Amplification and detection was performed using a 7700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA). The amplification ramp included an initial hold step of 10 min. at 95 °C followed by a two-step cycle consisting of 15 sec. at 95°C and 1 min. at 57°C, repeated 40 times. Each real-time PCR run included reactions with no template controls containing all PCR components but without template DNA to ensure that the reagents mix were free of contaminants.

**Statistics**

In **paper I**, the survival functions were estimated with the Kaplan-Meier product limit method and the median survival time with linear interpolation of the survival function. The backward stepwise logistic regression method was used for univariate statistic interpretation of the data, and Cox regression was utilized for the multivariate analysis. Throughout this study, a 5% significance level was used in the statistical tests.

In **paper II**, survival was estimated using the Kaplan-Meier product limit method, where univariate analysis was performed using a log-rank test. Cox regression analysis was performed to investigate whether certain continuous factors had a significant effect on survival and for multivariate survival analyses. Spearman’s rank order correlation was used for tests of associations between factors. The survival analysis, correlation analyses, and descriptive statistics were based on the first serum sample donated from each patient. In the descriptive statistics, range was defined as the minimum and maximum. Throughout the paper a 5% significance level was used.

In **paper III**, the HER-2 data was dichotomized into overexpression and no overexpression. Survival was estimated using the Kaplan-Meier product limit method, where univariate analysis was performed using a log-rank test. Cox regression analysis was performed to investigate if certain continuous factors had a significant effect on survival or to perform multivariate survival analyses. Spearman’s rank order correlation was utilized for tests of
associations between factors. Throughout the paper a 5% significant level was used.

In papers IV and V, the same statistical approach was used as in paper II.
Results and Discussion

During the last three decades, significant improvements in cancer treatment and survival have been achieved. For example, in the mid-1960s, the 5-year survival rate for testis non-seminoma was approximately 45%; but since the introduction of platinum based chemotherapeutics in 1978, together with improved radiotherapy, the 5-year survival rate has increased to approximately 95% [235]. During the 1990s, survival rates increased for solid tumors as cancer of the rectum, breast and cervical cancer [235]. During the past 5 years, several studies have reported increased median survival for patients with advanced stage cancers such as prostate cancer and non-small cell lung cancer (NSCLC) [236,237]. The recent improvements in survival for these patients have been achieved partly due to the introduction of targeted therapies. However, for esophageal carcinoma patients, the overall 5-year survival has not changed since the 1980s, remaining at approximately 10% despite treatment strategies involving surgery, chemotherapy, and radiotherapy, alone or in combination. Explanations for these poor survival rates might be sought in the biological characteristics and behavior of this malignancy as well as early tumor cell dissemination undetected by current tumor staging methods.

In paper I, the clinical characteristics of 126 patients treated for esophageal carcinoma at the Department of Oncology in Uppsala between 1990 and 2000 were investigated retrospectively. The number of patients in this study might seem small, but this is often the case in single-institution investigations of esophageal carcinoma in areas of low incidence such as Europe; one exception is a study from France in which 742 patients were included during a 20 year period [238]. In our study, patients with loco-regional disease and performance status 0-1 were considered candidates for treatment with curative intention and were thus referred for further consultations. We are aware that the resulting selection bias of patients is considerable and will have affected the survival rates.

87 patients (69%) were classified as having squamous cell carcinoma of the esophagus, 38 patients (31%) were classified as having adenocarcinoma of the esophagus, and the one remaining patient was classified as having mucoepidermoid carcinoma. While the two dominant histologies in esophageal carcinoma are squamous cell carcinoma and adenocarcinoma, their ratio differs between different geographical areas. The histological distribution found among our subjects is consistent with other studies reported from
Europe and Asia, where squamous cell carcinoma is still dominant [1]. Several authors have studied the prognostic significance of histology. Alidina et al. retrospectively investigated esophageal carcinoma patients in Pakistan and showed a longer survival time for patients with squamous cell carcinoma than for those with adenocarcinoma [239]. Siewert et al. reported a survival benefit for patients with adenocarcinoma who underwent esophageal resection with curative intention [240]. Our study did not find any prognostic value due to histology in the investigated population. However, it would not be unreasonable to speculate that treatment can be selected based on histology, by analogy with recent findings for NSCLC patients; patients with squamous cell NSCLC histology have been shown to have an increased survival when given antiangiogenic treatment (Avastin®) in conjunction with chemotherapy. It is thus possible that this treatment regime would also result in increased survival for patients with esophageal squamous cell histology.

Among our study subjects, the prognostic factors were performance status, smoking habits, swallowing function, localization of the tumor, leukocyte and albumin levels at first admittance, and disease stage; however, the only independent significant prognostic factors remaining in the multivariate analysis were performance status and disease stage. The univariate analysis showed that low albumin levels were a prognostic factor associated with poor survival. This might indicate an advanced stage of disease, as seen in other cancer patients at terminal stage of the disease [241]. Several plausible explanations have been suggested for low albumin levels in terminal cancer patients, including malnourishment, catabolic state, and an activated immune response resulting from the tumor. The activated immune response might also elevate leukocyte levels, thus explaining the presence of leukocytes as a prognostic parameter in our study and others. Alidina et al. also found low platelet count to be a prognostic factor, a finding not mirrored in western countries [239].

The clinical prognostic factors of esophageal carcinoma do not seem to have the same clinical characteristics in other parts of the world [239,242]. Among the 263 esophageal carcinoma patients studied by Alidinia et al., obstruction of esophagus was a favorable factor for survival [239], possibly reflecting an early detection and faster admission to hospital care in Pakistan.

An investigation by the Radiation Treatment Oncology Group (RTOG) enrolled a total of 416 patients, including patients from three previous trials, for analysis of clinical prognostic factors before radiation or chemotherapeutic treatment [243]. The aim was to determine the prognostic contribution of each investigated parameter and to make an attempt at classifying prognostic subgroups in a recursive partitioning analysis. The authors found that esophageal patients with a weight loss of more than 10% at the time of diagno-
sis benefited less from chemotherapy, suggesting that weight loss should be a parameter for stratifying patients in future randomized trials. Weight loss is known to be an prognostic factor in a number of malignancies such as non-small cell lung cancer, colon cancer, and head and neck cancer as well as esophageal cancer [7,244,245].

In the statistical analyses of our study, tumor involvement as determined by the staging procedure was classified into localized (and hence operable) and non-localized, i.e. stage IV disease with distant metastases. We found that localized disease was an independent factor for prognosis, thus supporting previous findings that metastasis is associated with poor prognosis.

A pattern of care study survey for patients in the USA receiving radiotherapy between 1996 and 1999 reports an increase in concurrent chemoradiation before planned surgical resection [246]. Only one randomized trial has shown that pre-operative chemoradiotherapy is superior to surgery alone; but it has been criticized for the poor surgical result, although surgery was involved in both treatment arms [79]. Although the pre-operative approach has been reported to be superior to chemoradiation alone, this might be explained by a selection bias for patients with poor performance and medical status.

Among our study subjects, 34 patients were admitted to a pre-operative protocol and 21 received definitive chemoradiation with curative intention. We found no significant survival benefit for patients admitted for surgery. A study comparing definitive chemoradiotherapy with pre-operative chemoradiotherapy reported that the addition of surgery was only beneficial in patients that did not respond to pre-operative chemoradiotherapy [105].

Performance status and stage of disease predicted outcome in esophageal patients admitted to the Department of Oncology in Uppsala. This finding indicates that the TNM staging system might not be sufficient, and that additional biological parameters are necessary to determine the best treatment approach for the individual patient.

In paper II we investigated the circulating pro-angiogenic factors VEGF and bFGF together with the protease inhibitor cystatin C in sera in order to determine their prognostic value and their relationship with each other. Serum analyses of the circulating pro-angiogenesis marker VEGF in esophageal carcinoma have only been performed in a limited number of studies. In a study by Shimada et al. investigating serum VEGF levels of 96 esophageal squamous cell carcinomas, the authors reported that serum VEGF correlated to tumor TNM stage, and that patients with partial or complete response to chemoradiotherapy had significantly lower levels of serum VEGF than non-responding patients [143].
The formation of new blood vessels has been postulated to be necessary for tumor growth and also to facilitate the tumor’s ability to form distant metastases [247]. Tumors growing beyond the capacity of the local oxygen and nutrient supply will initiate angiogenic signaling, and eventually angiogenic molecules will reach the bloodstream [182]. Previous investigations using IHC have found elevated levels of VEGF in esophageal tumors, with a reported range of 24%–65% [248,249]. The prognostic value of VEGF IHC has been investigated in a limited number of studies concerning esophageal carcinoma patients, with differing results [250,251]. Concerning pro-angiogenic factors as predictors of the results of radiation therapy, a study by Nomiya et al. investigated microvessel density and VEGF levels in patients submitted for curative radiation therapy and found a correlation between radio sensitivity and angiogenic factors. They hypothesize that tumors with high angiogenic activity might have insufficient oxygen supply, causing a higher resistance to radiotherapy [252].

In a study of head and neck carcinoma, the hypoxic tumor volume was found to correlate to the levels of serum VEGF, indicating a strong connection between tumor hypoxia and angiogenic signaling in circulating blood [253].

The use of circulating biomarkers is an appealing approach since such markers are easily accessed with a blood sample, but several pitfalls must be taken under consideration. Measurements of VEGF in plasma, serum, and blood represent different fractions of VEGF. Further, while plasma represents the free circulating fraction, serum represents the biological fraction of VEGF available in the coagulation cascade. Spence et al. compared plasma VEGF levels with serum VEGF levels in 23 patients committed to esophageal surgery and reported a correlation between platelet count and both serum and plasma VEGF; they also found that surgery elevates plasma and serum VEGF levels transiently [254].

The pro-angiogenic molecules VEGF and bFGF are secreted from tumor cells into the circulating blood stream and may then be degraded by the liver and immune cells or filtered by the renal circulation, thus affecting the level measured in a venous blood sample. Further, it has been suggested that infiltrating tumor macrophages could be the source of the elevated serum VEGF levels which have been observed in esophageal carcinoma patients after treatment with chemoradiation. Another factor is that tissue or immune cells surrounding the tumor can affect the released molecules, thus altering the amount reaching the systemic blood flow. VEGF and bFGF are stored by platelets and therefore pre-analytic handling of the blood sample must be performed following a standardized protocol, since clotting time and platelet count may affect measurement of the circulating molecules.

Our study found a serological overexpression of VEGF in 20% of the patients. Further, VEGF was positively correlated to tumor volume, weight reduction, platelet count, and leukocytes. It was positively correlated to cys-
tatin C but not to creatinine or bFGF. VEGF was associated with survival when considered as a continuous variable, but the statistical significance disappeared after stratification with platelet counts.

The cystatin C level was correlated to serum VEGF levels in our study but we did not find any prognostic value for cystatin C either alone or together with VEGF. Association between pro-angiogenic factors such as VEGF and extracellular matrix proteases (MMP) has been observed in a study of 134 esophageal carcinoma tumors [255]. The authors report different expression patterns between tumors depending on lymph node metastasis and tumor differentiation, indicating an important biological factor for selecting appropriate samples for angiogenesis marker investigations [255].

bFGF was elevated in 40% of the patients in our study and was correlated to platelet count and leukocytes but not to any other clinical parameter or to survival [255]. The lack of prognostic value for bFGF also indicates that the collection of samples are crucial since in vitro investigations have shown that bFGF is expressed in late stage esophageal carcinoma [167,256,257].

In paper II, neither of the angiogenic factors were associated with survival and they seem to have limited prognostic value for esophageal carcinoma patients.

In paper III we characterized paraffin-embedded tumor samples from 97 esophageal carcinoma patients regarding their HER-2 status. Only two other studies have included more patients; the largest included 205 patients with 78 squamous cell carcinomas and 127 adenocarcinomas, and the other included 104 esophageal squamous cell carcinoma patients [258,259]. HER-2 activation has been shown to mediate cell proliferation as well as differentiation, and overexpression of HER-2 has been reported as a predictor of poor prognosis in several human tumors such as non-small cell lung cancer [260], gastrointestinal tumors [261], and breast cancer [262].

The interpretation and selection of monoclonal antibody regarding HER-2 IHC were stratified according to HercepTest® with strict criteria for each level of HER-2 overexpression (between 0 to 3+ in breast cancer tissue samples). HercepTest® has also been applied in other types of cancer with varying results. It is difficult to make comparisons between studies investigating HER-2 status in esophageal carcinoma, since the reported overexpressions differ according to the interpretation of HER-2 status, and the HercepTest® criteria were only used in few of the studies. The choice of monoclonal antibody affects the result because they may differ in providing a positive staining at different gene amplification levels. Press et al. investigated 28 monoclonal antibodies targeting the HER-2 gene and reported that 12 antibodies showed positive staining when the gene was amplified 2–5-fold and six of the antibodies showed positive staining when the gene amplification was 5-fold or higher [263]. We found a HER-2 overexpression, defined as (3+) according to HercepTest®, in 17% of all tumors. Other studies investigating
HER-2 overexpression in esophageal carcinoma report overexpression ranging between 0% and 73% [264,265]. The differences between overexpression rates might depend on stage of disease and tumor histology included in the investigation as well as the methodology, i.e., interpretation of IHC results.

In an investigation of 80 patients with Barrett’s-related adenocarcinoma the authors report that HER-2 overexpression was correlated with the depth of tumor invasion as well as with lymph node metastasis and distant metastasis [266]. In our study, eight out of 29 patients with localized disease (28%) had overexpression of HER-2, as did 13% of patients with advanced disease.

Our study included both squamous cell carcinoma and adenocarcinoma. 17% of the investigated tumors overexpressed HER-2, and HER-2 was a prognostic factor in the univariate statistical analysis. Nine of the 70 patients with squamous cell carcinoma (13%) had HER-2 overexpression, as did eight of the 27 patients (30%) with adenocarcinoma.

We did not find a prognostic value for HER-2 overexpression for patients with adenocarcinoma; this might be due to the low number of observations in our study. Patients with HER-2 overexpressing squamous cell carcinoma tumors were found to be associated with poorer survival although this was not an independent statistically-significant prognostic factor according to the multivariate analysis. The selection bias regarding stage of disease must be considered when comparing results from investigations into HER-2 overexpression. The group of patients with advanced disease outnumbered the patients with loco-regional disease in our study and we could not find any significant survival differences between the groups regarding their HER-2 status.

A possible explanation for these results might be that HER-2 interferes with and enhances radiotherapy resistance. Among several signaling pathways, the PI-3K signaling pathway is activated by HER-2 and, together with downstream-activated factors, has been suggested to be part of the cellular response to radiation therapy [267]. Thus, our results might indicate that HER-2 overexpressing esophageal squamous cell carcinomas have a higher degree of radiation resistance than esophageal adenocarcinoma. In order to enhance radiosensitivity, Sato et al. incubated HER-2 overexpressing esophageal cell lines with trastuzumab, a monoclonal antibody targeting tumors with HER-2 overexpression, and reported a synergistic effect of trastuzumab together with radiation [268]. In breast cancer, trastuzumab has been shown to be effective in tumors with HER-2 (3+) overexpression and in tumors scored as (2+) in the HercepTest® with gene amplification determined with fluorescence in situ hybridization (FISH). In our study, we also investigated HER-2 overexpressing (3+) tumors as well as (2+) tumors according to HercepTest® with CISH to determine gene amplification. CISH was chosen primarily for practical reasons. CISH has previously been used to confirm
IHC results with almost as good accuracy as FISH [269-271]. HER-2 amplification, according to CISH, was present in five out of 17 HER-2 overexpressing (3+) tumors but of eight HER-2 weakly positive (2+) tumors no amplification according to CISH was seen. The lack of gene amplification in the majority of investigated tumors might indicate that either CISH is not the best method for determining HER-2 gene amplification in esophageal cancer, or that the tumors might have an additional mechanism than gene amplification for overexpression of HER-2. The fact remains that HER-2 is overexpressed; and some tumors might be influenced by the signaling network caused by the HER-2 overexpression, and might ultimately have a lower sensitivity to radiation. In paper III, we conclude that esophageal carcinoma tumors with HER-2 overexpression seem to be associated with poorer survival, especially in patients with squamous cell esophageal carcinoma.

In patients with non-small cell lung cancer, colorectal cancer [272], and gastric cancer [212], telomerase activity is up-regulated during cancer progression, making it a potential marker of prognosis [273]. Studies of telomerase activity in esophageal carcinoma have reported a correlation between telomerase activity and lymph node involvement, indicating that telomerase activity might be associated with a aggressive tumor phenotype [274]. Further, in a study of esophageal tumors a significantly higher telomerase activity was observed in tumors with extensive blood vessel invasion [275]. The prognostic value of telomerase activity has been reported for esophageal carcinoma, with poorer survival for patients with high levels of telomerase activity, although these results are contradicted by a study of esophageal adenocarcinoma in which no prognostic value was observed [212,213]. Telomerase activity has been observed to decline in cell lines treated with cytotoxic drugs, and has been suggested as a marker for cytotoxic drug killing [276]. In a study of telomerase activity and cytotoxic drug response in esophageal carcinoma, cells exposed to 5-FU showed a diminished telomerase activity preceded by a time-dependent decrease in the mRNA expression of the telomerase rate-limiting subunit hTERT [275]. Investigations regarding telomerase activity and cytotoxic drug resistance have suggested the apoptotic signaling pathway as a possible reason for altered chemosensitivity [277]. Inhibition of telomerase activity has also been shown to sensitize breast cancer cells to topoisomerase inhibitors [278] and malignant glioma cells to temozolomide [279]. High telomerase activity might be one of the routes by which tumor cells avoid apoptosis as they strive for independence from external growth stimuli and controls. Further, telomerase activity and hTERT expression have been assigned to the signaling network of apoptosis, and suggested as an anti-apoptotic factor [280].

Telomerase activity is repressed in most somatic cells, but has been observed in approximately 85% of all malignancies. Several transcriptional factors have been identified as part of the regulation of hTERT, including c-
Myc and p53, and thus it has been suggested that an activation of telomerase in malignant cells could depend on up-regulation of hTERT gene expression [281]. Further, the subunit protein hTERT has been associated with anti-apoptotic events such as repression of pro-apoptotic genes and up-regulation of growth-promoting genes such as EGFR [280].

Hypothetically, increased basal telomerase activity in tumor cells could represent a tumor cell phenotype with less ability to start the apoptotic suicide program after cytotoxic drug damage. Further, hTERT has been found to antagonize p53-dependent apoptosis independently of telomerase activity [280]. Certain cytotoxic drugs, including cisplatin, have been shown to cause DNA double strand break and subsequent induction of apoptosis [282]. Regulation of apoptosis involves both the mitochondrial p53 pathway and the death receptor pathway. Cytotoxic drugs probably activate both pathways, but the relative contribution to cell death of these and other pathways may depend on the drug’s mechanism or concentration or on the tumor cells’ threshold for apoptosis as altered by expression of pro- and anti-apoptotic molecules [283]. In paper IV, we investigated four different mechanistic classes including topoisomerase inhibitors (n=9), alkylating agents (n=4), tubulin active agents (n=5), and antimetabolites (n=2) regarding their cytotoxic activity according to FMCA and relationship to telomerase activity in ten esophageal cell lines. We found no significant association with malignant potential such as doubling time (p=0.57) or cell differentiation (p=0.87). Further, no correlation between telomerase activity and cytotoxic drug activity was observed for any of the cytotoxic drugs. The explanation for this might be the in vitro setting, the selection of cell lines, or the possibility that biological assumptions regarding the role of telomerase activity in inducing cell death are not valid for esophageal tumor cells.

In vitro settings have simplifications in their setup that most likely interfered with our results. The selection of cell lines might also have affected the results, since telomerase activity levels are generally higher in esophageal carcinoma cell lines than in clinical samples [213,284]. The relatively high telomerase activity in our cell lines might have interfered with the effect of telomerase on apoptosis, thus making it impossible to distinguish chemosensitivity between the cell lines and telomerase activity. However, the observed IC50 values for the standard drugs investigated covered a wide range of cytotoxic drug activity. This might well indicate that our cell lines are not representative of esophageal squamous cell tumors which are generally considered as resistant to chemotherapeutics. Further, the FMCA cytotoxic drug assay is a highly automated assay and suitable for cell line investigations [285], and has shown good results regarding the predictive value of chemotherapeutic sensitivity in vitro compared to the drug response in vivo. However, the methods and results achieved in in vitro studies cannot always be translated into the clinical reality. In a study by Berglund et al., the FMCA method was used for selection of chemotherapy for patients with
advanced cancer not amenable to standard treatment. The results showed a poor correlation between \textit{in vitro} and \textit{in vivo} prediction of tumor response indicating that there are factors disregarded in the \textit{in vitro} setup [286].

Besides apoptosis, necrosis has been suggested as a death mechanism for cells killed by cytotoxic drugs [287]. A study by Zong et al. suggested that necrosis in response to DNA damage depends on activation of the DNA repair protein PARP, and on ATP depletion [288]. Cell able to maintain ATP levels become resistant to death in response to PARP activation; this might explain how cytotoxic agents are able to selectively induce tumor cell death independently of apoptotic signaling proteins [288]. In paper IV, we conclude that basal telomerase activity level is not a key determinant of sensitivity to standard cytotoxic drugs in esophageal carcinoma cell lines.

HPV infection has been reported as a favorable prognostic factor in several investigations of head and neck cancer, in which HPV is present in approximately 50% of cases [289]. The transmission of HPV to the oral cavity has not been fully elucidated. Both perinatal transmission and sexual behavior have been suggested as possible modes of HPV virus transmission [290]. Incorporation of HPV virus DNA into the host cell DNA genome has been suggested as an important step of tumorigenesis [290].

HPV is detected in an average of 15% of investigated esophageal carcinoma cases. The variation in geographical distribution of esophageal carcinoma follows the large differences in the incidence of HPV infected tumors [2] and the role of HPV in the etiological role in esophageal carcinoma has been suggested to be more important in high incidence areas than in low incidence areas [291]. Tonsil cancer and tumors located in the oropharynx and hypopharynx have been shown to have a high rate of HPV positivity [292]. In tonsil carcinoma, approximately 50% of all cases are infected with a high risk HPV type, and several studies have shown HPV to be a favorable factor for survival [289]. HPV is also found in other head and neck tumors but the prevalence of high risk HPV types is lower, approximately 20-30%, and the prognostic value is not as strong as for tumors arising in the tonsils.

In paper V, the primary aim was to determine the spectrum of HPV types present and the secondary aim was to investigate whether HPV infection or the viral load had any effect on survival. Previous studies of HPV infection in esophageal carcinoma have mainly focused on the etiological role of HPV infections [2]. We investigated a total of 100 patients with esophageal carcinoma for the presence of HPV types 16, 18, 31, 33, 39, 45, 52, 58, and 67. These are the most prevalent high-risk HPV types and together account for more than 95% of all high-risk HPV types historically detected in malignant tumors [293]. Our study is, to our knowledge, the first study that includes this range of HPV types in esophageal carcinoma tumors. HPV 16 was the only HPV type that we detected; it was present in 16% of the tumors. The reason that only HPV 16 was detected despite the investigation of nine dif-
different high-risk HPV types might be related to HPV virulence factors such as virus adherence to the epithelial cells. Another possibility is the fact that HPV 16 survives in the oral cavity while other high-risk HPV types are diminished by the oral environment. HPV 16 has a clear dominance in HPV-positive head and neck tumors, indicating that the oral cavity rarely harbors any HPV infection other than HPV 16 [289]. In our study, HPV 16 was found in 10 out of 71 squamous cell carcinomas, 5 out of 27 adenocarcinomas, and one of two with mucoepidermoid histology.

Although there are several studies regarding HPV infection in esophageal carcinoma, its prognostic value is not well-investigated. One study of 60 esophageal tumors found that HPV was not an prognostic factor [294]. In a study of 71 esophageal tumors regarding their p53 status and presence of HPV infection, a better survival was seen among patients with functional p53 status and without HPV infection [295]. Other types of viruses associated with malignant transformation properties, such as herpes simplex virus (HSV), Epstein Barr virus (EBV), and cytomegalovirus (CMV), have not been present or only present at low rates in studies of esophageal tumors [296,297]. Our study did not find any association between HPV infection and survival, although a few of the patients with HPV infection showed a trend towards improved survival.

Integration of the oncogenic proteins E6 and E7 is connected with DNA instability and has been shown to be a key step in the carcinogenesis of cervical carcinoma, abrogating the functions of the p53 and Rb proteins [298]. The abrogation of p53 function have rendered into special interest regarding chemosensitivity as well as radiation sensitivity. HPV DNA integration is associated with chromosomal instability, and, theoretically, unstable DNA would be more sensitive to radiation [299]. One investigation of HPV-positive cell lines in cervical carcinoma reported an increased resistance to radiation as well as to the chemotherapeutics cisplatin and 5-FU compared to cell lines not infected by HPV [300]. Further, cisplatin has also been shown to restore p53 function and enhance the radiosensitivity in HPV 16-positive cell lines [301]. The treatment response in HPV positive tumors has also been investigated in tonsil tumor samples but without finding any association between HPV infection and radiotherapy resistance [302].

In tumor cells infected with HPV, HPV E6 protein interferes with p53; and this might lead to a defective apoptotic pathway and thus a decrease in chemotherapy-induced cell death. Further, cisplatin has been shown to cause less activation of the apoptotic caspase-8 pathway in resistant tumor cells, which might well be a plausible explanation for the impaired cell killing seen in HPV-positive cell lines [303]. Our study did not find a significant positive effect of HPV 16 infection on survival after radiation treatment (p=0.16) or chemotherapy (p=0.22).
In tonsil squamous cell carcinoma, the anatomical location is a strong determinant for both the course of the disease and the influence of HPV infection [304]. In our clinical record the anatomical sites of the esophageal tumors were divided into the upper, middle, and distal parts of the esophagus. We found no significant differences in clinical outcome between the different tumor locations. In head and neck cancer, especially in tumors of Waldeyer’s tonsillar ring, metastatic spread to the lymph nodes seems more frequent in HPV-positive tumors than in HPV-negative tumors, although patients with HPV-positive tumors do have better survival rates than those with HPV-negative tumors with lymph node involvement [304]. This finding has led to suggestions that HPV infections activate the immune response more strongly than is the case in tumors without HPV infection [289]. Our study did not show any significant association between HPV and stage of disease. Another factor is that immune system response has been shown to be affected by alterations of hormones [305]. Our study found no statistically-significant differences between men and women regarding HPV infection and survival time.

Survival has also been correlated to the viral load of HPV in the tumor cells, with better survival for patients with tumors with high rates of HPV DNA copies; this supports the theory of a stronger immune response, especially against tumors with high viral load [306]. Our study did not find any correlation between survival and HPV viral load of >1 genome equivalents or between survival and viral load of <1 genome equivalents. The number of viral DNA copies/ human DNA equivalents in HPV-infected cells ranged from <0.1 to 437. The median viral load was 2.1 viral copies per human DNA equivalents and cell. Although this is considerably lower than the viral loads found in tonsil carcinoma, it is in agreement with a previous study of viral load in esophageal carcinoma [289,307], which looked at HPV viral load in esophageal carcinoma tumors from five different regions in China with variation in both the incidence of esophageal carcinoma and seroreactivity for HPV 16. Low HPV infection rates and low viral load were observed in the area with the highest rates of esophageal carcinoma [307]. The low viral load might explain the absence of HPV influence on survival rates and may also shed doubt on the etiological role of HPV infection in esophageal carcinoma.
Conclusions

I. Stage of disease and the patient’s performance status are important factors for predicting outcome in patients with esophageal carcinoma but the results of our study imply that further characterization of tumor biology in esophageal carcinoma patients are needed to find additional predictive factors for survival and future treatment strategies.

II. The usefulness of angiogenic factors in serum as prognostic factors in patients with esophageal carcinoma was in the present study of limited value.

III. Esophageal carcinoma tumors with HER-2 overexpression seem to be associated with poorer survival, especially in patients with squamous cell esophageal carcinoma.

IV. Our results do not support a causal relationship between basal telomerase activity and cytotoxic drug sensitivity in the investigated human esophageal carcinoma cell lines.

V. Presence of HPV 16 in esophageal carcinoma tumors was in the present investigation not associated with increased survival but larger, prospective studies are needed to fully elucidate this issue.
Future experiments

Investigation into whether inhibition of telomerase activity can increase conventional cytotoxic drug efficacy.

Investigation of the expression of angiogenic (VEGF, PDGF) receptors in esophageal tumors.

Investigation of the gene expression profile in esophageal tumors associated with resistance to radiotherapy.

Investigation of the serum protein profile in esophageal tumors associated with response to radiotherapy.
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References


31 Mayne ST, Risch HA, Dubrow R, Chow WH, Gammon MD, Vaughan TL, Farrow DC, Schoenberg JB, Stanford JL, Ahsan H, West AB, Rotterdam H,


179 Rosen LS: Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. Cancer Control 2002;9:36-44.


203 Voldborg BR, Damstrup L, Spang-Thomsen M, Poulsen HS: Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. Ann Oncol 1997;8:1197-1206.


299 Wentzensen N, Vinokurova S, von Knebel Doeberitz M: Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dys-
305 Brabin L: Interactions of the female hormonal environment, susceptibility to viral infections, and disease progression. AIDS Patient Care STDS 2002;16:211-221.
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