Prognostication in esophageal cancer
Lagarde, S.M.

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Molecular prognostic factors in adenocarcinoma of the esophagus and gastroesophageal junction

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G Johan A Offerhaus
J Jan B van Lanschot

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Abstract

Introduction
Adenocarcinoma of the esophagus or GEJ is an aggressive disease with early lymphatic and hematogenous dissemination. Molecular pathology has revealed many molecular mechanisms of disease progression, which are related to prognosis. Some of these factors can be seen as prognostic factors per se. Better knowledge of molecular bases may lead to new paradigms, improved prognostication, early diagnosis and individually tailored therapeutic options. This review describes genetic and molecular changes related to adenocarcinoma of the esophagus and gastroesophageal junction (GEJ) with emphasis on prognostic value and possibilities for targeted therapy in clinical setting.

Methods
A review of recent English literature (1990-October 2005) concerning esophageal adenocarcinoma was performed. This review focuses on genetic and molecular changes as prognosticators of adenocarcinoma of the esophagus and GEJ.

Results
A bewildering number of biomarkers have been described. Many genes and molecules have prognostic impact (cyclin D1, EGFR, Her-2/Neu, APC, TGF-β, Endoglin, CTGF, P53, Bcl-2, NF-κB, Cox-2, E-cadherin, β-catenin, uPA, MMP-1,3,7,9, TIMP, Th1/Th2 balance, CRP, PTHrP).

Conclusions
Adenocarcinomas of the esophagus and GEJ show multiple genetic alterations, which indicate that progression of cancer is a multistep complex process with many different alterations. Presumably, it is not one molecular factor that can predict the biological behavior of this cancer. The combination of diverse genetic alterations may better predict prognosis. In future, gene expression analysis with microarrays may reveal important prognostic information and the discovery of new genes and molecules associated with tumor progression and dissemination will enhance prognostication and offers adjuvant therapeutic options.
Introduction

The incidence of adenocarcinoma of the esophagus is rising in the Western world and is now higher than that of squamous cell carcinoma of the esophagus\textsuperscript{1-5}. From 1975 to 2001, the incidence of esophageal adenocarcinoma rose sixfold\textsuperscript{4}. Adenocarcinomas are predominantly located in the distal esophagus and at the gastroesophageal junction (GEJ)\textsuperscript{1-7}. It is an aggressive disease with early lymphatic and hematogenous dissemination. Surgery is the best curative treatment option, but used to be accompanied by a high morbidity rate and a substantial mortality rate. Due to improvement of surgical technique and perioperative care, complications have been decreased to a more acceptable level. However, despite comprehensive preoperative staging to select patients for potentially curative surgery, many patients still present with recurrences within two years after operation\textsuperscript{8-10}. The majority of these patients develop hematogenous recurrences, but also locoregional, pleural and peritoneal recurrences are common\textsuperscript{8-12}. The 5-year survival rates after intentionally curative esophagectomy rarely exceed 30\%\textsuperscript{13-16}.

Well-known prognostic (pathological) factors of adenocarcinoma of the distal esophagus and GEJ are summarized in TNM staging\textsuperscript{17,18}. Also other (classical) pathological factors, not included in the TNM-system, such as the number of positive lymph nodes and the presence of extracapsular lymph node involvement have additional prognostic value\textsuperscript{19-23}. Molecular pathology has revealed an overwhelming number of genes and molecules, which are related to tumor invasion and metastasis (lymphatic and hematogenous dissemination). Many of these genes and molecules have prognostic impact per se. A better knowledge of these factors may not only improve prognostication, but may offer new individually tailored therapeutic options. Also selective medical intervention, especially if targeted at the level of those genes and molecules which have strong prognostic impact, holds a great therapeutic promise. Therefore, this review describes the recent advances in our understanding of genetic and molecular changes related to adenocarcinoma of the distal esophagus and GEJ with special emphasis on their specific prognostic value and possibilities for (future) targeted therapy in the clinical setting.

Methods

A review of the recent English literature (1990-October 2005) concerning esophageal adenocarcinoma was performed. This review focuses on genetic and molecular changes as prognostic factors in adenocarcinoma of the distal esophagus and GEJ.

Background molecular biology

Adenocarcinoma of the distal esophagus and GEJ is an aggressive disease with early lymphatic and hematogenous dissemination. To produce a tumor metastasis (lymphatic as
well as hematogenous), tumor cells must complete a multistep progression through a series of sequential and selective events (Figure 1). The metastatic process consists of tumor cell detachment (1), local invasion (2), angiogenesis and survival in the circulation (3), adhesion to endothelial cells (4), extravasation and regrowth in different organs (5). In each step, causative molecules have been identified: these include cell adhesion molecules, various growth factors, matrix degradation enzymes and motility factors. A recent concept is the so-called epithelial–mesenchymal transition. This is an important process during development by which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility, endowing the incipient cancer cell with invasive and metastatic properties.

Originally, molecular cancer research has focused on discovery of mutations in so called onco- and tumor suppressor genes in animal and human models. Oncogenes need to be activated and these “gain-of-function” mutations can be caused by point mutation, chromosomal translocation, and gene amplification. Similarly, the functional properties of tumor suppressor genes are opposite. Tumor suppressor genes are inactivated by “loss-of-function” mutations in cancer cells, loss of the remaining wild type allele when in heterozygous state (loss of
heterozygosity or LOH\textsuperscript{26,27} and epigenetic mechanisms like hypermethylation in promoter sequence of tumor suppressor genes.

However, cancer can not be summarized in “gain of function” or “loss of function” mutations, but is a complicated multistep process\textsuperscript{28-30}. To transform normal human cells into tumor cells, multiple genetic changes are necessary\textsuperscript{28,31}. Genetic changes can vary from subtle changes, such as point-mutations, to gross changes such as chromosome arm losses\textsuperscript{32}. For adenocarcinoma of the distal esophagus and GEJ it is generally accepted that specialized intestinal metaplasia, as a result of gastroesophageal reflux disease (GERD)\textsuperscript{33-35}, represents an intermediate step in a process through which cells evolve progressively from normal to premalignant lesions into invasive cancers\textsuperscript{29,36,37}. The development of cancer can be categorized in six essential alterations in cell physiology that collectively dictate malignant growth\textsuperscript{29,36} (Table 1): (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory (antigrowth) signals, (3) evasion of programmed cell death (apoptosis), (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis. Each of these physiologic changes represents the successful breaching of an anticancer defense mechanism and therefore harbors prognostic information. In the present review, the physiologic changes of each step will be summarized and subsequently the prognostic value of genes and molecules in adenocarcinoma of the distal esophagus and GEJ is discussed according to these six steps and an additional seventh group as proposed by Kyrgidis et al.\textsuperscript{38} (Table 2). The relationship between these prognostic factors at the cellular level is shown in Figure 2.

### Results

(1) Self-sufficiency in growth signals

Cells require exogenous growth signals to move into an active proliferative state. These growth signals (e.g. hormones, growth factors, and cytokines) bind to receptors on the cell membrane. The activated receptors communicate with the cell-cycle machinery in the nucleus via progrowth regulatory molecules (e.g. cyclins), which can inactivate the retinoblastoma

<table>
<thead>
<tr>
<th>Hallmarks of adenocarcinoma of the distal esophagus and gastroesophageal junction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Self-sufficiency in growth signals Cyclin D1 and E, Ki-67, PCNA, EGFR, TGF-α, EGF, Her2/neu, integrins</td>
</tr>
<tr>
<td>(2) Insensitivity to antigrowth signals Rb, p16, p21, APC, TGF-β, Smad4</td>
</tr>
<tr>
<td>(3) Avoidance of apoptosis p53, Bcl-2, Fas-fas ligand, NF-κB, COX-2,</td>
</tr>
<tr>
<td>(4) Limitless replicative potential telomerase</td>
</tr>
<tr>
<td>(5) Sustained angiogenesis VEGF</td>
</tr>
<tr>
<td>(6) Invasion and metastasis cadherins, integrins, CD44, serine protease system, MMP, TIMP</td>
</tr>
<tr>
<td>(7) Other factors DNA-content, Promotor hypermethylation multiple genes, Th1/Th2 balance, CRP, PTHrP,</td>
</tr>
</tbody>
</table>
### Table 2: Prognostic factors of genes and molecules in adenocarcinoma of the esophagus and gastroesophageal junction

<table>
<thead>
<tr>
<th>Pathologic factor</th>
<th>Prognostic impact</th>
<th>Negative survival benefit</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Self-sufficiency in growth signals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- cyclin D1</td>
<td>minor</td>
<td>amplification and nuclear staining</td>
<td>43,44</td>
</tr>
<tr>
<td>- TGF-α</td>
<td>dubious</td>
<td>elevated expression</td>
<td>46,47</td>
</tr>
<tr>
<td>- EGFR</td>
<td>minor</td>
<td>elevated expression</td>
<td>47,49</td>
</tr>
<tr>
<td>- HER-2/neu gene (c-erbB2)</td>
<td>considerable</td>
<td>amplification and overexpression</td>
<td>50,52,54</td>
</tr>
<tr>
<td>- Ki-67</td>
<td>none</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>(2) Insensitivity to antigrowth signals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Rb</td>
<td>none</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td>- p16</td>
<td>dubious</td>
<td>hypermethylated p16 (strong trend)</td>
<td>64</td>
</tr>
<tr>
<td>- p21</td>
<td>dubious</td>
<td>alteration of expression after chemoradiotherapy</td>
<td>65</td>
</tr>
<tr>
<td>- APC</td>
<td>minor</td>
<td>hypermethylated APC DNA in plasma</td>
<td>68</td>
</tr>
<tr>
<td>- TGF-β</td>
<td>considerable</td>
<td>elevated level in azygos vein</td>
<td>69</td>
</tr>
<tr>
<td>- endoglin</td>
<td>minor</td>
<td>elevated expression</td>
<td>71</td>
</tr>
<tr>
<td>- CTGF</td>
<td>minor</td>
<td>elevated expression</td>
<td>71</td>
</tr>
<tr>
<td>(3) Avoidance of apoptosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- p53</td>
<td>considerable</td>
<td>mutation</td>
<td>75-78</td>
</tr>
<tr>
<td>- bcl-2</td>
<td>minor</td>
<td>reduced expression</td>
<td>79</td>
</tr>
<tr>
<td>- NF-κB</td>
<td>minor</td>
<td>expression</td>
<td>82</td>
</tr>
<tr>
<td>- COX-2</td>
<td>considerable</td>
<td>reduced expression</td>
<td>83,84</td>
</tr>
<tr>
<td>(4) Limitless replicative potential</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- telomerase</td>
<td>none</td>
<td>-</td>
<td>86</td>
</tr>
<tr>
<td>(5) Sustained angiogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- VEGF</td>
<td>none</td>
<td>-</td>
<td>89,93</td>
</tr>
<tr>
<td>(6) Invasion and metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- E-cadherin</td>
<td>considerable</td>
<td>reduced expression</td>
<td>94,95</td>
</tr>
<tr>
<td>- β-catenin</td>
<td>considerable</td>
<td>reduced expression</td>
<td>94,95</td>
</tr>
<tr>
<td>- integrins</td>
<td>none</td>
<td>-</td>
<td>39,96</td>
</tr>
<tr>
<td>- uPA</td>
<td>considerable</td>
<td>uPA expression</td>
<td>98</td>
</tr>
<tr>
<td>- MMP-1</td>
<td>considerable</td>
<td>elevated expression</td>
<td>99</td>
</tr>
<tr>
<td>- MMP-3</td>
<td>minor</td>
<td>elevated expression</td>
<td>102</td>
</tr>
<tr>
<td>- MMP-7</td>
<td>minor</td>
<td>staining on invasive site</td>
<td>100</td>
</tr>
<tr>
<td>- MMP-9</td>
<td>minor</td>
<td>staining on invasive site</td>
<td>100</td>
</tr>
<tr>
<td>- TIMP</td>
<td>minor</td>
<td>reduced expression</td>
<td>104</td>
</tr>
<tr>
<td>(7) Other factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- DNA content</td>
<td>strong</td>
<td>DNA ploidy</td>
<td>105-107</td>
</tr>
<tr>
<td>- promotor hypermethylation</td>
<td>considerable</td>
<td>&gt;50%</td>
<td>64</td>
</tr>
<tr>
<td>- Th1/Th2 balance</td>
<td>minor</td>
<td>ability to produce IL-2</td>
<td>109</td>
</tr>
<tr>
<td>- CRP</td>
<td>minor</td>
<td>elevated level in serum</td>
<td>110</td>
</tr>
<tr>
<td>- PTHrP</td>
<td>minor</td>
<td>elevated level in serum</td>
<td>111</td>
</tr>
</tbody>
</table>

NOTE: Prognostic impact in clinical factors is scored as none (no evidence), dubious (conflicting evidence), minor (only evidence from univariate analysis), considerable (evidence from uni- and multivariate analysis) and strong (evidence of prognostic value in several multivariate analyses).
protein (Rb) by phosphorylation. Eventually, this mechanism results in promoting cell-cycle progression\textsuperscript{29,36}.

Other mechanisms in which cancer cells acquire self-sufficiency are mediated by the production of growth factors (independence on exogenous growth signals), or by receptor overexpression (hyperresponsive to growth signals) or by altering the growth-factor receptors (constant activation in the absence of growth factor stimulation).

Furthermore, tumor cells can switch the types of integrins they express. Integrins physically link cells to the extracellular matrix (ECM) and successful binding to the ECM enables the integrin receptors to transduce signals into the cytoplasm. These signals influence cell functions such as cell attachment, cell migration, cell cycle progression, and apoptosis\textsuperscript{29,39}.

**Cyclins**

Cyclins D1 and E are key cell cycle regulatory proteins and oncogenic proteins. Both are related with tumor progression. A polymorphism of CCND1, the gene which encodes cyclin D1 was associated with an increased risk for esophageal adenocarcinoma\textsuperscript{40}. Furthermore CCND1 amplification and cyclin D1 nuclear staining were found to correlate negatively with survival\textsuperscript{41,42}. The pathophysiological mechanisms behind it remain unclear. Although cyclin E is reported to be overexpressed in adenocarcinoma of the distal esophagus\textsuperscript{43,44} and overexpression has negative prognostic value in gastric cancer\textsuperscript{45}, the prognostic effect

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**Figure 2:** Relationship between the diverse prognostic factors in patients with adenocarcinoma of the esophagus and gastroesophageal junction.
is unclear in patients with adenocarcinoma of the esophagus. Cyclin-dependent kinase inhibitors (e.g. flavopiridol) are currently tested in clinical trials as an antitumor drug and may be used in future46,47.

Growth factors and their receptors
The epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) belong to the family of growth factors that bind to the epidermal growth factor receptor (EGFR, or HER1 or c-erbB1). EGF shows about 40% sequence homology with TGF-α and competes with TGF-α for binding to the EGFR. EGF has possible prognostic significance, however, there is no information regarding adenocarcinoma of the esophagus and GEJ. TGF-α is an important proliferation activator. The interaction between TGF-α and EGFR leads to autocrine tumor growth cascades, which lead to rapid tumor growth. TGF-α mRNA and protein can be detected in approximately 60% of adenocarcinomas of the GEJ. TGF-α protein expression is associated with tumor progression and in univariate analysis with survival48. In another study this prognostic effect was not confirmed49. Which can possibly be explained by the small sample size.

The family of EGF growth factor receptors consists of four members; HER1 (or EGFR), HER2, HER3 and HER4. However, there is no information available regarding HER3 and HER4 in adenocarcinoma of the esophagus and GEJ. EGFR is a transmembrane receptor and contains an external binding domain for epidermal growth factor (EGF) as well as transforming growth factor α (TGF-α). Many solid tumors overexpress EGFR and in adenocarcinoma of the esophagus, EGFR is expressed in 30-60% of the tumors49-51. A higher degree of EGFR immunostaining was significantly associated with a poor survival49 and another study showed a relation with poor differentiation grade and a trend for worse survival51. The precise relationship between increased EGFR and tumor growth remains unknown, but the interaction between growth factors and EGFR can lead to autocrine tumor growth cascades and thus play a role in various cellular functions including proliferation and differentiation.

The HER-2/neu oncogene is a proto-oncogene that encodes a tyrosine kinase growth factor receptor. Amplification of the HER-2/neu gene or overexpression of its encoded protein is associated with a worse survival in various cancers52. Increased expression of HER-2/neu is associated with inhibition of apoptosis and enhanced cell proliferation as well as increased mitogen-activated protein kinase (MAPK) activity. HER-2/neu signalling is also associated with increased matrix metalloproteinase activity and enhanced tumorigenic and metastatic potential. Additionally HER-2/neu is a potent inducer of vascular endothelial growth factor (VEGF) and enhanced tumor vascularity53. In adenocarcinoma of the esophagus there are contradictory data. Overexpression ranges from 0 to 73% in these tumors. However, most of the information available indicates that this marker has significant prognostic value52,54. In two studies, overexpression was even independently associated with a poor prognosis55,56. The development of selective inhibitors of EGFR and HER2 is changing cancer treatment and may enhance individual treatment options in EGFR en HER2 positive patients57. Two major approaches have been taken to target EGFR and HER2. The first approach is the
development of monoclonal antibodies that bind to the extracellular domain of the receptor and inhibit ligand binding and thus block receptor activation (e.g. Cetuximab). The anti-HER2 antibody Trastuzumab has shown favorable results among women with HER2-positive breast cancer\textsuperscript{58}. The second approach consists of small molecules that inhibit tyrosine kinase function by binding to and consequently blocking the ATP-binding site of the kinase (e.g. Lapatinib and Gefinitib). Clinical trials to treat Her2 positive patients with adenocarcinoma of the esophagus with Trastuzumab and Lapatinib are ongoing\textsuperscript{59}. The therapeutic effect in these patients is still unknown.

**Proliferation-associated antigens**

Cell proliferation can also be assessed through immunohistochemistry of the expression of proliferation-associated antigens in tumor tissues. Several monoclonal antibodies reacting with different proliferating cell nuclear antigens have been described, such as PCNA and Ki-67. This method is cheap and easy to perform in virtually every pathology department. However, it is also a time-consuming process and is subject to intraobserver variability. Patients with squamous cell carcinoma of the esophagus with a high Ki-67 labeling index had lower postoperative survival rates\textsuperscript{60,61}. However, in adenocarcinoma this prognostic effect has not been confirmed\textsuperscript{62}. Proliferating cell nuclear antigen (PCNA) is a requirement for cellular proliferation. It has been suggested that PCNA is involved in the neoplastic transformation of Barrett’s esophagus. However, no studies have been performed to investigate its prognostic value\textsuperscript{38}.

**(2) Insensitivity to antigrowth signals**

Apart from the exogenous growth signals that cells require to move into a proliferative state, also antiproliferative signals operate in normal cells to maintain cellular quiescence and tissue homeostasis. These growth-inhibitory signals are received by transmembrane cell surface receptors coupled to intracellular signaling pathways. Proliferation can be blocked by forcing a cell into the quiescent state or by inducing a permanent growth arrest. Most antiproliferative signals converge on the Rb pathway. Tumor cells can become insensitive for these antigrowth signals by inactivating tumor-suppressor genes like Rb. Other tumor suppressors, such as adenomatous polyposis coli (APC), block cellular proliferation by binding proteins that are involved in cellular signal transduction, and by inducing differentiation\textsuperscript{29,36}.

**Retinoblastoma protein (Rb)**

Rb normally blocks tumor progression and is inactivated via phosphorylation. Loss of the Rb gene itself is a rare event in Barrett’s metaplasia, but occurs in HGD and adenocarcinoma. Rb LOH occurs in adenocarcinoma of the esophagus, but its prognostic value has not been determined\textsuperscript{38}.
p16
Although not much evidence exists regarding Rb genome changes in adenocarcinoma of the esophagus and GEJ, there are data to support changes in genes that normally block Rb-phosphorylation such as p16\(^{63,64}\). The protein p16 regulates progression through a phase of the cell cycle by binding CDK4. This limits CDK4-cyclin D complex formation and phosphorylation of Rb\(^{64}\). The tumor suppressor gene p16 plays an important role in the molecular progression of Barrett’s epithelium to invasive esophageal adenocarcinoma\(^{63-65}\). Information about its prognostic value is limited to one study\(^{66}\). This study found that in 39\% of the patients, the promoter region was hypermethylated (and thus functionally repressed) for p16. This hypermethylation was related with a strong trend for shorter survival in patients with adenocarcinoma of the esophagus.

p21
This gene is a regulator of cell cycle progression and encodes a potent cyclin-dependent kinase inhibitor, which binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes. This protein can interact with PCNA (and thus inhibits cell growth) and plays a regulatory role in DNA replication and DNA damage repair as member of the p53 signalling pathway. There is no evidence regarding the prognostic value of p21 protein in patients with adenocarcinoma of the esophagus. However, p21 is activated after DNA damage by ionizing radiation, which causes cell cycle arrest and apoptosis. It may therefore be a causative factor of radioresistance and has been studied as a response predictor after neoadjuvant chemoradiation therapy. Tumors demonstrating a rise in levels after treatment had worse long term survival compared to those showing no rise\(^{67}\).

Adenomatous polyposis coli (APC)
APC is a tumor suppressor gene that blocks cell proliferation by binding cellular signal proteins and by inducing differentiation. Disruption of any of its components results in loss of cell-cell adhesion due to disruption of normal cell-cell adhesion through altered association of the E-cadherin-catenin complex\(^{38}\). The prevalence of mutations in the APC gene is low in adenocarcinoma of the esophagus compared with colon cancer\(^{68}\). In adenocarcinoma of the esophagus, the APC gene is inactivated by an alternative mechanisms. Allelic deletion (LOH) on chromosome 5q, the part where the APC gene is located, frequently occurs\(^{69}\). Besides LOH, hypermethylation of the promoter region of the APC gene occurred in 92\% of the patients with esophageal adenocarcinoma. Hypermethylated APC DNA was observed in the plasma of 25\% of adenocarcinoma patients. A high plasma level was significantly associated with reduced patient survival\(^{70}\). This can be explained by two possible reasons. First of all, tumors shedding large amounts of DNA into the blood are more aggressive or advanced, and second tumors containing a greater proportion of methylated cells are more aggressive or advanced\(^{70}\). Large-scale testing is warranted to confirm the potential value of plasma-hypermethylated APC DNA as a biomarker to support esophageal cancer staging.
Transforming growth factor β (TGF-β) en SMAD4

Cell cycle progression of normal epithelial cells is inhibited by exogenous TGF-β, whereas malignant epithelial cells are often insensitive to the growth-inhibitory effects of TGF-β. The SMAD protein family is a family of intracellular signal transducers that acts downstream of receptors for TGF-β and regulates gene expression in this pathway. TGF-β belongs to a family of cytokines and has effects on cell development, cell growth and various cell functions by binding to transmembrane receptors (TGF-β-receptor). TGF-β may have both negative and positive effects \(^71,72\). Early in carcinogenesis it acts as a tumor suppressor but later it acts as a stimulator of tumor invasion by prompting extracellular matrix production and angiogenesis, stimulating tumor proliferation, and inhibiting host immune functions. The growth stimulatory action of TGF-β on mesenchymal cells around the tumor is mediated via autocrine growth factors such as connective tissue growth factor (CTGF) \(^73\). There is evidence to suggest that the TGF-β signaling pathway is involved in the initiation and progression of esophageal adenocarcinomas. An elevated level of plasma TGF-β1 measured from the azygos vein was independently correlated with survival in patients with both squamous cell carcinoma and adenocarcinoma of the esophagus \(^71\). Loss of expression of the functional receptor for TGF-β is also associated with adenocarcinoma of the esophagus \(^63\). Endoglin is a member of the TGF-β-receptor complex. Endoglin staining showed positive correlation with the presence of angiolymphatic invasion, lymphatic metastases, tumor stage, and survival. Furthermore the presence of CTGF gene expression in adenocarcinoma of the esophagus influenced survival negatively \(^73\). TGF-β signaling offers an attractive target for cancer therapy. Several strategies to target this molecule have been developed and are currently being tested in clinical trials \(^74\).

The SMAD4 protein acts downstream of receptors for TGF-β. In gastric cancer and squamous cell cancer of the esophagus, a higher Smad4 expression was related with a better prognosis \(^75,76\). In patients with adenocarcinoma of the esophagus and GEJ, SMAD4 expression is a frequent event, but its prognostic effect remains unknown \(^77\).

(3) Avoidance of apoptosis

The ability of tumor cells to expand in number is not only determined by the rate of cell proliferation (see above), but also by the rate of programmed cell death (apoptosis). The apoptotic machinery comprises several signaling pathways. Once the apoptosis pathway is triggered, the cell is destroyed within 30-120 minutes. In this way apoptosis provides a protective mechanism by removing DNA-damaged, senescent or diseased cells. The functions of key molecules that either sense DNA damage or that commit cells to die are lost during cancer progression. Apoptosis has relations with the cell cycle molecules described above. Hence, cell cycle arrest is a survival mechanism that provides the tumor cell the opportunity to repair its own damaged DNA. Abrogation of cell cycle checkpoints, before DNA repair is complete, can activate the apoptotic cascade.
One mechanism through which cancer cells avoid apoptosis is by interfering with agents like p53 which normally activates apoptosis\(^\text{29,36}\). Other regulators of apoptosis such as members of the Bcl-2 family (e.g. Bcl-2, Bcl-XL, Bax, Bad) and inhibitors of apoptosis (e.g. survivin) have also been shown to play a pivotal role in allowing cancer cells to escape apoptosis. The members of this family are either pro- or anti-apoptotic, they interact with each other to regulate apoptosis. Inhibiting the heterodimerisation between pro- and anti-apoptotic members is sufficient to promote apoptosis.

Death receptors are a component of the death-commitment pathways that can be altered by cancer cells to avoid apoptosis. The binding of a death receptor on the cell surface (e.g. Fas) with a death-promoting ligand (e.g. FasL) activates the apoptotic cascade\(^\text{29,36}\). Cancer cells can also block Fas so that FasL cannot bind anymore\(^\text{38}\). Cells can also avoid apoptosis is by increased synthesis of agents that normally inhibit death pathways (e.g. cyclooxygenase-2 (COX-2))\(^\text{29,36}\).

**p53**

p53 is a nuclear tumor suppressor protein involved in the maintenance of genomic integrity. DNA damage results in increased expression of p53, and subsequently causes growth arrest. It can induce factors that allow DNA repair or, if the damage is too great, cause apoptosis. In cases of nonrepairable damage, p53 activates the caspase cascade, leading to apoptosis. p53 can also trigger apoptosis itself by shifting into mitochondria where it interacts with antiapoptotic Bcl proteins, and eventually induces cytochrome c, which is a potent catalyst of apoptosis\(^\text{38,63}\).

Mutations and deletions of the p53 gene are the most common genetic lesions in human cancers. In adenocarcinoma of the esophagus, p53 alterations have been detected in 52-93% of patients\(^\text{38}\). p53 protein overexpression measured with immunohistochemistry is related with survival\(^\text{78}\) and p53 mutations are related with a more aggressive malignant phenotype. p53 gene mutations within the tumor were associated with a worse prognosis in patients with curatively resected adenocarcinoma of the esophagus and GEJ\(^\text{79-81}\). In two studies it is even an independent prognostic factor\(^\text{79,81}\). Furthermore, alteration of p53 expression is also related to chemotherapy response and prognosis in patients treated with neoadjuvant chemoradiation therapy\(^\text{67,79}\). This can be explained by the fact that p53 plays a role in cellular response to radiation damage. Cells that either lack p53 gene expression or overexpress a mutant p53 do not exhibit a G1 arrest and function as some kind of a cell cycle “checkpoint.”

**The Bcl-2 family**

The Bcl-2 family consists of about 20 homologues of important pro- and anti-apoptotic regulators of programmed cell death (e.g. Bcl-2, Bcl-XL, Bax, Bad). This function is based on the preservation or disturbance of mitochondrial integrity, thereby inducing or preventing release of apoptogenic factors such as cytochrome c. The Bcl-2 proto-oncogene encodes a protein that blocks apoptosis. In adenocarcinoma of the esophagus, Bcl-2-positive patients...
Survivin
Survivin forms an inhibitor of apoptosis protein and is necessary for anti-apoptotic function. In a mix of both squamous and adenocarcinomas of the esophagus surviving immunostaining was detected in 95% of the tumors. The level of expression and the number of proliferating cells was related with each other. However, no correlation was seen with survival.

Nuclear factor kappa B (NF-κB)
NF-κB normally resides in its inactive form in the cytoplasm. If activated it can translocate into the nucleus and provoke a response. Signals such as cytokines (TNF-α, IL-1, and IL-18), viral and bacterial pathogens and cytotoxic agents induce activation and translocation of NF-κB into the nucleus. NF-κB is involved in the protection against apoptosis. The activation of NF-κB protects from cell killing and its inhibition enhances apoptotic killing by TNF, radiation and chemotherapy. Around 60% of adenocarcinomas of the distal esophagus and GEJ displayed NF-κB immunoreactivity. The absence of NF-κB activity significantly correlated with response to chemoradiotherapy and was associated with a better survival. Furthermore, celecoxib inhibits NF-κB activation through inhibition of Akt activation. Consequently COX-2 and other genes are down-regulated. NF-κB inhibitors may be used as a novel approach in the prevention and management of adenocarcinoma of the esophagus and GEJ.

Cyclooxygenase (COX)-2
COX-2 plays a central role in the production of prostaglandins and is a mediator of angiogenesis and tumor growth. It is inducible through the action of cytokines and endotoxins which normally block cellular death pathways. Cancer cells might avoid apoptosis by increased synthesis of COX-2. Downstream proangiogenic actions of these products include: VEGF; enhanced endothelial cell survival via Bcl-2 expression and Akt signaling; induction of MMPs; activation of EGFR-mediated angiogenesis; and suppression of IL-12 production.

Patients with adenocarcinoma of the esophagus with high COX-2 expression are more likely to develop distant metastases and local recurrences, and survival was independently associated with reduced survival. This effect was not so pronounced in adenocarcinomas that originated from the cardia. Selective inhibition of COX-2 activity suppresses angiogenesis and induces apoptosis. Therefore, COX-2 inhibitors can possibly be used for the treatment of adenocarcinoma of the esophagus and GEJ.

(4) Limitless replicative potential
Cells carry an intrinsic, cell-autonomous program that limits their multiplication. To prevent mutations, cells are allowed to undergo only a certain amount of multiplications. This program appears to operate independently of the cell-to-cell signaling pathways described above. At some point during the course of tumor progression tumor cells must breach this mortality
barrier to acquire unlimited replicative potential. The counting device for cell generations has been discovered over the past decade: the ends of chromosomes, telomeres, which are composed of several thousand repeats of a short 6 base pair (bp) sequence element. Replicative generations are counted by the loss of 50–100 bp telomeric DNA from the ends of every chromosome during each cell cycle. After a certain number of divisions, telomeres are too short to protect the chromosomes. The shortened telomeres trigger exit from G1 into a permanent growth-arrest (G0).

To reach a state of unlimited multiplication, tumor cells must stabilize their telomeres. Most tumors succeed in doing so by upregulating expression or reactivation of telomerase (which can counteract telomere erosion)\(^29,36\). Telomerase activity did not correlate with survival in patients with adenocarcinoma of the distal esophagus and GEJ\(^90\), but telomerase inhibition may give new therapeutic options in future\(^91,92\).

(5) Sustained angiogenesis
The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. The development of blood vessels (angiogenesis) is essential for the development, progression, and dissemination of malignant tumors.
Counterbalancing positive (VEGF) and negative signals (thrombospondin-1) encourage or block angiogenesis. The VEGFs are a family of potent angiogenic growth factors that stimulate endothelial cell proliferation and migration, and many human tumors sustain angiogenesis by increasing the expression of VEGFs. Specific VEGF/VEGF-R inhibitors (e.g. bevacizumab) are developed and may offer an attractive therapeutic option in future\(^29,36\).

The vascular endothelial growth factor (VEGF)
The vascular endothelial growth factor (VEGF) family consists of a few related glycoproteins. They contribute to tumor angiogenesis and presumably tumor growth and hematogenous spread of the tumor. VEGF-A is mainly associated with angiogenesis and induces endothelial sprouting through proliferation and migration of endothelial cells and protects endothelial cells from apoptosis. VEGF-C and D are mainly associated in the development and sprouting of lymphatic vessels (lymphangiogenesis)\(^93\). VEGF A and -C are possibly regulated by COX-1 en COX-2\(^94,95\).
Neovascularization is an early effect in the transition from Barrett’s metaplasia to cancer. This might be a reason for the early onset of local spread and distant recurrence. Neovascularization in adenocarcinoma of the esophagus is associated with survival. Patients with a low neovascularization coefficient had significantly better survival compared with patients with a high neovascularization coefficient\(^96\). However, as far as the VEGF prognostic value per se is concerned, VEGF fails to be of prognostic significance\(^93,97\).

(6) Invasion and metastasis
Adenocarcinoma of the distal esophagus and GEJ is known for its early lymphatic and hematogenous spread. Successful invasion and dissemination depend upon all of the five
(above mentioned) acquired hallmark capabilities. However, abnormalities in cell-cell adhesion molecules (CAMs) also play an important role. CAMs such as the cadherin glycoproteins normally function as glue that holds cells together and are mediators of cell-cell interactions. E-cadherin is expressed on the surface of epithelial cells, is linked to the actin cytoskeleton through interactions with catenins in the cytoplasm and forms bridges with other cells. Disrupted cell-cell adhesion might lead to metastases. Changes in expression of CAMs in the immunoglobulin superfamily also appear to play critical roles in the processes of invasion and dissemination. Changes in integrin expression are also evident in invasive and metastatic cells. Successful colonization of tumor cells at new sites (local and distant) demands adaptation, which is achieved by shifts in the spectrum of integrins.

The second general parameter of the invasive and metastatic capability involves extracellular proteases, which can destroy the surroundings of the tumor cell. Protease genes can be upregulated and protease inhibitor genes can be downregulated. Furthermore, inactive zymogen forms of proteases can be converted into active enzymes. Matrix-degrading proteases are associated with the cell surface and docking of active proteases on the cell surface can facilitate invasion by cancer cells into nearby stroma, across blood vessel walls, and through normal epithelial cell layers.

Cadherins
The cadherins are a family of integral membrane glycoproteins, which are the prime mediators of cell–cell adhesion in normal cells. When cadherins are expressed, the inactivation of other cell–cell adhesion molecules has little or no effect. The members of the cadherin family are involved in adherens junctions and are components of desmosomes. E-cadherin plays an important role in the induction and maintenance of normal architecture in human tissues. The cytoplasmic domains of E-cadherin bind to the actin skeleton of the cell through catenins (α- and β-catenin). These catenins are closely related with the cadherins. Genetic alterations of the genes encoding the catenins (particularly β-catenin) lead to reduced cell–cell adhesivity. Apart from cell adhesion molecular function, β-catenin participates in Wnt signaling (Wnt proteins involve three intracellular signaling pathways) and activates oncogene transcription by complexing with T-cell factors (TCF) and can influence the transcription of genes such as the cyclin D1 cell cycle regulator. The analysis of E-cadherin, α-catenin, and β-catenin expression in adenocarcinoma of the esophagus revealed that reduced expression of all three proteins correlated with decreased patient survival. The expression of E-cadherin and β-catenin was independently associated with poor prognosis.

Integrins
Integrins are transmembrane glycoproteins found at cell adhesion sites. The extracellular domains of integrins function as cell surface receptors for extracellular matrix (ECM) molecules. Their intracellular domains connect to the actin cytoskeleton. Integrins must be stimulated to undergo binding to the ECM. Activation of integrins may occur through local stimuli from soluble mediators (see above) or by solid interfaces (ECM or other cells). Inactivation
of integrin mediated adhesion is also necessary to prevent binding at inappropriate times or locations. In adenocarcinoma of the distal esophagus and GEJ no correlation between survival and the expression of integrins was found\textsuperscript{39,100}.

**CD44**
The CD44 transmembrane glycoproteins bind to ligands in the extracellular matrix such as collagen, and laminin. CD44 allows for the attachment of circulating lymphocytes to vascular endothelium, in addition to binding epithelial and stromal cells to each other or to the intercellular matrix\textsuperscript{98}. In patients with adenocarcinoma of the esophagus CD44s and a splice variant (CD44\textsubscript{v4}) showed a strong correlation with prognosis, which was independent in the case of CD44\textsubscript{v4}. Soluble forms of CD44 may be detected in the serum of patients with cancer and may correlate with prognosis\textsuperscript{101}. However in adenocarcinoma of the esophagus and GEJ this factor is still unknown\textsuperscript{98}.

**Serine protease system and urokinase-type plasminogen activator (uPA)**
The serine protease system plays an important role in the invasive potential of cancers, by breaking down components of the extracellular matrix. Elevated levels of urokinase-type plasminogen activator (uPA) have been implicated in this invasive process\textsuperscript{63,102}. A specific receptor binds uPA to cell surfaces and enhances plasmin generation. Plasmin can degrade most components of the extracellular matrix either directly or through the activation of some procollagenases and can activate latent growth factors. In patients with adenocarcinoma of the esophagus urokinase-type plasminogen activator (uPA) content was an independent prognostic factor for survival\textsuperscript{102}.

**Matrix metalloproteinases (MMPs)**
MMPs are involved in the degradation of different components of the extracellular matrix. These MMPs have been classified into collagenases, gelatinases and stromelysins. The MMPs are important in the initial stages of tumor invasion as they degrade components of the basement membrane and extracellular matrix. Furthermore they can degrade the basement membrane of vessels which is necessary to invade into blood and lymph vessels\textsuperscript{103-105}. In esophageal cancer MMP-1 is important in tumor spread. In a study, containing tissue samples of both squamous cell carcinoma and adenocarcinoma, MMP-1 positive staining was an independent prognostic factor\textsuperscript{103}. In another study MMP-3 was related with survival\textsuperscript{106}. In superficial carcinomas, MMP-7 and MMP-9 positive staining on the invasive site were related to survival\textsuperscript{104}. Inhibitors of MMPs have been developed and might restore normal balance of proteolytic activity. In patients with gastric cancer an MMP inhibitor showed a positive therapeutic effect\textsuperscript{107}.

**Tissue inhibitor of metalloproteinases (TIMP)**
TIMPs form a complex with MMPs leading to inactivation. Disruption of the balance between MMPs and TIMPs may influence tumor invasion and dissemination. TIMP-3 inhibits MMP-1,
MMP-2 and MMP-3 and a reduced expression of TIMP-3 was associated with increased tumor invasiveness and reduced patient survival\textsuperscript{108}.

(7) Other factors
There are some genetic alterations in adenocarcinoma of the esophagus and GEJ that do not fit into the above mentioned classification system of six oncogenic hallmarks.

DNA content and chromosomal abnormalities
A normal cell has a chromosome number of 2N, for which the term diploid is applied. Cells reproduce by duplicating their content (4N) and then dividing in two. A cell with numeric chromosomal aberrations is called aneuploid\textsuperscript{63}. This chromosomal instability is associated with defects in mitotic checkpoint genes. In patients with adenocarcinoma of the distal esophagus, DNA ploidy was independently associated with a worse survival\textsuperscript{109-111}.

Another study showed that allelic loss of both the short arm of chromosome 17 on which p53 is located and the long arm of chromosome 18 on which DCC is located in adenocarcinoma of the esophagus and GEJ were associated with a worse survival\textsuperscript{112}.

Promotor hypermethylation of multiple genes
Gene promoter hypermethylation of individual genes can have a prognostic effect as described in a few cases above. However, in one study, hypermethylation of promoters of multiple tumor suppressor genes was performed. Patients whose tumors showed methylation of their gene profile with >50% had a significantly worse survival in uni- and multivariate analysis\textsuperscript{66}.

Th1/Th2 balance
Prognosis of cancer may be affected not only by the biological behavior of the malignancy, but also by host defense mechanisms. Some studies have shown that a growing tumor burden correlates with progressive immunological changes. The balance between T-helper type 1 (Th1) and type 2 (Th2) lymphocyte functions is an important immunological parameter.

Patients with adenocarcinoma of the esophagus and GEJ demonstrated a shift in the Th1/Th2 balance—in favor of Th1—compared with healthy volunteers. The ability of T cells to produce IL-2 was related to survival in univariate analysis\textsuperscript{113}. Preoperative immunotherapy receives much attention and IL-2 in stomach cancer had a positive effect on overall and disease-free survival and can be a potential target in future\textsuperscript{114}.

C-reactive protein (CRP)
CRP is a prototype acute phase protein and is synthesized in hepatocytes in response to inflammatory changes, but can also be expressed by cancer cells. CRP can be detected in serum and its expression can be induced by a pro-inflammatory cytokine (esp. IL-6), which also acts as a growth factor in cancers. In adenocarcinoma of the esophagus, patients with an elevated CRP level in the serum have a worse survival\textsuperscript{115}. 
Parathyroid hormone-related peptide (PTHrP)

Hypercalcemia is among the most frequent paraneoplastic syndromes, and it is estimated that hypercalcemia affects approximately 20% of patients during the advanced stages of malignant disease. Parathyroid hormone-related peptide (PTHrP) is a tumor-derived circulating factor that has been associated with hypercalcemia in cancer patients. PTHrP mimics the actions of parathyroid hormone on the kidneys and bone to increase serum calcium concentration. Elevated serum PTHrP levels were present in 20% of patients with adenocarcinoma of the GEJ and was associated with markers of systemic inflammation and with an adverse prognosis116.

New concepts

Research in oncology is ever evolving and new concepts regarding dissemination are constantly developed. Many of these promising concepts have not (yet) been tested on adenocarcinoma of the esophagus and GEJ and their prognostic value is thus still unknown. One attractive concept is about the role of stem cells. Stem cells have the function to maintain the integrity of tissues such as the intestinal epithelium and have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation. Growing evidence suggests that pathways (Wnt-, Hedgehog and Notch signaling) regulate the self-renewal of normal stem cells and are deregulated in cancer stem cells117. This results in the continuous expansion of self-renewing cancer cells and cancer formation118. Wnt-5a impairs tumor cell migration and thus reduces invasiveness in diverse cancers119-121, but has never been tested in adenocarcinoma of the esophagus and GEJ. Hedgehog signalling is also activated in carcinogenesis122. Recently, it was shown that hedgehog signalling in esophageal cancer is activated by ligand expression123. Notch ligand genes such as JAG1 are expressed in esophageal and gastric cancers124 and are negatively associated with prognosis in breast cancer125. Although the stem cell field is embroiled with political and ethical controversy, it may hold a promise for the treatment of cancer in future. Another concept is epithelial-mesenchymal transition (EMT). This is a process by which epithelial cells acquire mesenchymal fibroblast-like properties and consequently show reduced adhesion and increased motility. Both properties are important in the development of dissemination126-128.

Discussion

Molecular cancer research has generated an immense amount of information concerning the progression of adenocarcinoma of the distal esophagus and GEJ. Many studies showed diverse prognostic molecular factors, but the prognostic effect of many known predictors in other cancers (e.g. adenocarcinoma of the stomach and or squamous cell carcinoma of the esophagus) are unknown in adenocarcinoma of the distal esophagus or GEJ. Molecular
prognostic factors are still not included in prognostic models such as the TNM classification. The main reason for this is that molecular biological research is rapidly evolving and an astonishing number of biomarkers have been described, but sufficiently large studies about the prognostic value of one specific gene or protein are still lacking. Another drawback of many studies is that squamous cell- and adenocarcinoma samples are combined to draw conclusions. These results may be difficult to interpret because squamous cell cancers form a different histological subtype and have a different pathogenesis and perhaps clinical behavior.

Adenocarcinomas of the distal esophagus and GEJ show multiple genetic alterations, which indicate that this progression of cancer is a multistep complex process. Presumably, it is not one molecular factor that can predict the biological behavior of this cancer. The combination of diverse genetic alterations may better predict the patient’s prognosis. In other cancers, gene expression analysis with microarrays revealed important prognostic information and discovered new pathways. However, in adenocarcinoma of the distal esophagus and GEJ, microarray research is (thus far) limited to the progression of Barrett’s metaplasia into (early) cancer.

Drug development has already been transformed with the identification and ability to direct treatment at specific molecular targets (e.g. COX-2-, VEGF- and tyrosine kinase inhibitors). In esophageal cancer these novel targeted treatments are in its infancy although many phase I trials are currently being conducted. In the near future, the targeting of major signaling pathways (e.g. mitogen-activated protein kinase signaling), the targeting of multiple molecules together and the discovery of new pathways associated with tumor progression and dissemination will enhance the therapeutic options of patients with this type of cancer and will give clinicians the ability, to not only give (neo)adjuvant chemoradiotherapy on basis of based on prognostic information, but also to tailor individually targeted therapy based on molecular biology. It is likely that therapeutic intervention at the level of genes and molecules with great prognostic power and its signaling pathways will have the largest impact on long term survival.

References


