The development of new treatment strategies for oesophageal cancer

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So far, the molecular pathways involved in the progression from premalignant Barrett’s oesophagus to invasive oesophageal adenocarcinoma remain largely unknown.

**Current insight into the molecular biology of Barrett’s oesophagus and its potential clinical application**

This report reviews some of the molecular pathways identified and assesses how insights in these genetic events can contribute to the development of potential molecular markers stratifying a patient’s risk of progressing to oesophageal adenocarcinoma, and the development of new therapeutic approaches.
INTRODUCTION

Since 1970, the incidence of adenocarcinoma of the oesophagus has increased in western countries at a rate that exceed that of any other malignancy. It is now generally accepted that oesophageal adenocarcinomas develop from a premalignant lesion of the oesophagus, also referred to as Barrett's oesophagus. Barrett's oesophagus is a metaplastic change of the normal squamous cell epithelium of the oesophagus to a columnar type as result of longstanding gastro-oesophageal reflux. Three subtypes of Barrett's have been described, but the specialised intestinal type is the only subtype clearly associated with malignant transformation. Endoscopic surveillance can detect oesophageal adenocarcinomas in an early and curable stage, but most tumours are detected at an advanced stage. Until now, surgery is the best curative option, but even after a potentially curative resection long-term survival rates rarely exceed 25%. Extensive research on how to improve survival has been performed. Over the past decades, surgeons have had an important role in improving the management of oesophageal cancer. However, more extensive resections seem to increase overall survival only to a limited extent and it is unlikely that the recently reported five-year survival rates of oesophageal cancer will be further improved by surgery alone. The addition of (neo-)adjuvant chemo/radiotherapy has shown some beneficiary effect in several trials but even with the current advances in multimodal therapy, the prognosis for invasive oesophageal adenocarcinoma remains poor. A better understanding of the molecular evolution of the Barrett's metaplasia to dysplasia to adenocarcinoma sequence may allow improved diagnosis, therapy and prognosis.

MOLECULAR BIOLOGY OF BARRETT'S ADENOCARCINOMA

Proliferation and apoptosis
The cell cycle comprises intracellular events that occur during the period between two mitotic divisions. The cycle is divided into four phases called G1 (first gap) in which RNA and proteins needed for DNA replication are synthesised, S (DNA synthesis) phase, G2 (second gap) in which the tetraploid cell prepares for the upcoming mitotic division, and M (mitosis) phase. Late in the G1 phase, there is a critical juncture called the 'restriction-point' (R-point) which is the final check-point before the cell either completes or exits the cell cycle. After mitosis, cells may withdraw from the cell cycle to enter a quiescent
state termed G0. Under certain conditions, such cells can be stimulated to leave the G0 phase and re-enter the cell cycle (Figure 1). Genetic alterations can cause the transformation of a normal cell into a malignant tumour cell. Most tumour cells are characterized by genomic instability, facilitating the accumulation of mutations. This genomic instability occurs in two forms: microsatellite instability (MSI) and chromosomal instability. The targets of genomic instability are protooncogenes, tumour suppressor genes, mismatch repair genes and mitotic checkpoint genes. These altered genes, which ultimately exert their effect via the cell cycle apparatus, render the cell independent of regulated proliferative and cell death pathways. Hyperproliferation has been demonstrated by immunostaining for Ki-67 and proliferating cell nuclear antigen (PCNA) in metaplastic Barrett’s epithelium with a significant increase in the number of Ki-67 positive nuclei in high-grade dysplasia and oesophageal adenocarcinoma. Apoptosis, or programmed cell death, is responsible for cell loss and provides a protective mechanism by removing DNA-damaged cells that may cause neoplastic proliferation.

**FIGURE 1**
Overview of the cell cycle. Mitosis (M phase) is the process of nuclear division. Replication of diploid 2n DNA into tetraploid 4n DNA occurs in the S (synthesis) phase. The interval between M phase and S phase is called the G1 (gap) phase, and the interval between the end of the S phase and the beginning of the M phase is the G2 phase. Cells can exit the cell cycle and enter the G0 phase, which is the quiescent state. R-point is the restriction point late in the G1 phase, which is the final check-point before the cell either completes or exits the cell cycle.
Apoptosis can be detected immunohistochemically by staining DNA fragmentations as markers for apoptosis. An increase has been detected in the apoptotic rate with increasing histologic severity in Barrett's dysplasia and adenocarcinoma, whereas others found few apoptotic cells in the Barrett's metaplasia-dysplasia-carcinoma sequence.

Genes involved in controlling the cell cycle
Various pathways involved in cell cycle progression and inhibition are illustrated in Figure 2.

FIGURE 2
Pathways involved in cell cycle progression and inhibition.
Abbreviations: EGF = epidermal growth factor; TGF-α = transforming growth factor α; EGFR= epidermal growth factor receptor; MAPK = mitogen activated protein kinase; COX-2 = cyclooxygenase-2; PGE-2 = prostaglandin E2; APC = adenomatous polyposis coli; GSK-3 = glycogen synthase kinase-3; TCF = T cell factor; LEF-1 = lymphoid enhanced binding factor-1; Cdk = cyclin dependent kinases; Rb = retinoblastoma.

RETINOBLASTOMA PROTEIN
The retinoblastoma (Rb) tumour suppressor gene appears to be the molecular switch that controls progression through the R-point. Rb protein that is not phosphorylated functions to block cell cycle progression. Phosphorylation inactivates the Rb protein, allowing the cell cycle
to advance through the R-point. So far, no studies have demonstrated mutation of the Rb gene in Barrett's oesophagus or Barrett's carcinoma, but the loss of allele 13q, the locus for the Rb gene, has been detected in up to 50% of oesophageal cancers.\textsuperscript{15}

**CYCLINS AND CYCLIN-DEPENDENT KINASES** Phosphorylation and inhibition of the Rb protein occurs by interaction with cyclin-dependent kinases (cdks) and cyclins. Cyclins D1 and E function as homing vehicles to carry the cdks4 and 6 to their Rb protein target thereby allowing the cell to enter the cell cycle. An increased nuclear expression of cyclin D1 protein has been observed in 22\%-64\% of oesophageal adenocarcinomas and is already present in Barrett's metaplasia.\textsuperscript{16, 17} Overexpression of cyclin E has not been detected in Barrett's oesophagus, but has been found in 18\% of the patients with high-grade dysplasia and in 14\% of the adenocarcinomas.\textsuperscript{18} The p16 and p15 genes encode proteins that form complexes with the cdk4 and 6, inhibiting their ability to phosphorylate the Rb protein. Thus, inactivation of these genes may lead to uncontrolled cell growth. P16 gene mutations with loss of heterozygosity have been reported in 23\% of oesophageal adenocarcinoma\textsuperscript{19} and two studies showed that p16 promoter methylation is a common mechanism of p16 inactivation during neoplastic progression in Barrett's oesophagus that is already present in metaplastic Barrett's epithelium.\textsuperscript{20, 21}

**P53** The tumour suppressor p53 can stop cell cycle progression by inhibiting the actions of cyclin D1 and E and preventing phosphorylation of Rb protein, thereby allowing the cell to repair its damaged genome. The prolonged half-life time of the mutant p53 protein and the concomitant increased cellular p53 concentration make visualization by immunohistochemistry possible. Immunostaining for p53 has been found in metaplastic Barrett's epithelium and is detected with increasing frequency up to 40\% as dysplasia progresses in severity.\textsuperscript{10, 22, 23} For oesophageal adenocarcinoma, p53 mutation is one of the most frequently detected alterations approaching 100\%.\textsuperscript{22, 23} Loss of heterozygosity (LOH) of 17p, the p53 locus, has been found at similar high rates (52-100\%) of oesophageal adenocarcinomas.\textsuperscript{24} LOH of 17p has also been detected in the nonmalignant metaplastic cells, suggesting that inactivation of p53 is an early step in the carcinogenesis of Barrett's carcinomas.\textsuperscript{25}

Growth factors and their receptors

**EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-\alpha** The binding of growth factors to the Erb family of receptor tyrosine kinases can promote cellular proliferation by activating signal transduction cascades that induce the
expression of cyclin D1. The growth factor ligands epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have been implicated in the development of adenocarcinomas in Barrett's oesophagus. Increased expression of TGF-α and the EGF receptor (also called ErbB-1) have been detected in metaplastic Barrett's epithelium as well as Barrett's carcinomas, whereas EGF overexpression was only found in oesophageal adenocarcinomas.26,27 The role of the oncogenic form of the normal receptor tyrosine kinase ErbB-2 (c-ErbB-2 or HER-2/NEU) is still debated but some studies found 10–30% overexpression in adenocarcinomas with an association to decreased overall survival rate.29,30 Overexpression was not demonstrated in dysplastic Barrett's oesophagus, suggesting that it is a late event in the dysplasia-carcinoma sequence.31

TRANSFORMING GROWTH FACTOR-β In contrast to TGF-a, transforming growth factor-β (TGF-β) can exert inhibitory effects on normal cell growth. One mechanism is by inducing transcription of the p16 and p15 genes (described above). TGF-b1 has two membrane receptors, type I and II, that have to be activated for appropriate signalling.32 Loss of expression of the TGF-β receptor II appears to be associated with Barrett's oesophagus and adenocarcinomas, but the exact role of TGF-β1 and its receptors needs to be clarified.33,34

RAS FAMILY Growth factors binding to their tyrosine kinase receptors often activate membrane-associated Ras proteins which activate signal transduction cascades. Ras signals can either induce cellular proliferation by activation of the Ras/Raf/mitogen activated protein kinase (MAPK) pathway that activates cyclin D1 or inhibit proliferation by inducing expression of p16 and p15 proteins that block cyclin D1-cdk complexes.35 Oncogenic Ras (H-Ras or K-Ras) was the first major class of cancer-related genes to be evaluated in Barrett's associated neoplasms, but an important role could not be established. H-ras mRNA expression has not been detected in intestinal metaplasia, and mutations in codons 12 and 13 of K-ras (areas frequently mutated in extra-oesophageal malignancies, especially pancreatic cancer) have only been found rarely in dysplasia and Barrett's carcinomas.36,37

CYCLOOXYGENASE-2 Another enzyme that can induce the activation of the Ras/Raf/MAPK pathway is the cyclooxygenase (COX) protein. Cyclooxygenase catalyzes the rate-limiting step in prostaglandin synthesis.38 There are two different isoforms of COX: COX-1 and COX-2. COX-1 is constitutively expressed and is involved in processes like cytoprotection of the gastric mucosa, vasodilatation, and platelet aggregation. In contrast, COX-2 is normally absent in normal tissues but can be readily induced in inflammation
and carcinogenesis where it produces prostaglandin E2, which is involved in many processes fundamental for tumour development (e.g. resisting apoptosis, increasing cell proliferation, stimulating angiogenesis, and modulating the invasive properties of cancer cells). COX-2 upregulation has been demonstrated to be an early event during the carcinogenesis of Barrett's carcinomas with progressively enhanced expression during the various stages of oesophageal carcinogenesis up to 80-100% in adenocarcinomas on both mRNA and protein level. In addition, COX-2 overexpression was demonstrated to be an independent prognostic variable for patients with Barrett's carcinomas together with tumour stage and radicality of resection.

Cell-cell adhesion

CADHERINS AND CATENINS Cadherins comprise a large family of cell adhesion molecules that mediate cell adhesion. Cadherins bind to cytoplasmic proteins called catenins that are linked to the cell's actin cytoskeleton. In non-malignant epithelia, E-cadherin and the β-catenin show a membranous localization at intercellular borders. In Barrett's adenocarcinomas, reduced membranous expression of E-cadherin and the catenins is observed in 60-80% of the tumours. Moreover, reduced expression of E-cadherin and β-catenin correlated significantly with reduced overall survival, but so far no mutations of E-cadherin have been demonstrated in oesophageal cancers despite frequent loss of heterozygosity at its locus 16q22.

Wingless/wnt-pathway

APC PROTEIN Besides establishing cell-cell adhesion, β-catenin also has a function in cell signalling. Under normal conditions, β-catenin is bound to the cytoplasmic tail of E-cadherin. Free, unbound β-catenin is degraded via phosphorylation by a complex of the adenomatous polyposis coli (APC) protein, glycogen synthase kinase-3 (GSK-3) and axin. Inactivation of the APC protein leads to free β-catenin accumulation in the cytoplasm which can enter the cell nucleus where it binds to and activates the T cell factor/lymphoid enhanced binding factor-1 (TCF/LEF-1) family of transcription factors that mediate the expression of growth related target genes such as the oncogene c-myc and cyclin D1. Besides inactivation of APC, mutations in the phosphorylation sites of the β-catenin gene itself can also lead to stabilisation of the protein. In oesophageal adenocarcinomas, increased cytoplasmic and nuclear localisation of β-catenin has been observed, implying activation of the signal transduction wingless/wnt-pathway. However, inactivation of APC is rare and no mutations in β-catenin have been detected in oesophageal adenocarcinomas. This may imply that other proteins that function in this pathway are involved.
Telomeres

While resistance to apoptosis, insensitivity to growth-inhibitory signals and growth signal autonomy all result in unregulated cell growth, a protective intrinsic mechanism exists which limits the proliferative capacity of normal human cells. This autonomous mechanism of physiologic telomeres shortening must be disrupted in order for a clone of abnormal cells to expand into a macroscopic tumour. Telomerase is a ribonucleoprotein complex that synthesizes telomeric DNA located at the chromosome ends, thereby maintaining telomere length. Only weak levels of telomerase RNA have been detected in normal squamous oesophageal epithelial cells by using in situ hybridisation. In contrast, moderate to strong levels of telomerase RNA expression was seen in 70% of the Barrett's metaplasias, in 90% of the low-grade dysplasias, and in 100% of the high-grade dysplasias and oesophageal adenocarcinomas.

The various genetic alterations involved in the progression from premalignant Barrett's oesophagus to invasive oesophageal adenocarcinoma are summarised in Figure 3.

**Figure 3**
Genetic alterations involved in the progression of Barrett's metaplasia toward Barrett's adenocarcinoma.
DEVELOPMENT OF NEW DIAGNOSTIC MODALITIES AND NOVEL TREATMENT STRATEGIES

Detection and treatment of early lesions
The prognosis of patients who present with symptomatic oesophageal cancer is poor. Due to endoscopic surveillance programs of patients with a Barret's oesophagus, an increased proportion of patients has lesions detected at an early stage (HGD or pT1) with a significantly improved 5-year survival rate of 80-100%. Unfortunately, detection of HGD or early carcinoma in a Barrett's oesophagus can be difficult due to heterogeneity of the Barrett's epithelium that cannot be discriminated with standard endoscopy. The detection of these early lesions by random biopsies is hampered by sampling error, and the intra- and interobserver variation of histological classification of observed abnormalities. Several new endoscopic techniques have been studied for their contribution to accurate detection of early lesions in a Barrett's oesophagus, but so far methylene blue staining and magnification chromoendoscopy are the only techniques that have shown to increase the detection rate of HGD.

The presence of genetic alterations in metaplastic and dysplastic Barrett's epithelium might be used in the future surveillance of patients with Barrett's oesophagus since these molecular markers might identify a subset of patients with an increased risk of malignant degeneration. In this context, DNA-flowcytometry has been described as a promising technique. With this technique, the relative amount of DNA can be analysed, hereby detecting aneuploid cell populations and identifying an increase in the number of cells in the S or G2 phase. Patients with an increase of tetraploid or aneuploid cell populations in their Barrett's biopsies, showed a significantly higher risk for carcinoma development during follow-up (RR 7.5).

So far, the diagnosis of HGD or adenocarcinoma has been an indication for surgical resection. However, since subtotal oesophagectomy is associated with high morbidity and substantial mortality, local endoscopic therapies have been developed to treat these early lesions. There are two techniques that can be used: tissue ablation and/or mucosal resection. The limited invasiveness in comparison to surgical resection results in decreased procedure related mortality and morbidity and the preservation of a functional oesophagus is associated with an increased quality of life. Recent studies demonstrated that these techniques are feasible and safe, but long-term follow-up results have to awaited before it can be considered as gold standard.
Pharmacodynamic strategies

TYROSINE KINASE INHIBITION Inhibition of tyrosine kinase receptors through the use of targeted small molecule drugs or antibody-based strategies has emerged as a promising approach to cancer therapy. An antibody directed against the ligand-binding domain of the EGF receptor has been demonstrated to decrease proliferation and increase apoptosis and Herceptin (antibody against HER-2/NEU) has already been successfully introduced in the treatment of breast cancer and leukemia. An in vitro study with a synthetic molecule that targets the EGFR-ATP binding site, showed selective inhibition of EGFR tyrosine kinase activity and a decreased proliferation rate with synergistic potentiation of tumour cell killing in combination with chemotherapy or radiation for squamous cell carcinoma cell lines. For oesophageal adenocarcinoma, no results have yet been published, but the EGFR tyrosine kinase inhibitor gefitinib (Iressa®) is currently tested in patients with irresectable Barrett's carcinoma in a phase II trial.

RAS-MAPK PATHWAY INHIBITION Farnesyltransferase inhibitors are a novel class of compounds that block the critical enzymatic step in the formation of active Ras proteins. Lonafarnib is a tricyclic nonpeptidomimetic compound that is active against a variety of tumours in vitro and in animal models of cancer. In a phase I study promising results were obtained with 40% of patients with solid tumours (e.g. non-small cell lung cancer) showing durable partial response. Although no studies have been performed on oesophageal cancer, it might be hypothesised that inhibition of the Ras-MAPK pathway could have a role in the development of new treatment strategies for this malignancy.

SELECTIVE COX-2 INHIBITION The suggestion that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) may have a role as anticancer agents first came from epidemiological studies. Of the five published observational studies on oesophageal cancer, four demonstrated a protective effect of NSAIDs. Although the precise anticancer actions of NSAIDs are yet not fully explained, they involve inhibition of the cyclooxygenase-2 (COX-2) enzyme and inhibition of the wingless/wnt-pathway. In vitro studies and animal studies have been published which support a possible chemopreventive effect of selective COX-2 inhibitors in Barrett's epithelium. In primary cultured endoscopic biopsy specimens from patients with Barrett's oesophagus, selective COX-2 inhibitors significantly decreased proliferation of epithelial cells, and in a rat model selective COX-2 inhibitors reduced the relative risk of developing oesophageal cancer. These findings led to the initiation of phase
II and phase III randomised clinical trials on the prevention of oesophageal adenocarcinoma in patients with Barrett's oesophagus, which are currently being performed. Since COX-2 upregulation has also been demonstrated to be an independent prognostic variable for patients with oesophageal adenocarcinomas, several clinical trials have been started with NSAIDs as (neo-) adjuvant therapy.

**Gene therapy**

Cancer gene therapy can be defined as the introduction of new genetic material into a tumour cell aiming at the correction of absent or mutated genes, or inducing selective cell death. This new genetic material is delivered to a cell by vectors that can be either viral or non-viral. One approach that has been analysed for oesophageal carcinoma is based on the introduction of wild-type tumour suppressor genes (e.g. p53). Gene replacement therapy with the tumor suppressor gene p53 transduced in several oesophageal adenocarcinoma cell lines by adenoviral vectors, resulted in efficient apoptosis and a significant reduction in cell growth. Another gene therapy strategy using immunotherapy with transduction of TNF-α by an adenoviral vector under control of the EGR-1 promoter resulted in regression of subcutaneously injected oesophageal adenocarcinoma cell line tumours in nude mice after radiation. Another promising new gene therapy strategy is the use of replicating viral vectors to induce cell death by viral oncolysis. To obtain selective elimination of tumour cells, a conditionally replicating adenovirus, only replicating in p53-deficient cancer cells, has been developed (ONYX-015). Although so far no results for oesophageal adenocarcinoma have been published with this vector, a remarkable clinical effect was achieved with the use of ONYX-015 in combination with chemotherapy in patients with head and neck cancer. Since for oesophageal adenocarcinomas p53 mutation is one of the most frequent alterations identified (52-100%), ONYX-015 potentially could have a role in the management of these cancers.

**FUTURE DIRECTIONS**

There is need for improved understanding of the molecular biology of Barrett’s oesophagus and oesophageal adenocarcinoma since the exact pathogenesis is still poorly understood. Despite enormous progress in the characterization of molecular changes in Barrett’s oesophagus, the clinical impact of this acquired knowledge is still limited. Recently developed microarray technology and proteomics will probably contribute significantly in the...
attempt to unravel more of the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. Novel insights into these genetic events are critical for the development of potential molecular markers stratifying a patient's risk for progression to oesophageal adenocarcinoma, and the development of new therapeutic approaches. With this increasing knowledge, a larger subset of patients might be treated with early stage lesions, resulting in improved overall survival, and new treatment strategies for advanced carcinomas will become more specific since they are directed towards characteristic features of the cancer cell. First attempts in this context have been made with the development of EGFR inhibitors, selective COX-2 inhibitors, and gene replacement therapy, and the results of various clinical trials are anxiously awaited. In the next years, it will become clear if the development of more targeted treatment strategies for oesophageal adenocarcinoma will ultimately lead to an improved clinical outcome for patients with this aggressive disease.
REFERENCES


