Signaling pathways in the molecular pathogenesis of adenocarcinomas of the esophagus and gastroesophageal junction

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Abstract

Esophageal adenocarcinoma develops in response to severe gastroesophageal reflux disease through the precursor lesion Barrett esophagus, in which the normal squamous epithelium is replaced by a columnar lining. The incidence of esophageal adenocarcinoma in the United States has increased by over 600% in the past 40 years and the overall survival rate remains less than 20% in the community. This review highlights some of the signaling pathways for which there is some evidence of a role in the development of esophageal adenocarcinoma. An increasingly detailed understanding of the biology of this cancer has emerged recently, revealing that in addition to the well-recognized alterations in single genes such as p53, p16, APC, and telomerase, there are interactions between the components of the reflux fluid, the homeobox gene Cdx2, and the Wnt, Notch, and Hedgehog signaling pathways.

Introduction

Esophageal adenocarcinoma is distinguished by having both a high case-fatality rate, with five year survival rates typically 15–20% in the community,1,3 and by having the fastest rising incidence of all cancers in many countries, with a more than 6-fold increase in incidence in the US in the past several decades.4 Although recent data suggest that the incidence rise may have slowed,5,6 the increase in the number of patients with adenocarcinoma of the esophagus or gastroesophageal junction has stimulated a corresponding increase in research into the biology of this cancer. Here we use the initialism EAC to denote both esophageal and gastroesophageal junction adenocarcinomas although it is recognized that some tumors classified as junctional may be gastric cancers that have extended proximally.

EAC is often studied in conjunction with Barrett esophagus (BE), the condition in which the normal squamous epithelium in the distal esophagus is replaced by a metaplastic columnar mucosa, often containing goblet cells (intestinal metaplasia, IM) in response to chronic severe gastroesophageal reflux disease (GERD). BE is the main predisposing factor for EAC, through a generally accepted multistep process in which IM in a very small proportion of individuals (probably less than 0.5%/year)7,8 progresses through low grade dysplasia and high grade dysplasia stages to invasive EAC. This review includes findings for both BE and EAC although the focus is on cancer.

Molecular Pathogenesis of EAC

Mechanisms underlying molecular abnormalities in the pathogenesis of EAC. In the development of cancer, both genetic and epigenetic mechanisms contribute to the activation or inactivation of key signaling pathways and acquisition of the cancer phenotype “hallmarks”.9 It is generally accepted that Barrett multistep carcinogenesis is characterized by genomic instability,10 which facilitates accumulation of lesions that target proto-oncogenes, tumor suppressor genes, mismatch repair genes, and mitotic checkpoint genes, thereby aiding tumorigenic progression.11 In addition, reflux components have been shown to induce DNA damage in esophageal cells.12,13 Although there are no data showing that reflux causes more permanent genetic (e.g., mutations) or epigenetic alterations, recent Next Generation sequencing data10 show a high overall mutation rate in EAC that is only exceeded by lung cancer and melanoma, both of which are known to be largely driven by mutagens (smoking and UV light, respectively). Epigenetic studies focused on CpG island promoter hypermethylation suggest that there may be “high” and “low” methylation epigenotypes,16 while genome-wide profiling not restricted to CpG sites indicates that the predominant epigenetic mechanism is widespread hypomethylation, which occurs before progression to HGD/EAC and acts in concert with gene amplification to upregulate expression of various genes.17

Confirming which of the many molecular alterations are essential to driving progression to EAC has so far largely eluded researchers. This may be partly due to an emphasis on non-mechanistic studies to identify clinically relevant biomarkers of progression to EAC in patients with BE. Another factor may be differences in tumors arising in the tubular esophagus, the gastroesophageal junction, and the proximal stomach including

Keywords: esophageal adenocarcinoma, Barrett esophagus, signaling pathways, receptor tyrosine kinases, leptin, adiponectin

References

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cardia,18-20 as well as a large degree of heterogeneity found within both individual cancers and segments of Barrett esophagus.21-24
This gap in knowledge has contributed to our persisting inability to identify which patients with BE are most at risk of progression.25 Baseline alterations including p16 and p53 loss combined with aneuploidy are strongly associated with the likelihood of progression to HGD/EAC in longitudinal studies (reviewed in refs. 20 and 25), but these findings require validation at other centers and are not currently suitable for routine use in clinical pathology laboratories. The lack of functional studies identifying drivers of disease has also hindered progress in the development of targeted therapies, including therapies aimed at preventing BE progression. While it is unlikely that all EAC will be treatable via inactivation of a single oncogene (as in the oncogene addiction model),26 an effective approach may involve the collective targeting of a small number of molecules, possibly via a pathway approach.

Mechanistic studies on the molecular pathogenesis of EAC. There are relatively few studies examining the effect that abnormalities present in BE and EAC tissues have on the acquisition of tumorigenic phenotypes in experimental models. Genetically manipulable animal models have only recently been described,27 and there is a paucity of appropriate cell lines.28 Due to a lack of cell lines representing early stages of this disease, many studies have used adenocarcinoma cell lines to model events that are likely to have occurred earlier in the neoplastic sequence. Furthermore, the majority of in vitro studies to date, rather than modeling the effect of genetic alterations discovered in vivo, have focused on the ability of reflux components such as acid and bile to induce the expression of specific proteins and/or activate relevant pathways. While these effects may play a role in tumorigenesis in BE, it is likely that more permanent genetic or epigenetic changes are required in the evolution of EAC. More promisingly, the step-wise neoplastic transformation of a hTERT immortalised, non-dysplastic Barrett cell line using the defined genetic manipulations of p53 knockdown and expression of oncogenic H-Ras (G12V) has been reported.29 These cells could prove useful to study the role of some of the molecular pathways (discussed below) in Barrett carcinogenesis and in the testing of novel therapeutic compounds targeting these pathways, particularly if combined with relevant in vitro 3-dimensional organotypic30,31 and organoid models32 and in vivo tissue reconstitution33 or xenograft models.34

In this review we highlight some of the signaling pathways for which there is evidence of a role in the development of EAC. Activation or inactivation of signaling pathways can occur at multiple levels from the growth factor/ligand that activates a pathway, to cell-surface receptors (often containing intracellular tyrosine kinase domains) and then to downstream kinases and intracellular effectors including transcription factors.

Growth factor and other cytokine-mediated signaling. Epidermal growth factor family. Epidermal growth factor (EGF) and the related family member transforming growth factor-α (TGFα) are two key ligands that have a stimulatory effect on epithelial cell proliferation via activation of the epidermal growth factor receptor (EGFR). There is evidence that signaling through EGFR may play a role in Barrett carcinogenesis to stimulate growth. Protein expression of EGF and TGFα is increased to similar levels in BE and EAC,35,36 suggesting that EGFR activation through these ligands via an autocrine signaling mechanism may be an early event in the BE metaplasia-dysplasia-EAC sequence. In BE, expression of TGFα was found to correlate with proliferation and TGFα immunoreactivity was found in the same areas as proliferating cells in BE glands showing high-grade dysplasia (HGD).37 Altered EGF expression in some cases may be due to the presence of the EGF A61G polymorphism, which is associated with an increased risk of EAC.38,39

Increased signaling through the EGFR pathway could also be a consequence of changes in expression or function of EGFR family members (e.g., EGFR and c-erbB-2/Her2). EGFR protein expression is reportedly increased in up to two thirds of EAC and has been associated with tumor (T) stage, lymph node metastasis, and a trend toward worse disease-free and overall survival.40-44 The gene for EGFR is also amplified in HGD and around one third of EAC,45,46 and activating mutations in exons 18 and 21 of the EGFR gene have been identified in approximately 15% of BE and EAC.47 Both EGFR overexpression and mutant p53 contribute to the enrichment of a subgroup of human esophageal epithelial cells which, after negating the oncogene-induced senescence induced by EGFR overexpression, undergo epithelial to mesenchymal transition (EMT) on TGF-β stimulation.48 The erbB-2/Her2 receptor is also amplified in approximately 10–50% of EAC with concomitant increased mRNA or protein expression.49-55 Amplification and overexpression of erbB-2 have been reported in HGD but not normal esophagus or BE with or without low grade dysplasia (LGD), suggesting that this lesion is a late stage event in BE carcinogenesis.50-52 Co-amplification of erbB-2 and EGFR occurs in approximately 15% of EAC in addition to increased immunoreactivity for erb-B2 in BE and EAC,46 which suggests the possibility of ligand independent activation of this signaling pathway via receptor hetero-oligomerization and subsequent enhanced tumor cell survival.

Despite the evidence above, the results of clinical trials targeting EGFR in the treatment of EAC (reviewed by Mukherjee et al.56) have not been very promising. This may be related to the presence of K-ras mutations, which are known to predict resistance to EGFR inhibition. These mutations are reported in up to a third of patients with HGD or EAC, but not in patients with non-dysplastic BE.57 In contrast, targeting erbB-2 in patients with HER2+ metastatic esophageal-gastric junctional adenocarcinoma has been more successful,48 and is being tested further in a clinical trial with earlier stage disease (RTOG-1010, National Cancer Institute, USA).

Vascular endothelial growth factor family. Vascular endothelial growth factors (VEGFs) are crucial to the formation of new blood vessels (angiogenesis, particularly VEGF-A) and lymph vessels (lymphangiogenesis, particularly VEGF-C) through binding to their cognate VEGF receptors (VEGFR-1, -2, and -3). Angiogenesis is important for the continued growth of a tumor as it outgrows the existing blood supply, and lymphangiogenesis is thought to be important for the metastatic spread of tumors. Evidence suggests that activation of signaling through these
pathways may be important early in the neoplastic progression of BE to EAC. A number of studies have reported an increase in the expression of VEGF-A across the sequence from non-dysplastic BE to dysplasia and EAC.\textsuperscript{59-62} However, correlation between VEGF-A expression, angiogenesis (in particular neo-vascularisation) and clinical outcome are unclear. Mobius et al.\textsuperscript{59} showed that EAC with a high level of neovascularisation did not have significantly increased VEGF-A expression, although low tumor neovascularisation correlates with better survival.\textsuperscript{63} In contrast, two other studies show a positive correlation between VEGF-A expression and high overall tumor vascularization,\textsuperscript{50} which also correlated with lymph node metastasis in one study.\textsuperscript{54} Co-expression of VEGF-C and VEGFR-3 on lymphatic vessels in EAC also suggests enhanced lymphangiogenesis and a potential facilitation of metastatic spread of this disease,\textsuperscript{65} although VEGF-C expression does not correlate with survival.\textsuperscript{66}

There are a number of possible mechanisms for increased VEGF-A expression in Barrett carcinoma including induction by human chorionic gonadotropin,\textsuperscript{67} which is increased in EAC,\textsuperscript{80} by prostaglandins\textsuperscript{68-70} or by bile acid.\textsuperscript{69} In addition, polymorphisms in the VEGF-A gene, which are linked with increased VEGF expression, are associated with an increased risk of EAC, particularly in smokers.\textsuperscript{75}

These studies suggest that angiogenic properties are acquired early in disease progression, perhaps at the dysplasia stage. Inhibition of VEGF-A signaling as a therapeutic option in the treatment of this disease warrants further investigation, particularly since current clinical trials using a VEGF inhibitor, bevacizumab, are mainly aimed at junctional and gastric AC rather than EAC.\textsuperscript{75}

**Insulin-like growth factor family.** Obesity is associated with an increased risk of developing a number of cancers including EAC and may also contribute to development of BE.\textsuperscript{76-77} In Barrett carcinogenesis, obesity, particularly central adiposity, is thought to contribute through both GERD-related (e.g., mechanical promotion of GERD) and GERD-independent mechanisms.\textsuperscript{78,79} In addition, a large proportion of BE patients have metabolic syndrome, and approximately a quarter of these have hyperinsulinemia.\textsuperscript{80} There is emerging evidence that GERD-independent mechanisms may include insulin-mediated production of insulin-like growth factor 1 (IGF-1) and decreased production of IGF binding proteins 1 and 3 (IGFBP-1 and -3).\textsuperscript{81} A consequence, increased bioavailability of IGF can potentially stimulate proliferation and cell survival by binding to the IGF receptor (IGFR) and subsequent activation of intracellular signal transduction pathways.

However, interpretation of IGF-1 bioavailability is complicated by differences reported in tissue vs. serum expression of the relevant molecules. Expression of IGFBP mRNA is increased in BE and EAC tissue compared with normal tissue and is also increased in BE tissue of EAC patients compared with BE tissue from tumor-free patients.\textsuperscript{82} In contrast, Greer and colleagues\textsuperscript{83} found that serum insulin and IGF-1 levels are associated with an increased risk and serum IGFBP-1 and -3 with a decreased risk of BE compared with screening colonoscopy controls, but not when compared with GERD controls. The BE cases had a significantly higher waist-to-hip ratio but not BMI compared with colonoscopy controls, suggesting that central adiposity might be an important factor.\textsuperscript{84} A longitudinal study of patients with BE also found no association with baseline serum IGF-1 and IGFBP-3 levels and risk of progression to EAC.\textsuperscript{85}

Activation of this pathway may also occur through modulation of the receptor, IGF-1R. Overactivation of IGF-1R has been implicated in several cancers in which it is thought to promote cell growth, survival, and angiogenesis, possibly via heterodimerization with EGFR. Protein expression of IGF-1R is increased in the sequence from BE to dysplasia and EAC, with around 80% of EAC showing positive expression.\textsuperscript{85,86} Increased expression may be a result of posttranscriptional regulation since there is no difference in IGF-1R mRNA expression between EAC and matched normal tissue, with the exception of individuals carrying a G1013A polymorphism in the igf-1r gene, suggesting that this polymorphism may enhance transcription or stabilize the transcript.\textsuperscript{87} This same polymorphism increases the risk of developing BE and EAC in obese individuals by 3- and 5-fold respectively.\textsuperscript{88} There is thus some evidence for a role of this complex signaling axis in EAC although the importance of tissue vs. serum bioavailability remains to be determined.

**Other receptor tyrosine kinases.** C-Met is the tyrosine kinase receptor for hepatocyte growth factor (HGF) and is normally expressed by epithelial cells, where it is essential for morphogenesis and wound healing in adults. In cancer its abnormal activation has been associated with tumor growth, angiogenesis, and invasion. C-Met is overexpressed in dysplastic BE and EAC,\textsuperscript{89-91} although probably only in lesions where c-Met is amplified.\textsuperscript{92} Stimulation of OE33 EAC cells with HGF results in reduced E-cadherin expression and stimulated ß-catenin transcriptional activity leading to enhanced anchorage independent growth,\textsuperscript{93} suggesting a role for c-Met signaling in the acquisition of an invasive phenotype in EAC.

The Axl receptor tyrosine kinase (RTK) was recently identified as being significantly upregulated in the progression of BE to EAC.\textsuperscript{94} The Axl receptor has been implicated in mediating progression, metastasis and drug resistance in several other tumor types. Overexpression of Axl in EAC is inversely associated with survival and RNAi knockdown in 2 EAC cell lines reduced in vitro invasion, migration, and anchorage-independent growth and completely abrogated in vivo engraftment in immunocompromised mice.\textsuperscript{95} This novel finding is intriguing given the recent development of small molecule inhibitors of Axl that have shown promising results in a mouse model of breast cancer.\textsuperscript{96} Treatment of OE33 EAC cells with Axl inhibitors reduced anchorage-independent growth, invasion, and migration and blocked phosphorylation of ErbB-2, suggesting potential transactivation by Axl.\textsuperscript{93}

Overall, RTK signaling pathways are likely to play an important role in EAC and serve as attractive therapeutic targets due to the plethora of available approved and “in development” inhibitors. In particular, EGFR family members, cMET, fibroblast growth factor receptor (FGFR) family members, insulin receptor and IGFR1, collectively, were recently shown to be frequently hyper-activated in EAC and EAC cell lines, although the mechanisms underlying RTK activation were not investigated in
this study. In vitro studies indicated that using an individualized approach to target activated RTKs could be an effective tactic in the treatment of EAC, although many cell lines showed complex RTK profiles and combinations of inhibitors were required to induce cytotoxicity.105

**Lepitin and adiponectin.** In addition to IGF bioavailability, cytokines produced by adipocytes (adipokines) such as leptin and adiponectin may also contribute to obesity-mediated effects in Barrett carcinogenesis. Leptin is found at increased levels in the serum of obese people, while adiponectin is decreased. Leptin has been shown to have mitogenic effects on some tumor cell lines in vitro, including colon cancer.96 In contrast, adiponectin is thought to induce apoptosis97 and low plasma levels have been associated with an increased risk for a number of cancers including gastric98 and colon cancer.99 Therefore, it has been hypothesized that altered adiponectin and leptin levels may contribute to the association between obesity and some cancers, including EAC.

While a role for leptin in the progression to EAC is undefined, there is evidence to suggest it may contribute to development of BE. Gastric leptin levels are increased in BE and are associated with increased risk of BE.100 In contrast, the association between serum leptin and BE is unclear with studies showing either an association with BE in men but not women that is independent of both GERD and obesity,101 or an association in women but not men.102 Similarly, there are conflicting reports on the association between serum adiponectin levels and BE, which may also be gender dependent.103-105 Serum adiponectin levels are lower in patients with EAC compared with controls,104 which may contribute to increased tumor cell survival. As with IGF-1 and IGFBPs, expression of leptin and adiponectin in BE and EAC patients deserves further investigation.

There are limited data on alterations in the receptors for leptin and adiponectin in the development of EAC. Receptors for leptin are highly expressed in normal and inflamed esophagus and BE,100 but expression in EAC has not been reported. In keeping with adiponectin playing a protective role in carcinogenesis, expression of adiponectin receptors is decreased in BE at mRNA level.105

While the studies described above may not provide a compelling case for the involvement of adipokines in Barrett carcinogenesis, there is additional support from in vitro studies. Leptin induces proliferation and inhibits apoptosis via activation of COX-2, leading to prostaglandin E2-mediated transactivation of EGFR and JNK activation in OE33 EAC cells.106,107 Increased proliferation may also be partly due to leptin-induced HB-EGF and TGFα expression and secretion leading to subsequent EGFR transactivation.106 In contrast, adiponectin attenuates leptin induced proliferation in EAC cell lines, at least partly by inhibiting AKT activity,108 and may induce apoptosis via modulating expression of pro- and anti-apoptotic Bcl-2 family members.105

**Transforming growth factor β.** Transforming growth factor β (TGF β) is central to epithelial homeostasis by regulating both proliferation and differentiation. Dysregulated response to TGFβ has been associated with a range of epithelial cancers. In normal cells, one of the functions of TGFβ is to induce a reversible cell cycle arrest and many epithelial tumors are refractory to this response. In contrast, TGFβ is implicated in an epithelial to mesenchymal transition (EMT) in tumor cells, particularly at the invasive edges, where this change in phenotype is thought to aid invasion and metastasis. Both of these mechanisms have been implicated in the progression of BE to EAC. Expression of TGFβ is upregulated in EAC compared with normal esophagus and BE and is associated with advanced stage.109,110 In addition, increased expression of TGFβ at the invasive margins of EAC correlates with markers of EMT.111

TGFβ signaling can be impaired through modulation of the downstream transcriptional mediators, particularly SMAD2 and 4. Loss of heterozygosity (LOH) at chromosome 18q, location of SMAD2 and 4 genes, occurs in BE carcinogenesis112 and SMAD4 is mutated in approximately 8% of EAC.13 In addition, expression of SMAD2 and SMAD4 is decreased in BE and EAC, possibly via promoter methylation in the case of SMAD4,113 suggesting that response to anti-proliferative signaling by TGFβ is impaired. This was confirmed in ex vivo organ culture of normal, BE, and EAC biopsy tissue via measuring p21/WAF1 and MCM2 (a proliferation marker) expression in response to TGFβ.113 Interestingly, expression of Ski and SnoN, negative regulators of SMAD transcriptional function, is also increased in BE, but is then decreased or lost in dysplasia and EAC,114 suggesting a further level of regulation of this pathway in the progression from BE to EAC.

**Ligand/receptor mediated apoptotic pathways.** Apoptosis induced through the tumor necrosis factor receptor (TNFR) superfamily by ligands such as FasL/CD95L and TNF-related apoptosis inducing ligand (TRAIL) is important in the regulation of the immune system. Signaling via these pathways is also often downregulated in cancer. In addition, some cancers upregulate expression of ligands, which is thought to have an effect against immune surveillance. The evidence that modulation of pro-apoptotic ligand/receptor signaling complexes plays a role in Barrett carcinogenesis is unclear. In fact, both increased proliferation index and apoptosis rate are linked with progression to EAC, suggesting that suppression of apoptosis may be less critical in Barrett carcinogenesis compared with other cancers.135

FasL expression may be increased in BE and further increased in dysplasia and EAC116,117 and correlates with depletion of CD45+ tumor infiltrating lymphocytes,115 suggesting that BE progression is associated with FasL-mediated avoidance of immune surveillance. In contrast, FasL is not expressed on the cell surface or secreted into the medium by EAC cell lines,118 which is inconsistent with a role in establishing immune privilege. In contrast to FasL, TRAIL is expressed in BE but is rarely and weakly expressed in dysplasia and EAC.120

Similarly, the evidence for receptor modulation is unclear. Fas expression may be either increased117,121 or decreased122 in dysplasia and EAC. In vitro data show that bile salts preferentially upregulate Fas expression in the normal squamous derived Het1A cell line but not in BE-derived BAR-T or EAC-derived FLO-1 cell lines, which may suggest that bile reflux could play a role in the selection of cells that have developed apoptosis resistance via dysregulation of Fas-mediated immune surveillance.123
In contrast the TRAIL receptor, DR5, is upregulated in up to 90% of EAC compared with matched normal tissue, which would be expected to sensitize tumors to TRAIL-induced apoptosis. Thus, there is little evidence that regulation of apoptosis at the level of ligands or TNFR family members is a major mechanism driving Barrett carcinogenesis.

Apoptosis signaling downstream of both extrinsic (e.g., death receptor ligands) and intrinsic (e.g., mitochondrial centric) stimuli is regulated by a number of proteins including caspsases and the pro- and anti-apoptotic members of the Bcl-2 family of proteins. Polymorphisms in the genes for caspase-7 and caspase-9 are significantly associated with an increased risk of EAC, and polymorphisms in caspase-7 and Bcl-2 modify the risk of EAC in smokers. Data regarding expression of Bcl-2 family members is controversial, with studies suggesting that anti-apoptotic Bcl-2 is not expressed in BE, dysplasia or EAC, or that it is increased in BE and LGD but decreases in HGD and EAC. This suggests that increased Bcl-2 may have a role early in the development of BE but not in progression to EAC. Indeed, loss of Bcl-2 in dysplastic BE and EAC has been associated with tumor progression and poor survival. In contrast, increased anti-apoptotic Bcl-XL and decreased pro-apoptotic Bax expression have been described in the progression of BE to EAC, possibly indicating Bcl-XL and decreased pro-apoptotic Bax expression have been described in the progression of BE to EAC, possibly indicating a switch to a more anti-apoptotic state. Together, these data suggest that the balance of pro- and anti-apoptotic signaling may impact on the effect of environmental factors in the development of EAC. For example, a more anti-apoptotic intracellular environment may result in the survival of potential neoplastic cells in the DNA damaging and potential mutagenic environments provided by GERD and smoking.

Kinases, transcription factors, and other effectors. RAS/Raf/MAPK and PI3-kinase/AKT pathways. RAS/Raf/MAPK and PI3-kinase (PI3K)/AKT are central downstream mediators of a number of signaling pathways, particularly tyrosine kinase receptors. Together, they control a myriad of cellular processes including cell growth, proliferation, differentiation and motility, all of which are involved in tumorigenesis. In particular, MAPK pathway components were found to be upregulated in around 40% of EAC, suggesting that using MEK inhibitors to target MAPK activation could be an effective treatment, possibly in combination with RTK inhibitors. Aside from the modulation of ligand/RTK activation as described above, there is evidence that alterations to these downstream mediators may also contribute to the progression of BE to EAC.

Expression of mutant oncogenic ras (K-ras or H-ras) is rarely found in non-dysplastic BE but is detected in up to 40% of dysplasia and EAC samples, suggesting that acquisition of this mutation is important in progression. Mutation of BRAF, downstream of Ras, is also found at low frequency (5–10%) in dysplasia and EAC, although never in combination with Ras mutation, and thus represents an alternative mechanism for activating downstream signaling. Introduction of H-ras together with RNAi knockdown of p53 in p16-deficient non-dysplastic BAR-T Barrett cells leads to tumorigenic transformation, demonstrating a mechanistic role for Ras activation in BE carcinogenesis. However, H-Ras or p53 knockdown alone were not sufficient, highlighting the need for multiple steps in the development of EAC.

PI3KCA, the gene that encodes for the p110α catalytic subunit of PI3K is mutated in approximately 6% of EAC but no activating mutations have been reported in BE. PI3KCA is also amplified in a small proportion of EAC, suggesting that acquisition of PI3KCA lesions may be involved in the progression to EAC in a small subset of patients. Phospho-Akt, an indicator of active Akt signaling, is increased along the progression from normal esophagus to BE, dysplasia, and EAC and is associated with tumor progression. However, this is likely due to increased upstream signaling since activating mutations in Akt have not been reported in this disease.

Perhaps not surprisingly, Ras/ERK/MAPK and PI3K/Akt activation also appear to be central to signaling pathways activated by a number of factors relevant to BE, including acid, bile, leptin, and gastrin, resulting in enhanced proliferation, inhibition of apoptosis, and upregulation of MUC1, 4 and 5AC and COX-2. Thus, there is a putative role for these central molecules in mediating signaling by multiple effectors relevant to Barrett carcinogenesis.

COX-2. Cyclooxygenase-2 (COX-2) is a key enzyme in the arachidonic acid pathway that acts to produce prostaglandin as part of the inflammatory response. Chronic inflammation is believed to potentiate neoplastic development at least partially due to mediators such as prostaglandins. It is in this context that COX-2 is thought to contribute to Barrett carcinogenesis. Increased COX-2 expression is detected in the progression from BE to EAC, and is associated with proliferation and reduced survival. However, COX-2 expression appears to be independent of the degree of inflammation, although it is highest in the distal part compared with proximal BE, which is also the most frequent location of EAC.

COX-2 expression may also be increased as a direct effect of reflux components. Acid and bile are well established to induce COX-2 expression and prostaglandin production in vitro in EAC cell lines, in ex vivo organ cultures of BE tissue and in animal models, via a mechanism that involves reactive oxygen species-mediated PI3K/AKT and ERK/MAPK activation. COX-2 may also be upregulated by p53 via a NFκB-dependent mechanism.

There is controversy surrounding the presence of two polymorphisms in the promoter of the COX-2 gene, which have been linked with increased expression and activity of COX-2 as well as the risk of developing EAC. Two separate studies each found different haplotypes of the same polymorphisms as being more common in EAC than controls. An intragenic polymorphism has also been associated with an increased risk of EAC.

There are functional data indicating a causative role for COX-2 mediated inflammation in Barrett carcinogenesis. Selective COX-2 inhibition in primary cultures of BE cells and ex vivo organ cultures of BE reduces COX-2 activity, prostaglandin production and proliferation and in primary cultures this could be reversed by addition of prostaglandin E2. Similar effects are seen in EAC cell lines, suggesting a dependence of EAC on COX-2-mediated prostaglandin production. A xenograft model...
study suggests that targeting COX-2 may also be a viable therapeutic option in the treatment of established EAC.\textsuperscript{162}

Use of aspirin and NSAIDs is associated with reduced esophageal cancer risk in population-based studies. Taken together, these data highlight the possibility of COX-2 inhibition as a chemopreventive strategy. Unfortunately, a small (100 patients) celecoxib COX-2 inhibition trial failed to show a benefit in preventing progression of dysplasia to EAC\textsuperscript{160} and the large AspECT chemoprevention trial also seems to have found no clear benefit from daily aspirin to prevent esophageal cancer.\textsuperscript{164}

**NF\textsubscript{κ}B.** NF\textsubscript{κ}B controls the transcription of a large number of genes in response to a range of stimuli including intracellular stresses and cytokine mediated activation of receptor signaling pathways. NF\textsubscript{κ}B is intimately linked with regulation of the host inflammatory and immune response by regulating the expression of a number of key cytokines including TNF\textsubscript{α}, IL-1\textbeta, IL-6 and IL-8, which themselves can activate NF\textsubscript{κ}B. Overactivation of NF\textsubscript{κ}B has been linked to neoplasia, including EAC, through promoting cell survival, particularly in the context of chronic inflammation.

NF\textsubscript{κ}B is located on chromosome 4, which is frequently amplified in Barrett carcinogenesis,\textsuperscript{159} and is frequently expressed in the progression from BE to EAC.\textsuperscript{166,167} NF\textsubscript{κ}B is activated by acid and bile in EAC cell lines, possibly via production of reactive oxygen species,\textsuperscript{166,168,169} and thereby provides evidence for over-activation of NF\textsubscript{κ}B in the progression to EAC as a consequence of GERD. This is supported by recent data showing that bile induced activation of NF\textsubscript{κ}B in non-dysplastic BE cells leads to apoptosis resistance in the face of concomitant bile-induced DNA damage.\textsuperscript{170,171} Upregulation of COX-2 by acid and bile is also thought to be mediated by NF\textsubscript{κ}B,\textsuperscript{153,172,173} which may further enhance esophageal tumorigenesis via upregulation of additional inflammatory mediators.

**Cell cycle regulators.** Control of progression through the cell cycle is pivotal to regulating cellular proliferation. Much of that control is exerted through the action of cyclins and cyclin dependent kinases (CDKs) that act at different stages of the cycle. Dysregulation of cell cycle mediators appears to be central to development of EAC. Cyclin D is expressed in response to extracellular signals that promote cell proliferation, such as growth factors and forms a complex with CDK4 to phosphorylate and inactivate Rb. Nuclear cyclin D1 expression is increased in BE and is even more frequent in dysplasia and EAC.\textsuperscript{174-176} This may be at least partly due to the G870A polymorphism in the gene, which results in protein stabilization and a longer half-life. However, there are conflicting results regarding the presence of this polymorphism in EAC. Studies have demonstrated an association between this polymorphism and the risk of reflux disease, BE, and EAC,\textsuperscript{177} as well as earlier age of onset of EAC, poorer survival and distant metastasis.\textsuperscript{178,179} However, these associations have not been observed in other studies.\textsuperscript{180,181} Cyclin E expression is also increased in a proportion of dysplastic BE and EAC and correlates with amplification of 19q12, the location of the gene for cyclin E.\textsuperscript{182,183}

Rb, p27 and p21/WAF1 are tumor suppressor genes that block progression through the cell cycle by inhibiting cyclin-CDK complexes. Loss of heterozygosity (LOH) of the Rb locus and loss of Rb protein expression is common in EAC\textsuperscript{182,184-186} and is thought to represent a target for inactivation in the latter stages of EAC development. In contrast, inactivation of p16, which indirectly negates the function of Rb, occurs frequently in nondysplastic BE\textsuperscript{188,190} or at the non-dysplasia to LGD interface,\textsuperscript{191} and seems to represent one of the key early molecular events driving BE carcinogenesis. p27 expression is downregulated in the majority of EAC.\textsuperscript{192} In a mouse surgically induced reflux model, development of both BE and EAC are increased in p27 knockout mice compared with wild-type,\textsuperscript{193} demonstrating that loss of p27 can enhance Barrett carcinogenesis, possibly at an early stage. In contrast, p21 is increased in dysplastic BE and EAC but not nondysplastic BE.\textsuperscript{194}

**p53.** p53 is a well-known tumor suppressor that is frequently inactivated in most cancers. The function of p53 is central to controlling both cell cycle progression and initiation of apoptosis in response to extrinsic and intrinsic signals. p53 LOH and p53 gene mutations, occur in the majority of EAC cases\textsuperscript{95-198} and these lesions are associated with poor outcome.\textsuperscript{199-202} Mutations often result in stabilized p53 protein and increased staining for p53 has been detected in non-dysplastic BE and more frequently in dysplasia and EAC.\textsuperscript{196,203-206} However, mutations do not appear to account for the majority of p53 protein accumulation in BE carcinogenesis.\textsuperscript{207,208} Increased p53 expression is correlated with increased proliferation in the progression of BE to EAC in some studies,\textsuperscript{209,210} and may be a valuable biomarker predicting increased risk of disease progression in patients with BE.\textsuperscript{211}

**Wnt, Notch, and Shh.** The Wnt, Notch, and sonic hedgehog (Shh) signaling pathways are important for regulating cellular differentiation and proliferation during embryogenesis and normal tissue homeostasis in adults. These signaling pathways have also been implicated in tumorigenesis including development of BE and EAC.

Central to the Wnt pathway is stabilization of β-catenin and nuclear relocalization to form the β-catenin/TCF transcription complex. Nuclear accumulation of β-catenin has been commonly described in Barrett carcinogenesis and is independent of activating mutations in exon 3,\textsuperscript{212,214} Nuclear accumulation may be a consequence of APC LOH, which is a frequent late event in EAC.\textsuperscript{215,216} via APC promoter methylation\textsuperscript{217,218} or via upregulation of Wnt ligands and epigenetic silencing of Wnt inhibitory factor (WIF1).\textsuperscript{219,220} Significantly, increased Wnt signaling in organotypic cultures of squamous esophageal cells promoted expression of intestinal-type proteins that are also expressed in BE.\textsuperscript{20}

β-catenin can also be found in complex with cadherins, such as E-cadherin and downregulation of E-cadherin can lead to increased signaling through β-catenin. Reduced membranous E-cadherin is common in BE and EAC,\textsuperscript{213,215} possibly due to promoter methylation.\textsuperscript{221} Stimulation of OE33 EAC cells with HGF induces nuclear β-catenin, possibly as a consequence of E-cadherin downregulation.\textsuperscript{219} Similar findings have been described following stimulation with TNFα, which is upregulated in the progression from BE to EAC and also results in β-catenin mediated c-myc transcription.\textsuperscript{222} Loss of E-cadherin in EAC may also be a consequence of overexpression of the transcriptional repressor, Slug.\textsuperscript{226}
In the intestine, the Notch pathway controls intestinal cell fate determination through promoting expression of the Hes1 transcription factor. Hes1 negatively regulates expression of Hath1/Atoh1, which in the absence of Hes1 promotes differentiation of intestinal progenitor cells into secretory cell lineages, including goblet cells. Notch signaling appears to play a similar role in the development of BE. Intestinal-type BE with goblet cells show lower expression of Hes1 and upregulation of Hath1/Atoh1 and MUC2 compared with the non-goblet cell, proliferative BE crypts. Interestingly, the bile acid DCA suppresses Hes1 in EAC cell lines, possibly via upregulation of Cdx2, leading to increased Hath1/Atoh1 expression and expression of Muc2. In contrast, progression of BE to EAC is associated with activation of Notch signaling and expression of Hath1 in patient tissue, and a mouse model of BE/EAC. This activation of Notch signaling, with increased SOX9 expression, is associated with dysfunctional TGFβ signaling through loss of TGFβ adaptor β2SP.

Shh signaling is important in the embryonic development of the gastrointestinal epithelium, including the esophageal epithelium and in intestinal epithelial homeostasis, but is not active in the normal adult esophagus. Abnormal activation of Shh signaling by acid and bile reflux has been implicated in the pathogenesis of BE, possibly through activation of the bone morphogenic protein-4 (BMP-4) signaling pathway and the downstream transcription factor SOX9. Hedgehog signaling and upregulation of the downstream GLI1 transcription factor may also contribute to EAC tumorigenesis, including through interaction with the mammalian target of rapamycin (mTOR) pathway, which is itself activated by chronic inflammation in the esophagus. Therefore, targeting this pathway could be an effective approach to treat BE and/or EAC, especially in combination with mTOR inhibitors.

C-myc. The c-myc transcription factor is a proto-oncogene important for regulating the expression of several genes with roles in cell proliferation and thus over-activation of c-myc has been implicated in tumorigenesis, including development of EAC. Uregulated c-myc expression increases in the progression of BE to EAC, possibly as a result of c-myc gene amplification, although this is not found in non-dysplastic BE. Acidified bile, but not bile or acid alone, can induce c-myc expression in OE33 EAC cells, demonstrating that non-genetic mechanisms may also activate c-myc-mediated transcription in Barrett carcinogenesis. Interestingly, c-myc, cooperating with causal-type homeobox 1 (cdx1), has been implicated in the development of metaplasia, suggesting it may also act early in Barrett carcinogenesis.

Summary

The signaling pathways reviewed above are shown schematically in Figure 1, with the RTK pathways in Figure 1A and non-RTK pathways in Figure 1B. Much of the data for these pathways is descriptive, there has been a deficit of suitable cell lines and animal models and human studies have tended to compare separate cohorts of individuals rather than the same cohorts followed longitudinally. Partly for these reasons, the critical driver aberrations involved in BE/EAC pathogenesis have not been confirmed. However, activation of Notch and Hedgehog pathways, through mechanisms that include aberrant TGFβ signaling, cdx2 activation by the bile components of the refluxate and interactions with the TNFα/mTOR pathway, seem increasingly important. Obesity, especially central obesity due to visceral adipose tissue, is a highly important risk factor for EAC and BE. With one of the strongest associations with obesity of all human cancers, EAC provides a valuable opportunity to investigate the causal relationship of adiposity with cancer. Many of the pathways reviewed here are also activated in obesity but the results for obesity-related areas such as the IGF family and adipokines have so far been mixed or conflicting.

In conclusion, this review demonstrates that considerable recent progress has been made to unravel the pathways involved in EAC pathogenesis. As for other cancers, EAC research is entering an exciting era of discovery searching for associations between variations in massive scale data and disease, as exemplified by several completed and ongoing next generation sequencing (NGS) studies and the BE genome-wide association study (GWAS). Ultimately, the functional importance of these variations will need to be assessed. It has been shown that computational algorithms and metaanalysis can identify perturbed signaling pathways in disease, but laboratory-based pathway studies such as those reviewed here remain essential.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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Figure 1. Signaling pathways in the development of esophageal adenocarcinoma. Both receptor tyrosine kinase (A) and non-receptor tyrosine kinase (B) signaling pathways have been implicated in the progression of Barrett esophagus to esophageal adenocarcinoma. Black up or down arrows indicate changes in expression (usually at the protein level), Pol indicates gene polymorphism implicated in disease, Mut indicates gene mutation (usually activating), Amp indicates gene amplification, LOH indicates loss of heterozygosity, and Met indicates promoter methylation. Blue (dotted) arrows indicate effects on expression, red (dashed) arrows indicate effects on activity, gray (solid) arrows indicate translocation. ROS, reactive oxygen species.
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