

Molecular Analysis of Barrett's Esophagus

Lawrence Zukerberg, MD
Department of Pathology, Warren 2
Massachusetts General Hospital
Boston, MA 02114
lzukerberg@partners.org

Molecular understanding of Barrett's esophagus can be separated into 2 parts: 1) promotion of metaplasia and 2) neoplastic progression of the metaplastic epithelium. The metaplastic epithelium is derived from epithelial stem cells that undergo altered differentiation related to local milieu rather than mutation. Thereafter, progression from metaplasia to dysplasia/carcinoma requires a number of altered circuits. There are at least five circuits that must be altered in order for a cell to become dysplastic and eventually malignant: 1) requirement for a continuous or autonomous growth stimulus; 2) development of resistance to growth inhibitory signals; 3) avoidance of apoptosis; and 4) ability to transcend the limits imposed by the generational clock; 5) increased angiogenesis.

Molecular Pathogenesis of Metaplasia

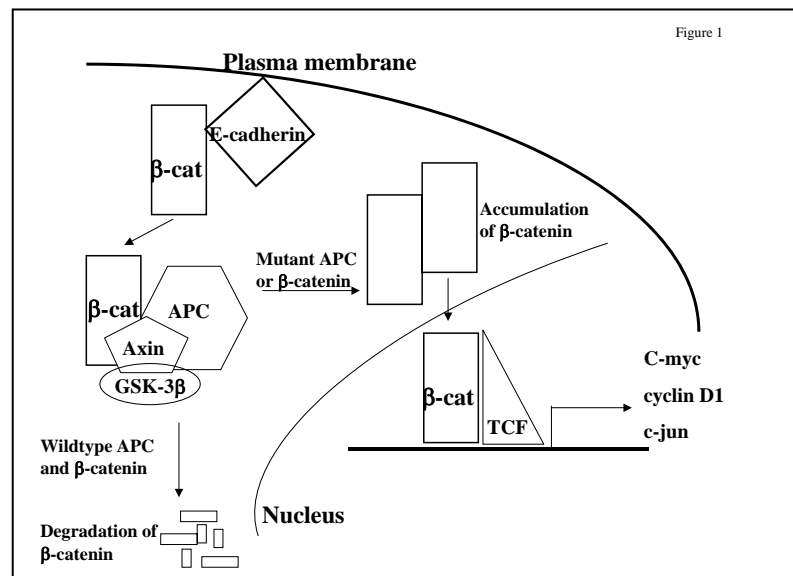
Little is known about the molecular events involved in the metaplastic pathway. Recent discussion has focused on the Cdx genes, which are transcription factors expressed by epithelial cells of the small and large intestine, but not in the normal esophagus or stomach. Cdx2 immunostaining is found in all cases of Barrett's with or without dysplasia but in only 30% of junctional type epithelium. Chronic acid exposure leads to activation of the Cdx2, which may initiate intestinal metaplasia. For example Cdx2 expression activates the intestinal mucin gene MUC2 which is expressed in goblet cells. Acid and bile acid exposure in the esophagus also appear to activate MAPK pathways,

which mediate an increase in induction of cyclo-oxygenase-2 (COX2), an enzyme which is important in cell survival and induction of angiogenesis. Similarly gastrin is a mitogen that binds to CCK2 receptor on Barrett's cells and this binding triggers the MAPK pathway. Thus the MAPK pathway may lead to continued proliferation and survival of metaplastic cells.

Growth Stimulatory Circuit

The requirement for a continuous or autonomous growth stimulus is usually the result of overexpression or mutation of oncogenes. Oncogenes are mutated versions of normal cellular genes, known as proto-oncogenes, that encode proteins involved in stimulating cell division. These proteins include 1) growth factors or receptors for growth factors (i.e. HER-2/neu); 2) cytoplasmic relay proteins that carry stimulatory signals from the plasma membrane to the nucleus (i.e. K-Ras); 3) transcription factors that activate growth-promoting genes (i.e. c-myc) and 4) other growth stimulatory factors (i.e. cyclins, phosphatases, etc.).

Increased cyclin D1 expression has been found in about 50% of metaplastic cases and it is known to be a proliferative factor in many tumors. Overexpression of cyclin D1 has been shown in some studies



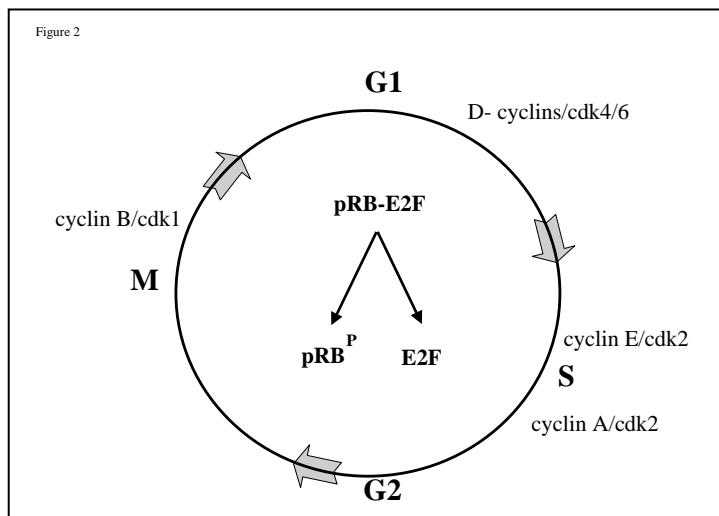
to be a predictive factor for tumor development. Another tumor suppressor/oncogenic pathway that leads to growth stimulation and is important in gastrointestinal dysplasia and carcinoma is the adenomatous polyposis coli tumor suppressor gene (APC)/ β-catenin pathway. Disruption of the APC/β-catenin pathway leads to excess free β-catenin, which is translocated to the nucleus and results in transcription of many genes

necessary for proliferation and transformation of gastrointestinal epithelium (Figure 1). Normal cellular levels of β -catenin are maintained by forming complexes with APC and GSK-3 β . This leads to β -catenin phosphorylation and degradation. Mutations of APC (tumor suppressor) or β -catenin (oncogene) that prevent the two proteins from interacting, leads to free β -catenin and cellular proliferation. promoter methylation of APC occurs in 83%-92% of high grade dysplasias. Among the growth stimulatory agents, *c-erbB2* gene encodes for a receptor for growth signals and is located at 17q21 which is amplified in esophageal adenocarcinoma.

Growth Inhibitory Circuit

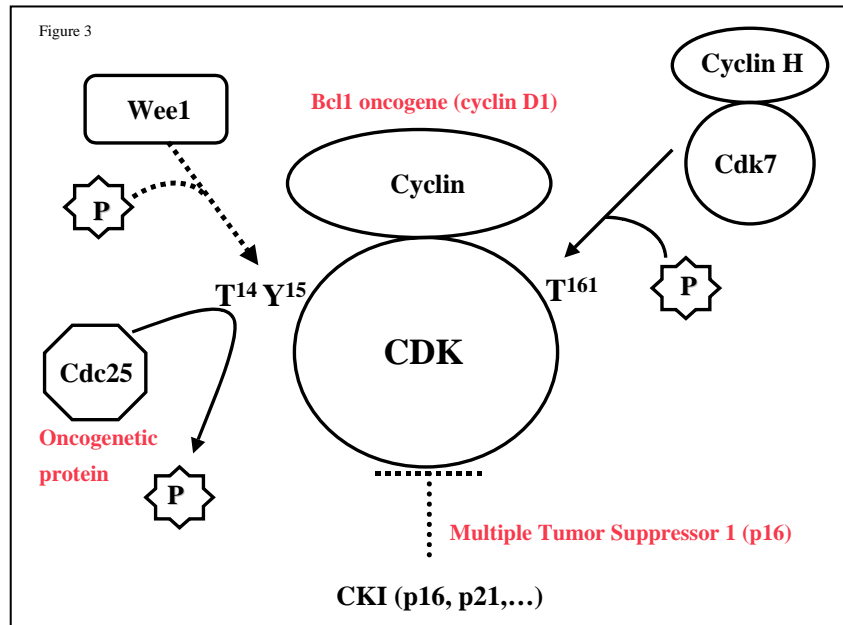
The development of resistance to growth inhibitory signals usually occurs by functional loss of genes that restrict cell growth, also termed tumor suppressor genes. Functional loss of tumor suppressor genes occurs by a number of mechanisms including point mutations, allelic loss, and DNA methylation. In sporadic lesions, one allele undergoes an inactivating mutation (a chance event in a single cell) and the other allele is deleted from the chromosome, probably related to genomic instability. Allelic loss (also known as “loss of heterozygosity” or LOH) is a common event in gastrointestinal dysplasia. Another mechanism of gene inactivation is abnormal DNA methylation. Many patterns of DNA methylation have been described but there is often a regional increase in DNA methylation of the promoter of specific genes, which leads to transcriptional silencing or gene inactivation.

The genes involved altering this circuit almost always relate to the *retinoblastoma gene product*, pRB. It is thought that this pathway must be disrupted in almost every dysplastic/malignant cell. In resting or non-proliferating cells pRB sits



on the DNA bound to a transcription factor, E2F, and blocks transcription of many genes important for DNA synthesis and cell division. During normal cell division pRB becomes phosphorylated by cyclin dependent kinases (cdks) (Figure 2). Phosphorylation of pRB renders it temporarily inactive leading to its dissociation from E2F. Free E2F leads to transcription of many genes involved in DNA synthesis and cell division. Alteration of this circuit may be by: 1) mutations that occur in pRB itself, such that it can

no longer bind to E2F; 2) viral proteins, such those encoded by human papilloma virus or adenovirus, which bind and inactivate pRB; or 3) alteration of genes that normally regulate pRB. Since pRB is normally regulated

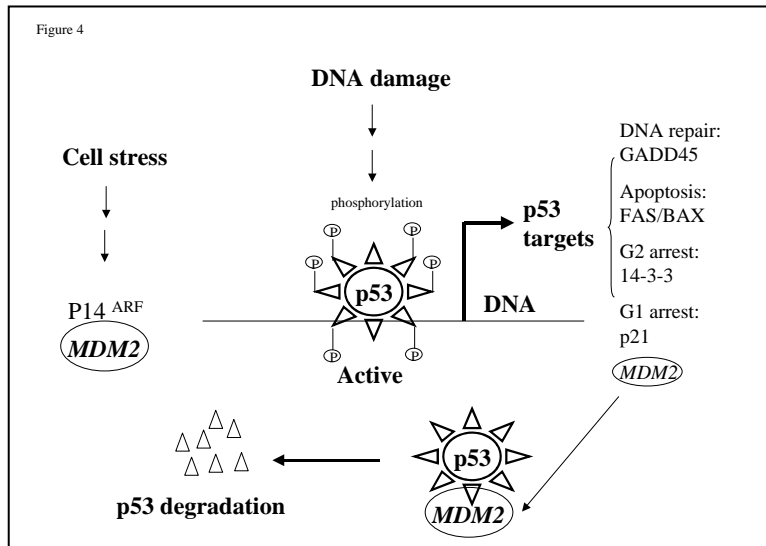


by the cdk's, cdk regulation is tightly controlled by a number of mechanisms including transcription of the activating subunit or cyclin, inhibition of the cdk by small inhibitory molecules (CKI's), and a series of phosphorylation and dephosphorylation events. Many oncogenes and tumor suppressor genes are found in this pathway (Figure 3). Overexpression of genes (such as cyclin D1 or Cdc25) which lead to increased cdk activity or loss of function of genes (such as p16 or Wee1) that lead to inhibition of the cdk activity, results in inappropriate pRB phosphorylation (functional pRB inactivation).

Increased cyclin D1 expression has been found in about 50% of metaplastic cases and it is known to be a proliferative factor in many tumors. Overexpression of cyclin D1 has been shown in some studies to be a predictive factor for tumor development. Inactivation of p16 through mutation or hypermethylation is an early event in the progression to adenocarcinoma. P16 abnormalities were found in 80% of patients with Barrett's esophagus, even when there is no apparent dysplasia.

Avoidance of Apoptosis

Apoptotic pathways are normally latent in healthy cells, but are initiated after cellular stress or DNA damage. The central theme of apoptosis in most tumors, including gastrointestinal dysplasia and carcinoma, is the p53 tumor suppressor gene which is located on the short arm of chromosome 17 (17p13). P53 functions as a transcription factor for

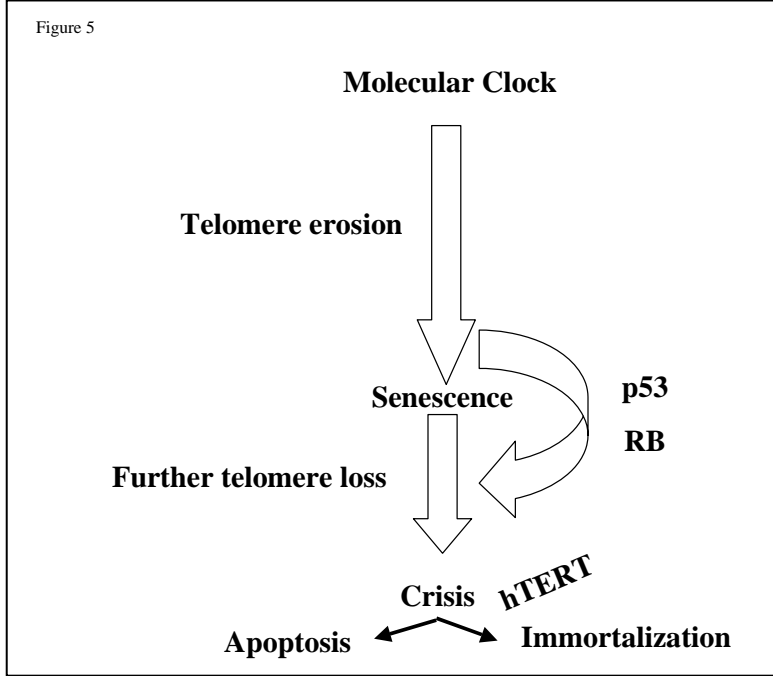


genes involved in DNA repair, growth arrest, and apoptosis (Figure 4). Loss of p53 function occurs by mutation, viral inactivation, overexpression of MDM2 (which leads to p53 degradation), and by allelic loss (LOH). Mutant p53 protein, usually shows mutations in the core DNA-binding domain, and most of these are single missense mutations, rather than deletions, insertions, or frameshifts. Thus the mutant p53 protein is predominantly a full-length protein with a single-amino acid substitution in its core domain. Although biologically inactive, it is more stable than its normal counterpart and is typically overexpressed in gastrointestinal dysplasia, where it can be detected by immunohistochemical methods. P53 protein overexpression is reported early in the progression to adenocarcinoma and overexpression noted in 15-60% of cases classified as indefinite/low-grade dysplasia and 45-89% in high grade dysplasia. Another mechanism by which cells may avoid apoptosis is by increased synthesis of agents that normally block the death pathway such as COX2. Overexpression of COX2 is detected in both esophageal adenocarcinoma and Barrett's metaplasia.

Cheating the Generational Clock

Human cells must overcome two barriers to continued cellular proliferation (Figure 5). The first barrier, referred to as senescence minimally involves the p53 and

pRB tumor suppressor pathways (discussed above). Inactivation of these pathways results in some extension of lifespan, but not immortalization. As normal cells undergo repeated rounds of DNA replication, their telomeres shorten between 50-200 base pairs per round of DNA replication, due to



the inability of traditional DNA polymerases to completely replicate the ends of the chromosomal DNA. This shortening continues until the cells reach a crisis, which is characterized by chromosomal instability, chromosomal end fusion, and cell death. Stabilization of the telomeres by telomerase activation or an alternative method is essential if cells are to survive and proliferate indefinitely. The recent cloning of the human telomerase (hTERT), which encodes the catalytic subunit of the telomerase holoenzyme, has shown that overexpression of hTERT results in telomerase-positive cells that maintain their telomeres, and avoid crisis. As expected hTERT overexpression is found in most forms of dysplasia and carcinoma.

Increased Angiogenesis

Tumors are required to have increased angiogenesis to support their metabolic need. A relationship between vessel density and vascular endothelial growth factor (VEGF) has been reported. COX-2 has been shown to stimulate angiogenesis through the production of VEGF. VEGF is found in endothelial cells and epithelial cells from Barrett's adenocarcinomas and surrounding dysplasia.

Molecular Tools for Diagnosis

There is no molecular tool that is used routinely in predicting metaplasia or the development of dysplasia/carcinoma in metaplastic epithelium. The most useful tools that are in use include: 1) Nuclear staining of p53 implies a p53 mutation and may support a diagnosis of dysplasia. However overexpression of p53 has been noted in non-dysplastic mucosa and not all cases of dysplasia will show positive staining. Also not all p53 mutations lead to protein overexpression. 2) Increased proliferation detected by immunohistochemical staining for PCNA or Ki67 parallel the progression from metaplasia through dysplasia, with increased proliferative compartments extending from the crypt base toward the surface. However, due to large overlaps of Ki67 stainings between histologically defined groups the diagnostic use in clinical practice is not much. Future studies that look at KI67 staining and progression may better define proliferative groups. 3) Nuclear staining for Cdx2 may become a sensitive and accurate marker for intestinal differentiation. Currently intestinal differentiation is made by morphologic evidence of goblet cells. However, Cdx2 is a marker of intestinal differentiation that is expressed in all cases of Barrett's but not in normal stomach or esophagus. Approximately 30% of junctional mucosal biopsies show staining. If these are more prone to develop or associated with Barrett's than Cdx2 might become an early objective marker for Barrett's esophagus.

4) DNA content flow cytometry has been used to study neoplastic progression in Barrett's esophagus. The prevalence of DNA aneuploidy and increased tetraploidy and S-phase fractions increased with increasing histologic grade. A few cases of histologically negative biopsy specimens who have aneuploid cells detected by flow cytometry have been reported.

In some cases, cell with the same aneuploid DNA content were found extending a large distance of 6 to 12 cm suggesting that abnormal clones spread over large areas of mucosa. In other cases, multiple different aneuploid clones were found in different biopsies suggesting multiple clones.

Diagnosis	Number	S>7%	G2>6%	Aneuploid
GERD	44	5%	0	0
Metaplasia	70	24%	0	4%
Low grade dysplasia	32	41%	22%	6%
High grade dysplasia	8	63%	88%	63%
Cancer	28	86%	64%	89%

In one study, sixty-two patients with Barrett's esophagus were prospectively followed by flow cytometry and histology. Nine of 13 patients who had aneuploidy in the initial specimens developed high grade dysplasia or carcinoma during follow-up. None of the remainder without aneuploidy developed a high grade lesion. Thus ploidy analysis in Barrett's esophagus appears to be a sensitive marker for the development of dysplasia and carcinoma. It may become a useful adjunct to biopsies and histologic screening. Additional studies, including cost analysis are sure to clarify its role in routine care.

Selected References

1. Spechler SJ. Barrett's esophagus: a molecular prospective. *Current Gastroenterology Reports* 2005, 7:17-181.
2. Flejou JF. Barrett's esophagus: from metaplasia to dysplasia and cancer. *Gut* 2005;54:6-12.
3. Morales CP, Souza RF, Spechler SJ. Hallmarks of cancer progression in Barrett's esophagus. *Lancet* 2002;360:1587-1589.
4. Lundberg AS, Weinberg RA: Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell. Biol.* 18:247-253, 1998.
5. Hinds PW, Weinberg RA: Tumor suppressor genes. *Curr. Opin. Genet. Dev.* 4:135-141, 1994.
6. Hunter T, Pines J: Cyclins and cancer II: cyclin D and cdk inhibitors come of age. *Cell* 79:573-582,1994.
7. Morgan DO: Principles of cdk regulation. *Nature* 374:131-134, 1995.
8. Cordon-Cardo C: Mutation of cell cycle regulators: biological and clinical implications for human neoplasia. *Am. J. Pathol.* 147:545-560, 1995
9. Donnellan R, Chetty R: Cyclin E in human cancers. *FASEB J.* 13:773-780, 1999.
10. Vogelstein B, Lane D, Levine A: Surfing the p53 network. *Nature* 408:307-310, 2000.
11. Sherr CJ: The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res.* 60:3689-3695, 2000.
12. Bullock AN, Fersht AR: Rescuing the function of mutant p53. *Nature Reviews Cancer* 1:68-76, 2001.
13. Stewart SH, Weinberg RA: Telomerase and human tumorigenesis. *Seminars Cancer Biology* 10:399-406, 2000.

14. Morales CP, Lee EL, Shay JW: In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. *Cancer* 83:652-659, 1998.
15. Jankowski JA, Wright NA, Meltzer SJ, Triadafilopoulos G, Geboes K, Casson AG, Kerr D, Young LS: Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am. J. Pathol.* 154:965-973, 1999.
16. Wu TT, Watanabe T, Heitmiller R, Zahurak M, Forastierre AA, Hamilton SR: Genetic alterations in Barrett's esophagus and adenocarcinomas of the esophagus and esophagogastric junction region. *Am. J. Pathol.* 153:287-294, 1998.