

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Issue: Barrett's Esophagus: The 10th OESO World Congress Proceedings

Barrett's esophagus: genetic and cell changes

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The following includes commentaries on how genetic code of Barrett's esophagus (BE) patients, the mechanisms for GERD-induced esophageal expression of caudal homeobox, and the development of Barrett's metaplasia are increasingly better known, including the role of stromal genes in oncogenesis. Additional lessons have been learned from *in vitro* models in nonneoplastic cell lines, yet there are limitations to what can be expected from BE-derived cell lines. Other topics discussed include clonal diversity in Barrett's esophagus; the application of peptide arrays to clinical samples of metaplastic mucosa; proliferation and apoptosis of Barrett's cell lines; tissue biomarkers for neoplasia; and transcription factors associated with BE.

Keywords: Barrett's esophagus; IGF-1R genotype; intestinal metaplasia; Hox genes; bile; mitogen-activated protein kinase; adenocarcinoma; p16; p53; CDKN2A; TP53; villin; mAb Das-1; BAR-T cells; CDX-1 gene; CDX-2 gene; NF- κ B; Hedgehog pathway; BMP-4

Concise summaries

- The mechanisms for GERD-induced esophageal expression of caudal homeobox and development of Barrett's metaplasia are better known, as is the genetic code of Barrett's patients, but there is not a specific genetic code predictive of Barrett's esophagus (BE). There is an upregulation of embryological pathways that are silenced in the late and postembryonic phase; the role of stromal genes in oncogenesis, as shown in rat models, as well as the lessons learned from *in vitro* models in nonneoplastic cell lines are helpful to understand the natural history of the disease. The clonal diversity in BE must be emphasized, but there are limitations to what can be expected from BE-derived cell lines. *In vitro* experiments with

cells in culture should be viewed as preliminary, and will need to be confirmed by *ex vivo* studies with whole Barrett's tissues that include elements of the stroma. Ultimately, *in vivo* longitudinal studies will need to confirm the pathways to neoplasia. Novel *in vitro* models demonstrate that benign Barrett's epithelial cells can change phenotype expression following exposure to acid and bile. The study of proteomics will probably result in an unparalleled understanding of BA carcinogenesis and will not only be helpful in identifying BE patients at risk for progression, but they will also be critical in better defining tumor stage. The complete transformation of normal esophageal squamous cells into intestinal type cells might be through a cooperative interaction of BMP-4 and CDX-2.

doi: 10.1111/j.1749-6632.2011.06043.x

- In nondysplastic Barrett's cells, acid exposure decreases proliferation and causes a slight increase in apoptosis. In contrast, bile salt exposure does not induce apoptosis in *ex vivo* cultures of nondysplastic Barrett's cells. Levels

of the tumor suppressor and transcription factor are frequently increased with progression to HGD and may be useful markers for adenocarcinoma, but this is a mutated form that is transcriptionally inactive.

1. Is there a specific genetic code predictive of BE to be considered as a useful tool in epidemiologic studies?

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BE develops through metaplasia, which is the process whereby one adult cell type replaces another. In the esophagus, the normal esophageal squamous epithelium becomes replaced by specialized intestinal epithelium that is characteristic of BE. In a general Swedish population, the prevalence rate of BE was found to be 1.6%.¹ Data from the United States suggest that familial Barrett's accounts for 7.3% of cases, whereas the vast majority of cases are considered sporadic.²

Familial Barrett's esophagus

Familial BE is defined as having a first- or second-degree relative with BE, esophageal adenocarcinoma (EAC), or adenocarcinoma of the gastroesophageal junction.² Recent data generated from 881 "familial Barrett's" families suggest that there is inheritance of one or more rare autosomal dominant susceptibility alleles in these families.² However, no specific genetic code indicative of familial BE has been identified yet.

Sporadic Barrett's esophagus

The main risk factors for BE are advanced age, male gender, white ethnicity, obesity, and gastroesophageal reflux disease (GERD). Of these risk factors, the ones that have been investigated for genetic variation as a predictor of BE include GERD and obesity. GERD is a major risk factor for BE.

So maybe rather than BE being an inherited condition, perhaps GERD is the inherited condition in these patients. In support of such a hypothesis, a number of studies have found a clustering of symptomatic GERD among relatives of patients with BE, suggesting that in Barrett's families there may be a genetic component for GERD. However, one study has also reported a clustering of symp-

tomatic GERD among relatives of GERD patients without BE, which refutes this hypothesis.³

Regardless, no specific genetic code indicative of familial GERD has been identified yet. Even if there was an inherited predisposition to GERD, this would still not explain why only a minority of GERD patients develop BE. So perhaps there is another mechanism whereby GERD causes a minority of patients to develop BE. Gastroesophageal reflux clearly leads to reflux esophagitis. In a minority of GERD patients, this inflammation can heal with the development of Barrett's metaplasia (BM). So it is conceivable that genetic alterations in the reflux-mediated inflammatory response may predict which GERD patients develop BE. In fact, there are a number of studies suggesting that patients with BE maybe genetically predisposed to more severe inflammation in response to reflux.⁴

Obesity is the other risk factor, which has been investigated for genetic variation as a predictor of BE. Although it is not entirely clear exactly how obesity contributes to the development of BE, one way might be to increase GERD. Another way may be to mediate signaling through the pro-proliferative insulin and insulin-like growth factor pathways. In fact, obese patients with BE may be genetically predisposed to enhanced signaling via these pathways by alterations in the IGF-1R. In one study, blood from obese patients with and without BE was analyzed for the presence of a pro-proliferative IGF-1R genotype.⁵ The investigators found that there was no difference between obese patients with BE and those without GERD or BE in the frequency of the wild-type genotype for IGF-1R.⁵ In contrast, they found that obese patients with BE were more likely to have a pro-proliferative IGF-1R genotype than obese patients without GERD or BE.⁵

Conclusion

Is there a specific genetic code predictive of BE to be considered a useful tool in epidemiological studies? The answer is NO.

2. What is the role of developmental signaling pathways in the mechanisms by which GERD may induce the esophageal expression of Caudal homeobox (Cdx) genes that mediate the development of BM?

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To determine the molecular mechanisms responsible for BM, it is important to understand how tissue types are specified during normal development. A specific set of transcription factors, like Hox genes or Cdx genes specifying intestine and signaling molecules, are important regulators of tissue type during embryogenesis.

Particularly CDX2, a homeobox gene, has a role in the development of the gastrointestinal tract. In fact, CDX2 has been shown to be an important transcriptional regulator in maintenance of normal adult small intestine and colonic epithelium and has been shown to activate other intestinal differentiation genes, including MUC2.

Cdx genes in BE

CDX2 is not expressed in normal esophageal mucosa but is abundantly re-expressed in intestinal metaplastic mucosa in the esophagus, and immunohistochemical staining studies have confirmed that the CDX2 protein is overexpressed in human BM.⁶ Animal studies have suggested that gastroesophageal reflux may enhance CDX2 expression in rat esophageal keratinocytes and studies of CDX2 gene expression in human esophageal biopsy specimens reveal an increase at each step in the development of BE.⁷

Although *in vitro* studies have demonstrated increased CDX2 promoter activity, RNA and protein expression, and upregulation of downstream target genes, Cdx regulation is an incompletely understood process, but it probably involves complex interactions among key signaling pathways, morphogenetic factors, and transcription factors involved in regulating embryonic development and in maintaining the homeostasis of adult tissues. Literature suggests that many developmental signaling pathways, like Wnt, BMP, transforming growth factor- β , hedgehog, notch, NF- κ B, and

other growth factor pathways play an important role in this process.⁸

Bone morphogenetic proteins (BMPs) are a group of growth factors now considered to constitute a group of pivotal morphogenetic signals, orchestrating tissue architecture throughout the body http://en.wikipedia.org/wiki/Bone_morphogenetic_protein-cite_note-1. They are an important factor in the progression of colon cancer and, conversely, overactivation of BMP signaling following reflux-induced esophagitis provokes BE and is thus instrumental in the development of adenocarcinoma in the proximal portion of the gastrointestinal tract. Supporting this contention, recent studies suggest that GERD may cause esophageal stromal cells to express BMP-4, one of the key players in early morphogenesis of the esophagus, which promotes the change from squamous to columnar epithelium.

Notch

Notch is translocated to the nucleus where it interacts with transcription factors to become a transcriptional activator and then can modulate the expression of Notch target genes that regulate cell fate decisions. The hypothesis is that exposure of esophageal cells to the bile acid, deoxycholic acid (DCA), results in inhibition of the Notch pathway, with alterations in its downstream effectors and induction of CDX2 expression.

NF- κ B

Recent evidence suggests that the transcription factor nuclear factor κ B may be a candidate factor linking inflammation to cancer because it plays a central role in the inflammatory cascade and has been linked to cancer development. NF- κ B is found at increasing levels from normal esophagus to esophagitis and from BM to EAC. NF- κ B has been shown to be integral to the regulation of two homeobox genes, caudal type homeobox transcription factors (CDX) 1 and 2. There is now increasing evidence of a link between NF- κ B and the CDX genes, suggesting a mechanism by which inflammation could induce metaplasia.⁹

Wnt

The Wnt-signaling pathway is essential in many biological processes and numerous studies of this pathway over the past years have led to the identification of several novel components. CDX2 has been shown to be a downstream target of WNT, and the

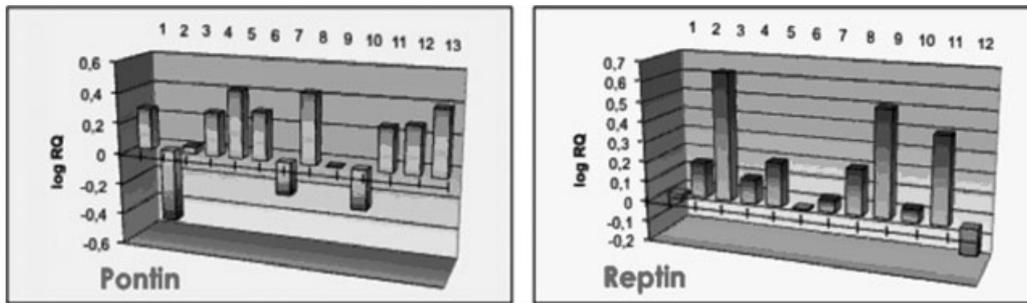


Figure 1. Relative expression of pontin and reptin genes in BM with respect to normal esophageal mucosa.

cytoplasmic accumulation and nuclear translocation of β -catenin, a transcription factor, represents a key step in the activation of the pro-oncogenic canonical WNT pathway.

Here, in comparison to normal, GERD, or BM tissues, β -catenin was found to be overexpressed in about one-third of EA tissues. Of particular interest were several significant associations between the expression of β -catenin and CDX2 in esophageal tissues, suggesting a central role for the WNT/CDX2 pathway in the molecular pathogenesis of EA. In the absence of the signal, action of the destruction complex (CKI α , GSK3 β , APC, and Axin) creates a hyperphosphorylated β -catenin, which is a target for ubiquitination and degradation by the proteasome. Binding of Wnt leads to stabilization of hypophosphorylated β -catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription.¹⁰ β -catenin activity is further modulated by other factors. Among these there is pontin and reptin.

Pontin and reptin

Pontin (Ruvb1) and reptin (Ruvb2) are highly conserved components of multimeric protein com-

plexes important for chromatin remodeling and transcription. They interact with many different proteins, including c-myc and β -catenin, and thus potentially modulate different pathways. In other words, pontin and reptin are Wnt-signaling interaction partners that antagonistically modulate β -catenin transcriptional activity.

Personal data

From our previous analyses using real-time PCR on rectal carcinoma samples, we observed that the expression of pontin is clearly more prevalent than that of reptin. We performed the same study on BE samples, and the results were inconsistent for pontin, whereas reptin expression appeared to be more prevalent in the various BM samples (Fig. 1).

We have begun to check BM biopsies for the expression of other key transcription factors known to effect intestinal differentiation, especially HOX genes (HoxD locus in particular). Significant differences exist for some of the genes, both in BM compared to a normal esophagus and compared to simple esophagitis (Fig. 2). This confirms our opinion, as well as that of many other researchers, that a single transcription factor like CDX2 is probably

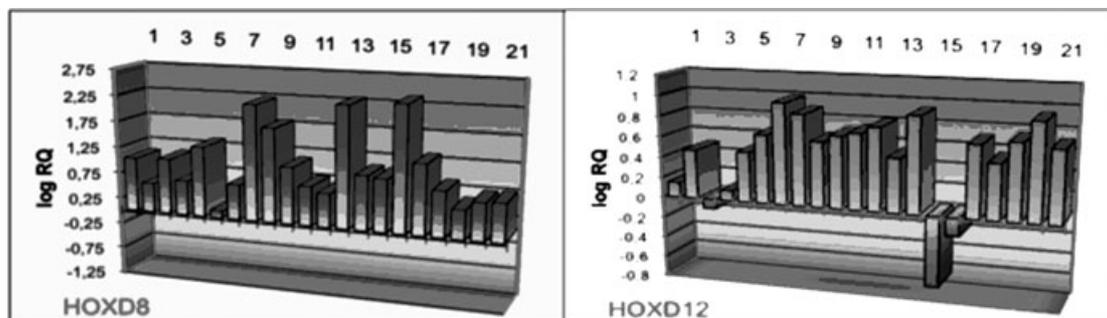


Figure 2. Relative expression of HOXD8 and HOXD12 genes in BM with respect to normal esophageal mucosa.

not solely responsible for a change as dramatic as that of BE, and that the homeobox gene network (HOX and CDX) acts just like an integrated circuit, with yet unexplored potential.

Although these data are very interesting, they are merely speculative and observational and obviously needs to be backed up with functional correlations with other types of experiments.

Conclusions

From recent findings, it has become apparent that in BM there is upregulation of embryological pathways that are silenced in the late and postembryonic phase. Identification of CDX2 as a key transcriptional regulator, and studies dissecting its activation are rapidly evolving, as is our understanding of its potential pathogenesis. The interaction of different components (Wnts, BMPs, Shh) is required for proper morphogenesis of the intestine but also for *cdx* gene expression and thus for BM. Future investigation of these factors is fundamental to further delineate the enigma of BM and to develop novel molecular therapeutic strategies aimed at preventing or reversing this premalignant condition. These additional studies may be warranted in several areas, especially how signaling pathways and transcription factors (such as CDX1, CDX2, and HOX genes) may interact with each other to mediate the development of BM (Fig. 1).

3. What model systems are likely to show the impact of acid and bile on gene expression of the esophagus *in vivo*?

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Thus far there have been two settings in which research has been done to address the question of acid and bile on gene expression. The first setting has been various cell culture systems, the latter, an *ex vivo* tissue culture Barrett's tissues, and both have been extensively used.

In one of many examples using cell lines, and to explore mechanisms whereby acid reflux might contribute to carcinogenesis in BE, Souza *et al.*, studied the effects of acid on the mitogen-activated protein kinase (MAPK) pathways, cell proliferation, and apoptosis in a Barrett's adenocarcinoma cell line (SEG-1).¹¹ SEG-1 cells were exposed to acidic media for three minutes, and the activities of three MAPKs

(ERK, p38, and JNK) were determined. Proliferation was assessed using flow cytometry; cell growth and apoptosis were assessed using cell counts and an apoptosis ELISA assay. They found that acid-exposed SEG-1 cells exhibited a significant increase in proliferation and total cell numbers, and a significant decrease in apoptosis. These effects were preceded by a rapid increase in the activities of ERK and p38, and a delayed increase in JNK activity.

In a classic example of *ex vivo* experiments, and because acid is a major component of refluxate, Fitzgerald *et al.*¹² investigated its effects *ex vivo* on cell differentiation as determined by villin expression, and on cell proliferation, as determined by tritiated thymidine incorporation and proliferating cell nuclear antigen expression. To mimic known physiological conditions, endoscopic biopsies of normal esophagus, BE, and duodenum were exposed, in organ culture, to acidified media (pH 3–5) either continuously, or as a one hour pulse and compared with exposure to pH 7.4 for up to 24 hours. Before culture, villin expression was noted in 25% of metaplasia samples, and increased after 6 or 24 hours of continuous acid to 50% or 83% of metaplasia, respectively. Increased villin expression correlated with ultrastructural maturation of the brush border. In contrast, an acid-pulse followed by culture at pH 7.4, did not alter villin expression in BE. Moreover, continuous acid exposure blocked cell proliferation in BE, whereas, an acid-pulse enhanced cell proliferation, as compared to pH 7.4. Based on their *ex vivo* findings, the authors proposed a model in which the diverse patterns of acid exposure *in vivo* might contribute to the observed heterogeneity and unpredictable progression to neoplasia of BE. However, both these types of models carry inherent limitations, and they do not address acid and bile reflux on metaplasia *in vivo*. One of their key limitations is the noninvolvement of the stroma in such experiments, because it plays an increasingly important role in Barrett's carcinogenesis.¹³

A small animal endoscopy in a rat model of BE has been recently described and it appears promising in the *in vivo* study of gene expression in this disease.¹⁴ The model allows the performance of surveillance, classification of mucosal patterns, for observation of the onset of intestinal metaplasia, and monitoring the progression of neoplastic transformation. Advantages of the model include (1) the

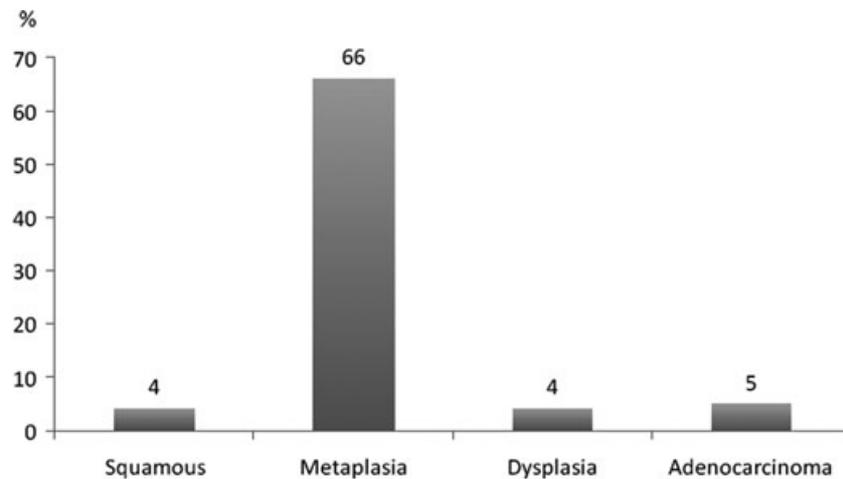


Figure 3. Esophageal histology at 36 weeks in an animal model of Barrett's carcinogenesis that allows for dynamic assessment of acid and bile exposure on gene expression *in vivo*.

esophago-gastro-jejunal anastomotic procedure is easy to perform; (2) it allows reflux of both acid and bile to occur and alter the biological behavior of BE; (3) gastric function, weight, and nutritional status are preserved; and (4) there is a high rate of animal survival and ability to achieve rapid gain in body weight. Figure 3 reveals esophageal histology at 36 weeks in the model.

Souza *et al.* also examined the ability of acid to activate the MAPK pathways *in vivo* in patients with BE.¹¹ MAPK activation was studied in biopsy specimens taken from patients with BE before and after esophageal perfusion for three minutes with 0.1N HCl. In these patient experiments, acid exposure significantly activated the MAPK pathways in the metaplastic epithelium.

The clonal evolution model in BE has recently evolved.¹⁵ The concept of an initial single clone that evolves and accumulates genetic defects, has now been replaced by another model, in which multiple defective clones progress independently or together into malignant transformation. This latter model has been substantiated by recent *ex vivo* studies by Leedham *et al.*,¹⁶ who aimed to assess clonality at a much higher resolution by microdissecting and genetically analyzing individual crypts. Determination of tumor suppressor gene loss of heterozygosity patterns, p16 and p53 point mutations, were carried out on a crypt-by-crypt basis. Cases of contiguous neosquamous islands and columnar metaplasia with esophageal squamous ducts were identified. Tissues were isolated by laser cap-

ture microdissection and genetically analyzed. Individual crypt dissection revealed mutation patterns that were masked in whole biopsy analysis. Dissection across esophagectomy specimens demonstrated marked clonal heterogeneity, with multiple independent clones present. The authors identified a p16 point mutation arising in the squamous epithelium of the esophageal gland duct, which was also present in a contiguous metaplastic crypt, whereas neo squamous islands arising from squamous ducts were wild-type with respect to surrounding Barrett's dysplasia. They concluded that by studying clonality at the crypt level they demonstrated that Barrett's heterogeneity arises from multiple independent clones, in contrast to the selective sweep to fixation model of clonal expansion previously described. They also suggested that the squamous gland ducts situated throughout the esophagus are the source of a progenitor cell that may be susceptible to gene mutation resulting in conversion to Barrett's metaplastic epithelium. Additionally, these data suggested that wild-type ducts might be the source of neo squamous islands.

In conclusion, *in vitro* experiments with cells in culture should be viewed as preliminary and will need to be confirmed by *ex vivo* studies with whole Barrett's tissues that include elements of the stroma. Ultimately, *in vivo* longitudinal studies will need to confirm the pathways to neoplasia. Further, tissue heterogeneity is common in Barrett's epithelia and reflects evolution of multiple mutated clones.

4. What are the limitations of currently available Barrett's cell lines?

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Cell lines serve as useful preclinical models of human disease and play an important role in research on BE and EAC because of the limited availability of patient samples and animal models. There are currently ten authenticated EAC cell lines (FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26, and ESO51) that have been verified to be derived from human EACs.¹⁷ Recent technology using telomerase has now allowed for the immortalization of Barrett's cells. There are currently three cell lines derived from patients with BE and high-grade dysplasia and two cell lines derived from patients with nondysplastic BE.^{18,19}

One of the major limitations of cell lines has been contamination. It is estimated that up to one-third of all cell lines have an origin other than what was expected due to cross-contamination and mislabeling of cultures. Recently, four commonly used EAC cell lines were identified as being contaminated and confirmed to be other tumor types.¹⁷ In fact, these cell lines actually represented lung cancer, colorectal cancer, gastric cancer, and esophageal squamous cell carcinoma cell lines. Therefore, it is very important to always authenticate cell lines by using DNA fingerprinting techniques such as short tandem repeat profiling or mutation analysis.

Another limitation is that we do not know how similar or representative the cultured cells are compared to the original cells. Barrett's mucosa *in vivo* is likely composed of a mosaic of different clonal populations. Over time, clonal evolution and the dynamics between these multiple clones may be important in the neoplastic progression to adenocarcinoma.²⁰ However, with a cell line, it is not possible to evaluate the interaction between multiple clones.

Furthermore, artificial selection pressure during the creation of the cell line may result in the propagation of rare clones that have adapted successfully to the cell culture conditions, and it is possible that these clones may not be representative of the biology of the original esophageal tissue. The immortalization procedures alone may affect the growth behavior of cells. In addition, during the creation and propagation of the cell line, there may be *ex vivo* acquisition of genetic or epigenetic alterations.

Finally, another limitation with cell lines is that it is difficult to replicate the natural *in vivo* environment. BE is thought to develop through a multifactorial process that involves not only genetic and epigenetic factors, but also interactions between other important contributors in the microenvironment such as chronic inflammation and the complex physiology behind acid and bile reflux in conjunction with esophageal dysmotility. It is also difficult to simulate the impact of various lifestyle factors such as obesity, diet, and smoking, which may interact and contribute to the development of BE or EAC. Other unknown factors not yet discovered within the microenvironment may also interact with the Barrett's cells and evaluating cell lines alone would not allow for the study of these interactions.

To overcome some of the disadvantages of standard monolayer cell cultures, recently there has been the development of innovative 3D culture systems. These systems are multilayered structures which mimic the tissue microenvironment by using a monolayer of epithelial cells overlaid on a special ECM gel enriched with collagen and fibroblasts.²¹ These models allow for the assessment of tumor cell interaction with stromal components and are a promising new technology that may help to overcome some of the limitations seen with standard cell lines.

5. How is clonal diversity in BE recognized?

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While a number of molecular events have been identified in the neoplastic progression from BM to dysplasia and thence adenocarcinoma, the cellular origin of BM, as well as the mechanisms that drive the development of tumor heterogeneity, remain a matter of considerable debate. Clarification of the extent of clonal diversity that exists within dysplastic or malignant Barrett's epithelium might provide further insight into these questions.

Tumor genetic heterogeneity may arise from the development of genetic instability, which can lead to alterations that confer an advantage in terms of survival or proliferation. While clonal diversity appears to correlate with genetic instability, such diversity is not equivalent to genetic instability but, instead, is "a function of both the generation and selection

of mutations.”²² Assessment of clonal diversity at different stages of tumorigenesis can provide an indication of the origins of tumor heterogeneity.²³

Premises for the development of tumor heterogeneity include the cancer stem cell hypothesis and clonal evolution.²³ In the cancer stem cell hypothesis pluripotent epithelial stem cells differentiate into either squamous or intestinal-type columnar epithelium, depending on the exposure to local stresses such as gastroesophageal refluxate.²⁴ If there is more than one progenitor cell population, tumor heterogeneity may develop as a result of ongoing and diverging cellular proliferation and differentiation. In the process of clonal evolution,²⁵ malignancy arises in a population of cells as a result of progressive accumulated genetic changes. Such changes can confer either an advantage or disadvantage in terms of survival or proliferation during natural selection.²³

Although tumor heterogeneity has been evaluated widely in terms of specific genetic events or histologic descriptors, in 2006, Maley *et al.* quantified the clonal diversity of esophageal biopsies obtained from patients with BE using methods of molecular evolutionary biology.²⁶ Using systematic sampling across segments of BE, tissue biopsies from 268 subjects were analyzed for DNA content, changes in microsatellite length (shifts), and/or loss of heterozygosity (LOH) at 9p and *CDKN2A* or *TP53* sequence mutations. In addition to segment length and number of clones, diversity was measured by mean pairwise divergence, defined as the number of loci showing molecular differences (e.g., LOH) divided by the number of informative (normal heterozygote) loci, or by the Shannon diversity index to integrate the number and abundance of clones.²⁶ During mean follow-up of 4.4 years, EAC developed in 37 subjects.

A number of diversity measures were predictive for the development of EAC, particularly the number of clones (RR 1.40, 95% CI 1.13–1.73), mean pairwise divergence (RR 1.45, 95% CI 1.08–1.95), and Shannon index (RR 3.10, 95% CI 1.37–7.01), as determined by 9p LOH, correcting for *TP53* LOH and abnormal DNA content. In multivariate analysis, the best predictive model for the development of adenocarcinoma included mean pairwise divergence and the number of clones.

To explore this further, this research group demonstrated that increasing clonal diversity, as de-

termined by several other diversity measures, was associated with increased risk for developing EAC. In particular, they found that diversity found even at evolutionarily neutral loci, specifically 9q and 17q, was as strongly associated with progression to adenocarcinoma as at loci where genetic alterations might confer a selective advantage, suggesting that clonal diversity alone can be predictive for the progression to adenocarcinoma regardless of the underlying molecular defect.²²

Merlo *et al.* caution that novel but low abundance “minority clones” might not be readily detectable when utilizing tissue biopsies to determine clonal diversity.²² Leedham *et al.* suggest that precise compartmental localization (i.e., laser-capture microdissection) can be used to evaluate more precisely the clonal nature of individual crypts.²⁷ This group observed considerable crypt-to-crypt clonal heterogeneity that otherwise might be obscured by analysis of whole biopsy samples. Their findings lend further credence to the concept that BE may develop as a result of a number of molecular events occurring in parallel, rather than being due to progressive stepwise accumulation of genetic heterogeneity.

While the steps that lead to the development of tumor heterogeneity and clonal diversity remain obscure, further elucidation of these mechanisms for the pathogenesis of Barrett's-associated adenocarcinoma could direct more specific antineoplastic therapy and improved clinical management.

6. How can the clonality study by individual crypt dissection demonstrate that Barrett's epithelium heterogeneity results from multiple individual clones?

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Despite years of active research, the histogenesis of BE, a metaplastic condition of the lower esophagus, remains essentially unknown. Over many years, hypotheses have been generated and explored. One area of direct interest are the esophageal gland ducts. These are lined in the proximal two-thirds by a cuboidal epithelium, and undergo a transition to a stratified squamous epithelium as they approach the lumen. It has been suggested that pluripotential stem cells may be located distally in the duct. These become exposed, inflamed with ulceration, and then heal with selective differentiation into an

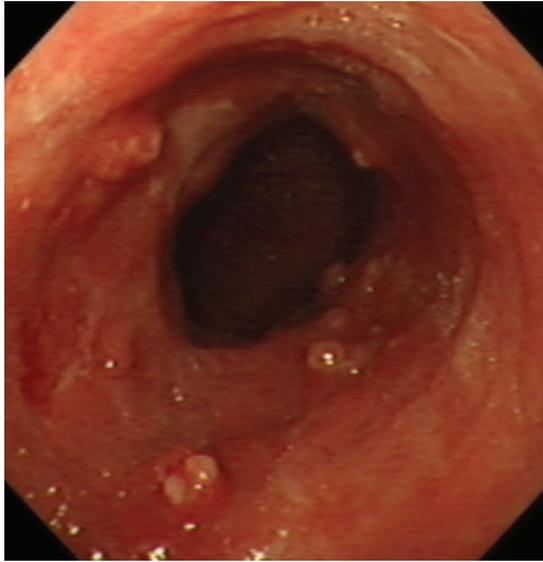


Figure 4. An endoscopic picture of multifocal high-grade dysplasia in Barrett's esophagus.

acid-resistant columnar phenotype. They then migrate to the surface replacing the squamous lining. This is similar to Wright's "ulcer-associated cell lineage" (UACL), a glandular differentiation occurring at sites of intestinal ulceration.²⁸

In addition, there is yet to be a consensus on the the clonal evolution, clonal interaction, and neoplastic degeneration, and these subjects remain very controversial issues. It may be that neoplasia develops through clonal selection which sweeps through the Barrett's segment.^{29,30} Nevertheless, BE is an excellent area to examine and develop models and systems to examine the progression to cancer. However, much of the work has been based on large biopsy samples—heterogeneous samples taken at multiple segments (Fig. 4).

Recently, Leedham *et al.*³¹ have contributed to the histogenesis debate by examining in detail individual crypts for genetic changes. The specimens were obtained from endoscopic resection specimens and whole esophageal resection specimens from endoscopic resection and esophagectomy specimens. The Figure shows an endoscopic picture of multifocal high-grade dysplasia. Individual laser capture micro-dissection was performed and tumor suppressor genes, loss of heterozygosity, p16 and p53 point mutations were examined on individual crypts within these resected specimen. In addition they examined neosquamous islands and metapla-

sia within the esophageal gland ducts. They clearly identified mutation patterns that had been hidden in whole biopsy analysis.

Clonal heterogeneity was identified, and p16 mutations were seen in the esophageal gland duct and its associated metaplastic crypt. It appears that BE may arise from multiple independent clones and is very heterogeneous.

They also suggested that squamous gland ducts could be the source of the metaplastic cells. This confirms earlier morphological three dimensional histological studies that have found continuity between human esophageal gland ducts and Barrett's mucosa. There was a gradual transition of the morphological features of the cells lining the duct: normal cuboidal cells, lining the basal aspect of the duct, into metaplastic cells as the duct opens onto the mucosal surface. This previously demonstrated a definite interrelationship between the two structures. However, identifying the direction of migration is difficult.³²

7. What lessons can be learned from the influence of environmental factors on various *in vitro* models and the cellular phenotype(s) of the nonneoplastic Barrett's cell line about pathogenesis of Barrett's epithelium and its progression to neoplasia?

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Epidemiology indicates a strong relationship between gastroesophageal reflux disease (GERD) and EAC. Barrett's epithelium is an intestinal type of columnar metaplasia that replaces normal squamous epithelium of the distal esophagus secondary to chronic GERD.

Barrett's epithelium is a major risk factor for development of EAC, causing a 30–125-fold increased risk in GERD patients complicated with BE. In BE, such morphologic changes can be recognized with a spectrum by pathologists as metaplasia–dysplasia–adenocarcinoma (MDA). The esophageal squamous epithelium is exposed to a dynamic environment where the differentiation process is modulated by the gastro-duodenal refluxate and GERD.

Various *in vitro* (nonneoplastic Barrett's cell lines, Barrett's adenocarcinoma cell lines, normal human esophageal epithelial cells, and *in vivo*

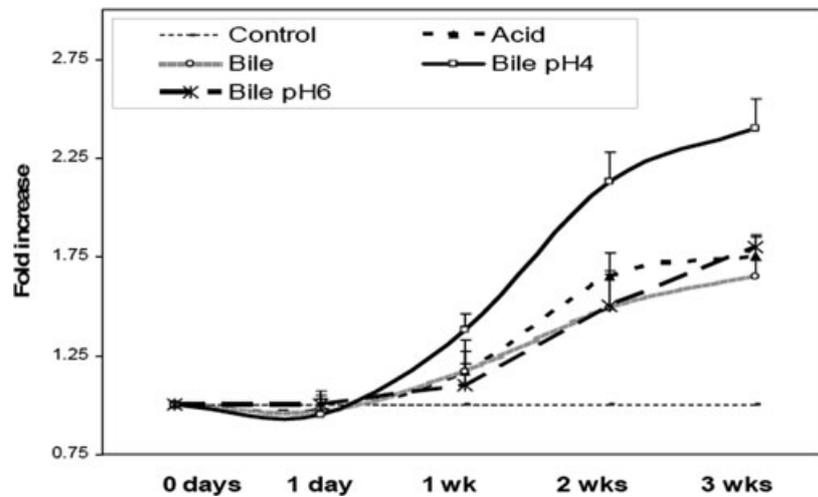


Figure 5. Effect of continued treatment of BAR-T cells with acid and/or bile salt on colonic phenotype expression.

[rodent]) models have been used for the study of pathogenesis of Barrett's epithelium to columnar metaplasia. Acid and assorted components of bile have been implicated in the process of metaplasia of native esophageal squamous epithelium. Presence of pluripotent stem cells, induction of NF- κ B, CDX-2 (caudal homeo-box gene) expression, villin expression, and mucin-secreting cells, all favor the resemblance of metaplastic Barrett's epithelium to intestinal epithelium. However, there is limited knowledge regarding the cellular phenotype(s) of this metaplastic process.

We utilized a nonneoplastic, telomerase-immortalized Barrett's cell line (BAR-T) that has key histochemical features of benign Barrett's epithelium.³³ With intact p53 and p21 cell cycle checkpoints, this cell line is ideal for studies on morphologic, phenotypic, and molecular changes under the influence of environmental factors such as acid and bile.

It is a heterogeneous cell line positive for CK4 a marker for squamous, CK 8/18, mAb Das-1, villin, and mucin, all markers indicative of intestinal type of epithelium. Monoclonal antibody Das-1 is a specific marker for colonic epithelium and incomplete type of gastricintestinal metaplasia, type II and III³⁴, and it does not react with small intestinal enterocytes and complete type or type I (intestinal metaplasia). However mAb Das-1 reacts with Barrett's epithelium with almost 100% sensitivity and speci-

ficity suggesting that Barrett's epithelium is colonic phenotype of metaplasia.³⁵

We observed an increase in the columnar, and particularly colonic phenotype (mAbDas-1 positive) cells (Fig. 5),³⁶ when BAR-T cells were exposed to acid and/or bile (glycocheno-deoxycholic acid, GCDA 200 μ M) individually or in combination at different pH, particularly at pH4, for five minutes each day for up to three weeks. The CK4 phenotype did not change.³⁶

We hypothesized that prolonged, repeated exposure of BAR-T cells to A + B may further induce intestinal phenotype and lead to tumorigenicity. In a systematic, prospective analysis over the course of 65 weeks, we demonstrated that following daily exposure to A + B for a brief period of five minutes per day, BAR-T cells showed progressive morphological, molecular, and biological changes.

Morphological changes between untreated and A + B treated cells were evident from 34 weeks. The treated cells grew as round or oval cells in clumps and displayed acini-like formation (Fig. 6B). Untreated cells remained spindle shaped and evenly dispersed on the culture plate (Fig. 6A). Changes in p53 expression as well as p53 target genes, MDM2, PERP, and p21, were consistent with a transformed phenotype. Loss of anchorage dependence was observed around 54 weeks of A + B treatment. The A + B treated cells could form foci after overconfluent on culture dishes (Figs. 6C and D), grow on

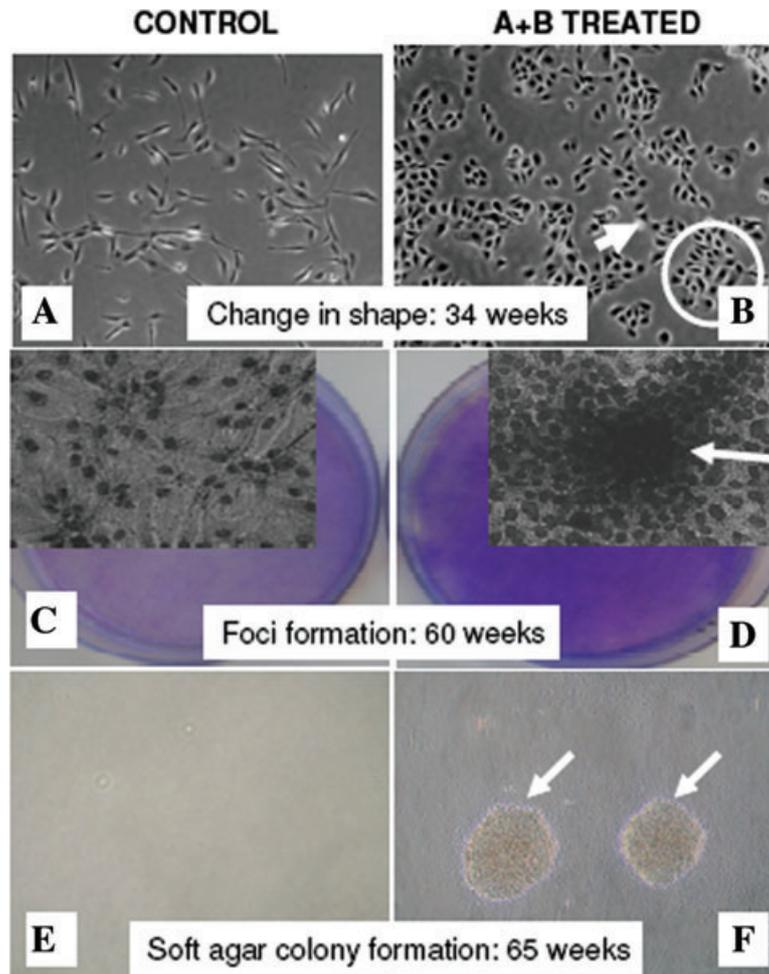


Figure 6. Progressive tumorigenic changes in BAR-T cells upon chronic exposure to A + B.

soft agar (Figs. 6E and F), and form tumors in nude mice.³⁷

Conclusion

The novel *in vitro* model demonstrates that benign Barrett's epithelial cells can change phenotype expression following exposure to acid and bile. This phenotype change occurs in favor of columnar phenotype and particularly colonic (incomplete-type) of metaplasia in BAR-T cells similar to *in vivo* situation.³ This *in vitro* model further demonstrates that continued exposure to acid and bile for longer duration may cause transformation of benign Barrett's epithelium to neoplasia.³⁷ BAR-T cells seem to closely reproduce the pathophysiologic progression of Barrett's epithelium and thus can be utilized as an "*in vitro*" model to study the phenotypic

and molecular changes in the pathogenesis of Barrett's epithelium /EAC, to evaluate gene targets and chemo-therapeutic candidates for treatment of Barrett's epithelium and to impede its progression to EAC.

8. What insight into the molecular process of Barrett's esophagus can be expected by applying peptide arrays to clinical samples of metaplastic mucosa?

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BE development is a multistep process that starts with the mucosal injury of the squamous epithelium of the distal esophagus by gastroesophageal

reflux disease (GERD) and progresses through intestinal metaplasia and dysplasia to invasive Barrett's adenocarcinoma (BA). Approximately 10% of patients diagnosed with BE ultimately progress from metaplasia to dysplasia and subsequently to BA. Routine endoscopic surveillance of patients with BE is an expensive practice due to the low rate of progression to EAC in patients without dysplasia. Identification of factors predicting progression to EAC would substantially help to improve screening and surveillance programs especially for patients with BE without dysplasia. While most efforts have been directed at genetic and epigenetic changes within the MDA sequence, little is known about the protein changes that occur in the progression of the disease.

Genomic-based approaches to biomarker development include the measurements of expression of full sets of mRNA and genomic DNA, such as serial analysis of gene expression (SAGE) levels,³⁸ large-scale gene expression arrays,³⁹ and genomic polymorphisms.⁴⁰ As proteins are often subject to proteolytic cleavage or posttranslational modifications, such as phosphorylation or glycosylation, studies of differential mRNA expression are informative, but do not necessarily correlate with associated protein expression or activity within a given cell. Even though several potential biomarkers have been described over the last decades, none of them have been validated in a population based-study.

It is becoming increasingly apparent that disease progression is largely driven by complex pathways, and analysis of one single genetic or protein marker is unlikely to precisely predict progression of disease with sufficient resolution and reproducibility. The human proteome—like the proteomes of all organisms—is dynamic, changing constantly in response to the needs of the body and differs widely between people depending on factors such as age, gender, diet, level of exercise, and sleep cycle. The proteome also changes in response to cancer and other nonmalignant diseases, making the proteome of great interest to cancer researchers. The science of proteomics—the study of the totality of proteins within a given cell, tissue, or organism—may therefore provide novel insights for the next level of molecular inquiry that is represented by functional genomics and proteomics.

Two-dimensional gel electrophoresis has been the mainstay of electrophoretic technology for a decade

and is a commonly used tool for separating proteins. In many cases, two-dimensional gel electrophoresis evaluates whole-cell or tissue protein extracts. The use of narrow, immobilized pH gradients for the first dimension has increased resolving power for the detection of low-abundance proteins. Radioactive or fluorescent labeling and silver staining allows visualization of hundreds of proteins in a single gel.

Over the past decade, advances in mass spectrometry (MS) and bioinformatics have improved our ability to discriminate cancer-specific peptides. As such, an MS-enhanced, high-resolution, two-dimensional (2D), polyacrylamide gel electrophoresis approach has been applied to further improve detection and separation of proteins at a wide range of pH gradients maximizing the number of separated proteins to up to 2000 proteins using a matrix-assisted laser desorption/ionization, time-of-flight and tandem mass spectrometry (MALDI TOF MS) technology.

Utilizing this technology, a recent study by Peng *et al.* identified protein upregulation of ErbB3, Dr5, cyclin D1, as well as several members of the zinc finger protein family, in eight BAs and four normal mucosal control samples.⁴¹ Interestingly, these proteins were validated in an independent set of 39 BA tissue samples by reverse-transcriptase PCR (RT-PCR) and immunohistochemistry (IHC), suggesting a critical role of these proteins in BA carcinogenesis.⁴¹ Another recent study by De Godoy and coworkers, used advanced computational proteomics to compare essentially all endogenous proteins in haploid yeast cells to their diploid counterparts, suggesting that system-wide, precise quantification directly at the protein level will help to open new perspectives in postgenomics and system biology.⁴² Although standards need to be agreed upon for what determines the validity of a biomarker, now that the draft of the human genome has been completed, the field of proteomics is emerging to tackle vast protein networks that both control, and are controlled by, the information encoded by the genome.

The study of proteomics will probably result in an unparalleled understanding of BA carcinogenesis and will not only be helpful in identifying BE patients at risk for progression, but will also be critical in better defining tumor stage, identifying novel therapeutic targets, and measuring response to therapy, thus tailoring a targeted and effective

therapy to the molecular profile of both the patient and the tumor while minimizing and avoiding life-threatening toxicity.

9. Can active cell-signaling pathways in BE be delineated?

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Several research groups have been performing studies to elucidate the role of CDX-2, a key intestinal transcription factor involved in both physiological and aberrant processes, such as intestinal metaplasia, in BE. One relevant study was recently performed by H. Kazumori and others, where it was demonstrated that administration of bile and acids to esophageal squamous cell lines increases the CDX-2 gene transcriptional activity. In this study they found, as well as in a rat surgical model, that expression of CDX-2 and mucin-2, a direct target of CDX-2, is increased upon bile and acid injury. They hypothesized that this increased transcriptional activity is mediated by NF- κ B.⁴³ In a study last year, the group of R. Souza *et al.* confirmed these findings in esophageal primary cultures of BE patients.⁴⁴

A few years after the 2006 study, the group of Kazumori *et al.* performed another study where they observed that CDX-1, belonging to the same family of CDX-2 transcription factors, is expressed in a surgical rat model. Bile and acid exposure of esophageal cell lines and rat esophageal primary keratinocytes increased the promoter activity of CDX-1 transcription factor, and overexpression of CDX-1 in HET1A esophageal squamous cell line induced expression of mucin-2, an intestinal specific marker. The conclusion from this study was that CDX-1 and CDX-2, in the case of bile and acid injury in the esophageal wall, autoregulate themselves and interregulate each other.⁴⁵

In 2008, Stairs and colleagues published interesting data in the journal *PloS One*, where they showed that EPC2-hTERT normal esophageal keratinocytes, after transfection with *c-myc* and CDX-1, show upregulation of mucin 5A, another pivotal BE marker, and of intestinal metaplasias, in a subset of cells. Moreover, clonal gene expression of several markers of BE, such as CDX-1, CDX-2, mucin2, mucin 5a, and CK20, is observed after microarray performed on patient material.⁴⁶ In none of these studies, however, was it demonstrated which mech-

anism determines exactly the development of an intestinal phenotype.

Interestingly, D. Wang and others published important data about the involvement of the hedgehog pathway in the development of BM. Namely, they looked at the expression of this pathway by microarray in BE and found expression of sonic hedgehog (SHH), namely. In functional studies, they observed that treatment of HET1A cells with BMP-4 induces expression of SOX-9, a transcription factor found in paneth cells and stem cells of the intestinal crypt. SOX-9, in turn, can upregulate expression of deleted in malignant brain tumors 1 (DMBT1), an extracellular matrix protein sufficient to induce a columnar-like phenotype in mice. They concluded the study with the hypothesis that bile and acid injury in the esophageal wall determines the upregulation of aberrant SHH expression, which, in turn, upregulate BMP-4, and subsequently SOX-9.⁴⁷ However, once more, it was not possible to observe the development of a specialized type of intestinal epithelium, as seen in BE.

In 2005, in a study performed by our group, we used SAGE and found that BMP-4 is uniquely expressed in BE as compared to squamous epithelium.⁴⁸ Here, we performed another study, whose results were published in 2007. We found that exposure of primary esophageal keratinocytes to BMP-4 induces upregulation of pSMAD 1, 5, 8, a downstream target of BMP-4, and by carrying out microarray on these cells, we could observe a shift of the gene expression pattern of the normal esophageal cells toward that of BE cells. At the protein level, we observed that a switch of cytokeratins expression pattern, toward those specifically expressed in BE columnar cells, could be achieved. However, we could not observe upregulation of intestinal specific genes, such as mucin 2 and villin 1.⁴⁹

The concluding hypothesis of this study was that BMP-4 is needed to initiate the transformation of the normal esophageal cells into columnar cells, but it is not sufficient to determine an entire shift of the cells into an intestinal phenotype. Presently, we are working on further defining these mechanisms, and we hypothesize that the complete transformation of normal esophageal squamous cells into intestinal type of cells might be through a cooperative interaction of BMP-4 and CDX-2.

10. What characteristics of Barrett's cell lines are related to proliferation and apoptosis?

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Traditional models that have been used to study proliferation and apoptosis in BE include human adenocarcinoma cell lines grown in culture, and human esophageal biopsy specimens grown in *ex vivo* organ culture. However, neither of these models is ideal for studying proliferation and apoptosis in benign Barrett's epithelial cells. Human adenocarcinoma cells have sustained numerous poorly characterized genetic abnormalities, and they are cancer cells, which have altered rates of proliferation and apoptosis. Human esophageal biopsies grown *ex vivo* contain diverse and uncharacterized cell types, so they are not useful for studies to address proliferation or apoptosis specifically in Barrett's epithelial cells. To improve upon the traditional models of BE, we and others have established telomerase-immortalized nondysplastic Barrett's epithelial cell lines from endoscopic biopsy specimens from patients with BE.⁵⁰ These cells are immortalized by telomerase expression but are not transformed.⁵⁰

Characteristics of proliferation in Barrett's cells

Proliferation of Barrett's epithelial cells has been studied in response to acid exposure using cultures of Barrett's-associated adenocarcinoma cells and Barrett's esophageal biopsies. In these *in vitro* models, acid exposure has been shown to increase proliferation.^{51,52} However, using nondysplastic Barrett's epithelial cells (BAR-T), we have reported very different effects of acid on proliferation. Cell number was significantly decreased by a 10-min exposure to acid in three Barrett's epithelial cell lines.⁵³ Using flow cytometry, we determined the effects of acid specifically on cell proliferation. At two hours following a 10-min exposure to acid, we found that there were slightly more cells in G1 and significantly less cells in S phase in the acid treated group compared to controls.⁴ By four hours, we found significantly more cells in G1 and significantly less cells in S phase in the acid treated group compared to control suggesting that acid decreases proliferation by causing a delay in cell cycle progression at the G1-S cell cycle checkpoint.⁵³ Subsequent data from our laboratory have demonstrated that acid caused

DNA double strand breaks due to generation of intracellular reactive oxygen species suggesting that the antiproliferative effects of acid are in response to genetic damage.

Characteristics of apoptosis in Barrett's cells

We have also reported that a 10-minute exposure to acid induces a small (1%), but statistically significant increase in apoptosis in Barrett's epithelial cells.⁵³ Apoptosis of Barrett's epithelial cells has also been studied in response to bile acid exposure using *ex vivo* cultures of esophageal squamous and Barrett's esophageal biopsies.⁵⁴ In these models, exposure to the unconjugated bile acid DCA increased apoptosis in the esophageal squamous biopsies, but not in the BE biopsies, suggests that the Barrett's epithelial cells resist apoptosis in response to bile acid exposure.⁵⁴

Conclusions

In nondysplastic Barrett's cells, acid exposure decreases proliferation and causes a slight increase in apoptosis. In contrast, bile salt exposure does not induce apoptosis in *ex vivo* cultures of nondysplastic Barrett's cells.

11. Are there tissue biomarkers that can stratify risk for future neoplasia in BE?

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BE is one of the most significant known risk factors for development of EAC. The proportion of patients with BE progressing to EAC is small, thus there are continuous efforts to stratify these patients accurately to focus rigorous surveillance on a high risk group in a cost effective manner. Clearly, the available clinical and endoscopic criteria are not highly predictive and this has led to increasing interest in biomarkers. Early Detection Research Network (EDRN) has proposed five phases of biomarker evaluation⁵⁵ as illustrated in Table 1. Various biomarkers have been studied in context of Barrett's progression and are in different phases of development.

DNA content abnormalities (aneuploidy/tetraploidy)

Multiple studies have evaluated DNA content abnormalities due to structural and or numerical changes in chromosome numbers. They have shown

Table 1. Phases of biomarker development

1	Identification
2	Cross-sectional studies for validation and standardization of assay
3	Case-control studies to confirm expression
4	Prospective longitudinal studies
5	Population-based studies

variable relative risk of progression with one phase 4 study showing a five year cumulative cancer rate of 28%.⁵⁶ DNA content abnormalities are one of the most widely studied markers of progression in subjects with BE, but technical challenges with flow cytometry, in addition to need for special media, has limited widespread application.

Tumor suppressor loci abnormalities

p53, a well-known tumor suppressor, has been shown to be frequently inactivated in Barrett's carcinoma progression. There is convincing evidence that supports p53 LOH as a fairly accurate predictive marker.⁵⁷ Evaluation of p53 LOH requires genotyping that is currently limited to the research setting. Immunohistochemistry has been proposed to be an alternative means of evaluation but is not as accurate. Thus, despite high predictive power, clinical use has been a challenge.

p16 hypermethylation

Epigenetic silencing of p16 is one of the most common abnormalities reported in BE. Its high prevalence has led to its evaluation in multiple studies, but predictive power has been found to be low.⁵⁸ It has been proposed that this may be the initial event creating an environment conducive for further accumulation of genetic changes and eventually leading to the progression to carcinoma.

Other biomarkers of interest

Cyclin D1, aberrant methylation of tumor suppressor genes: RUNX3 and HPP1, HER2/Neu, c-myc, COX2, EGFR, survivin, caspase 3, and E-cadherin. To address the initial question, there are tissue biomarkers that may help in risk stratification, but none of them are equipped for widespread clinical use.

Future directions

With increasing availability of compelling information on various biomarkers, there has been an interest in evaluation of panels combining clin-

ical features, endoscopic criteria, and molecular biomarkers as tools of risk stratification.

12. Is there one transcription factor that shows a significant increase in the progression from BM to adenocarcinoma?

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The basis for this question likely emerges from what is currently understood or suspected regarding the pathogenesis of BE.⁵⁹ Presently, one model for BE holds that ectopic expression of transcription factors and growth factors normally associated with the intestine and colon contributes to the emergence of the intestinal metaplasia. A number of transcription factors have been identified with prominent roles in BE pathogenesis including C, Gli (SHH), Smad (BMP4), Sox9, and NF- κ B. The criteria by which these factors were identified include (1) they are not normally expressed in the esophagus; (2) they are nearly universally detected in BE tissues; (3) they have prominent roles in intestinal development or cellular responses to inflammation; and (4) for many of these factors there is experimental data from cell culture and animal models demonstrating their contribution to promoting intestinal metaplasia.⁵⁹

The question is, then, can we identify a transcription factor or factors that meet similar criteria and that are equally important for transforming BE cells into neoplastic adenocarcinoma cells? A review of the literature finds a number of microarray and other genetic studies of EACs that have identified candidate factors. Most of these studies compared gene expression patterns in BE tissues and EACs, and suggested *increased* expression of Sox9, ERG3, ERG4, c-Myc, COX2, DNMT3b, RARa, SPARC, and Wnt/ β -catenin are all associated with progression from BE to EAC.⁶⁰ One problem with these candidates is that all are expressed in BE without dysplasia, only their levels are increased with progression to cancer. This seems unsatisfactory, since none would appear to have the same dramatic transforming effect as those factors associated with BE pathogenesis; none are presently being utilized as a marker predicting disease progression.

However, one transcription factor does meet these stringent criteria in an unexpected way, and

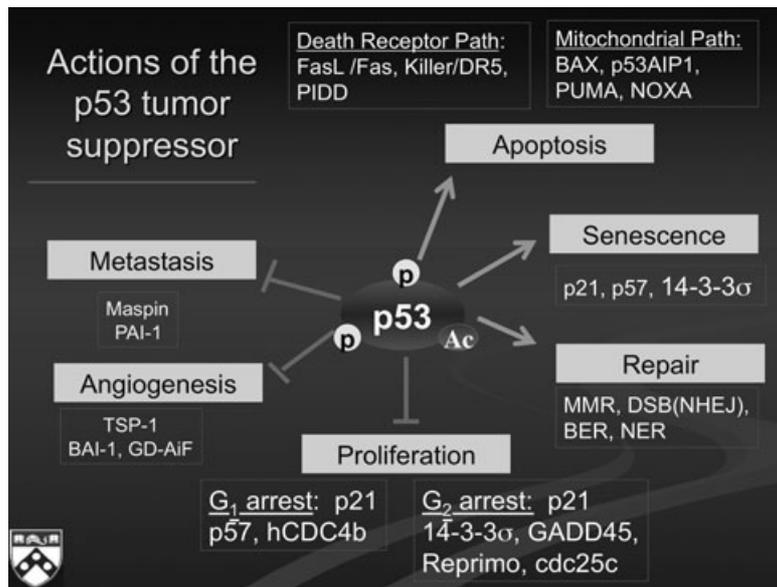


Figure 7. Functions of the tumor suppressor and transcription factor p53. The p53 gene targets are indicated with their associated tumor-suppressor functions.

that factor is p53. p53 is a transcription factor and a well-known tumor suppressor. The genes targeted by p53 perform many tumor-suppressor functions, including growth arrest, induction of DNA repair, induction of apoptosis, induction of cell senescence, and the prohibition of cell metastasis and angiogenesis (Fig. 7).⁶¹ The actions of these many gene targets serve to inhibit five of the six hallmark features of cancer cells identified by Hanahan and Weinberg in their seminal review of the subject.⁶² Thus, mutation and inactivation of p53 disrupts these many tumor-suppressor qualities and advances the neoplastic transformation of a cell. Consistent with this, p53 is typically normal in BE cells but frequently mutated in BE with high-grade dysplasia and in EAC.⁶³

One other feature of p53 should be noted here. MDM2 is a ubiquitin ligase and an important p53 target gene. MDM2 normally ubiquitinates p53, shunting it to the proteasome for degradation. In cells with normal p53, induction of MDM2 acts to feedback and limit p53 levels. However, when p53 is mutated, it cannot induce MDM2, and therefore levels of mutant p53 remain elevated. This is why immunohistochemistry for p53 in BE tissues is being studied as a marker for predicting disease progression.⁶³ Those tissues that have acquired a mutant p53 will often have elevated p53 levels eas-

ily detected by immunohistochemistry. And those cells with p53 mutations are at an advanced stage in their transformation to cancer.

In summary, with regard to the question: is there one transcription factor that shows a significant increase in the progression from BM to adenocarcinoma? The answer to this is: it is a trick question. Levels of the tumor suppressor (and transcription factor) p53 are frequently increased with progression to HGD and EAC, and may be a useful marker predicting likely progression to adenocarcinoma. However, this is a mutated form that is transcriptionally inactive. Loss of p53 function confers many hallmark features for neoplasia, and undoubtedly contributes to progression of BE cells to EAC.

Conflicts of interest

The authors declare no conflicts of interest.

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