Histol Histopathol (1999) 14: 553-559

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Invited Review

The molecular pathology of Barrett's esophagus

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Summary. The incidence of adenocarcinoma of the distal esophagus is rapidly increasing in the Western world. The histopathological sequence of (Barrett's) metaplasia, which develops as a consequence of chronic reflux, to dysplasia and then to carcinoma is well established for these tumors. In Barrett's esophagus a variety of molecular changes have been characterized and correlated with tumor initiation and progression. Among the early changes in premalignant stages of metaplasia are alterations of the transcripts of FHIT, a presumptive tumor suppressor gene which spans the common fragile site FRA3B. Mutations of p53 seem to accumulate mainly in the transition from low to high grade dysplasia. Inactivation of other tumor suppressor genes by mutation (APC, p16) or hypermethylation (p16) as well as amplification of oncogenes such as c-erbB2 are relatively late events in the development of adenocarcinoma. Among the phenotypic changes in Barrett's esophagus are an expansion of the Ki67 proliferation compartment which correlates with the degree of dysplasia. Moreover, accumulation of rab11 molecules which are involved in membrane trafficking has been reported to be specific for the loss of polarity seen in low grade dysplasia. Reduced expression of the cadherin/catenin complex as well as increased expression of various proteases develop chiefly in invasive carcinomas. Despite the progress that has been made in the identification of molecular markers in Barrett's carcinoma, to date the histopathogical diagnosis of high grade dysplasia in endoscopic biopsies remains the best predictor of invasive cancer. Immunohistochemistry applying a panel of antibodies including p53, Mib-1 or rab11 can be helpful to diagnose regenerative metaplastic epithelium or low and high grade dysplasia.

Key words: Esophagus, Metaplasia, Dysplasia, Carcinoma, Molecular genetics

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Introduction

As a result of chronic duodeno-gastro-esophageal reflux the normal squamous epithelium in the distal esophagus is replaced by a columnar or intestinalized epithelium with goblet cells (Spechler and Goyal, 1986; Stein and Panel of experts, 1998). This metaplastic (Barrett's) epithelium is a predisposing condition for the development of adenocarcinoma, through a well defined histopathological sequence from metaplasia to low grade dysplasia to high grade dysplasia and then to carcinoma. The risk for developing adenocarcinoma is estimated to be increased 30-125-fold in patients with Barrett's metaplasia vs. comparison populations without Barrett's metaplasia. In the Western world, Barrett's adenocarcinoma has the most rapidly increasing incidence among all malignancies (Blot et al., 1991; Altorki et al., 1997).

The esophagus is easily accessible for endoscopic surveillance, and periodic examination with systematic biopsies has been proposed in Barrett's esophagus patients to identify those with an increased risk for cancer (Levine et al., 1993). So far, the best predictor of carcinoma is the histopathological detection of high grade dysplasia, since it is known that there is a high incidence of invasive cancer which is either coincident or develops within a short time (McArdle et al., 1992). However, the histopathological grading of dysplasia in endoscopic biopsies from Barrett's esophagus is subjective and has a relatively high interobserver disagreement. Therefore, high-grade dysplasia, which in most centers is an indication for "prophylactic esophagectomy", should be confirmed by two experienced pathologists (Pera et al., 1992; Rusch et al., 1994). Recently, the assessment of cytometric and morphometric features has been proposed as a valuable adjunct for clinical decision-making in cases of Barrett's esophagus with dysplasia (Polkowski et al., 1998).

In addition to histomorphology there may be other criteria for recognizing Barrett's esophagus patients with a significant risk for developing cancer. During the multistep process of neoplastic transformation, gross chromosomal changes occur. Accompanying genetic alterations lead to abnormalities in gene expression and

cell cycle regulation. The precise timing and significance of most of these molecular genetic changes during tumorigenesis in Barrett's carcinoma remain unclear. This review summarizes the molecular events believed to play a major role in the neoplastic transformation of Barrett's epithelium (Table 1).

DNA ploidy and cytogenetics

In the majority of Barrett's adenocarcinomas aneuploidy can be detected by flow cytometry (Giaretti, 1997). Aneuploidy is also observed in pre-neoplastic Barrett's esophagus, and has been found to rise markedly in prevalence from low- to high-grade dysplasia and adenocarcinoma (Reid et al., 1992). Flow cytometry has been proposed to be helpful as a screening method in addition to histopathology for identifying patients at high risk for developing malignancy (Lewin and Appelman, 1996). In investigations of the spatial distribution of DNA abnormalities in Barrett's epithelium, multiple aneuploid clones have been noted in dysplasia and associated adenocarcinoma suggesting that the genome of the metaplastic mucosa of these patients is unstable (Rabinovitch et al., 1989).

Chromosome analyses have revealed complex karyotype changes in esophagus carcinomas. Among the recurring abnormalities seen are structural aberrations involving 1p, 3p, 3q, 11p, 22p (Menke-Pluymers et al., 1996).

Tumor suppressor genes

p53

The p53 gene is located on 17p and encodes a nuclear protein that functions as a guardian of the genome. DNA damage stimulates the expression of wild-type p53 protein. Normal p53 protein mediates cell cycle arrest and induces DNA repair. If DNA damage is severe, p53 can drive the cell into apoptosis. During carcinogenesis p53 is often inactivated and loses its protective function, resulting in genomic instability and tumor progression. p53 is inactivated by two discrete steps, i.e. mutation of one allele and deletion of the second allele (Hartwell, 1992; Levine, 1992). Mutated p53 accumulates in the nucleus and becomes detectable by immunohistochemistry. However, proof of the mutation requires molecular genetic techniques such as SSCP (single strand conformation polymorphism) in combination with sequencing (Ireland et al., 1997).

Although p53 is the best studied molecule in Barrett's esophagus, only a few papers have compared molecular genetic data for p53 with the histopathological sequence of metaplasia, dysplasia and carcinoma. Occasional cases of intestinal metaplasia or low grade dysplasia have been reported to harbor p53 mutations. In high grade dysplasia a prevalence of p53 mutation of approximately 60%, similar to invasive cancer (Casson et al., 1994; Hamelin et al., 1994; Neshat et al., 1994; Gleeson et al., 1995b; Audrezet et al., 1996; Schneider et al., 1996; Ireland et al., 1997) has been reported. So far, it is not clear whether patients with p53 mutations have an increased risk for progression to adenocarcinoma.

Most studies investigating the role of p53 in Barrett's esophagus have applied immunohistochemistry to paraffin sections. In general, these data confirm the results of molecular genetic studies, but the prevalence of accumulation of p53 protein is higher than the genetic abnormalities found by mutation analysis. Paralleling the molecular genetic analyses, an increase in immuno-

Table 1. Molecular biological factors involved in carcinogenesis in Barrett's esophagus.

FACTOR	COMMENT	POTENTIAL DIAGNOSTIC USEFULNESS
DNA Ploidy	Aneuploidy in high grade dysplasia	+
Cytogenetics	Multiple abnormalities in carcinoma	-
Tumor suppressor genes		
p53	60% mutation - high grade dysplasia and carcinoma	++
APC	Late in dysplasia-carcinoma sequence	-
FHIT	Common, early abnormalities	?
p16	Late in dysplasia-carcinoma sequence	-
Growth factors		
fas	Shift to cytoplasm in carcinoma	-
EGFR	Expressed in 60% carcinomas, gene amplification	-
c-erbB2	Co-amplified with EGFR	-
Cell adhesion		
E-cadherin	Loss of expression in high grade dysplasia, carcinoma	+
Catenins	Similar loss of expression to E-cadherin	+
Proteases		
UPA	Prognostic factor in carcinoma	±
Proliferation		
Ki-67	Abnormal distribution in high-grade dysplasia	++
Membrane trafficking		
rab11	High expression in low-grade dysplasia	++

reactivity with progression to high-grade dysplasia and invasive cancer is seen. Therefore, p53 immunohistochemistry has been proposed as a progression marker in Barrett's esophagus (Jones et al., 1994; Krishnadath et al., 1995). For the clinically relevant distinction between low- and high-grade dysplasia, immunohistochemistry for p53 may be helpful if used in a panel with other antibodies (Polkowski et al., 1998). In adenocarcinoma, p53 immunostaining has been reported to be prognostically significant but this was not independent of stage (Moskaluk et al., 1996).

APC

The APC (adenomatous polyposis coli) gene is a tumor suppressor gene located on chromosome 5q and is frequently mutated in colorectal carcinoma (Fearon and Vogelstein, 1990). In Barrett's esophagus, mutations of APC seem to develop late in the progression to invasive cancer (Gonzalez et al., 1997). Allelic loss of APC is frequently found in adenocarcinoma, but there are conflicting results concerning the presence of LOH in premalignant stages. Using microdissection techniques, Zhuang et al. (1996) were able to demonstrate allelic loss at the APC locus in the same metaplastic monoclonal populations which had apparently progressed to dysplasia and adenocarcinoma due to their spatial proximity. Gonzalez et al. (1997) failed to detect LOH of APC in dysplasia, but microdissection to enrich the cells of interest was not used. Allelic loss at the APC locus is believed to be concurrent (Gonzalez et al., 1997) or subsequent to allelic loss at p53 (Blount et al., 1993).

FHIT

Located on 3p14.2, FHIT (fragile histidine triad gene) has recently been identified as a presumptive tumor suppressor gene (Ohta et al., 1996). So far, the precise function *in vivo* of the enzyme encoded by FHIT is not known. The FHIT gene spans the common fragile site FRA3B. Fragile sites are genomic regions which predispose for structural chromosome aberrations such as translocations or deletions, especially following exposure to toxins (Sutherland, 1991).

In Barrett's esophagus, alterations of FHIT mRNA, for example, the deletion of one or more exons, have been detected by RT-PCR in up to 86% of cases of metaplasia as well as in 93% of adenocarcinomas (Chen et al., 1997; Michael et al., 1997; Zou et al., 1997). Homozygous deletions have been found only in invasive cancer and involve large regions (Michael et al., 1997). In some paired non-neoplastic and neoplastic samples, sequence analysis has revealed different patterns of transcript aberration, while in others aberrant transcripts were only found in the non-neoplastic tissue sample (Chen et al., 1997). These data suggest that the aberrant transcripts in the metaplastic and carcinomatous areas may have been induced by exposure of the entire field to toxic agents that affect the fragile FRA3B site, possibly involving other target genes in the vicinity of FHIT (Chen et al., 1997).

p16

The cyclin-dependent kinase inhibitor p16 (also CDKN2A, INK44A or MTS1) gene is mapped to 9p21 and encodes for a 156-amino-acid protein characterized as a cyclin-dependent kinase inhibitor. This protein is capable of forming complexes with cyclin-dependent kinases which then lose their ability to phosphorylate the RB (retinoblastoma) gene. As a consequence, cells are prevented from entering the cell cycle and proliferation is inhibited (Serrano et al., 1993). Inactivation of p16 by mutation, deletion or hypermethylation allows uncontrolled cell proliferation (Lukas et al., 1995).

Inactivation of p16 seems to occur quite late in the progression to Barrett's adenocarcinoma. Wong et al., (1997) detected hypermethylation of the p16 promoter and LOH at 9p21 in 8/21 patients with invasive cancer, and in 3 patients with only premalignant Barrett's epithelium. Mutations or homozygous deletions of p16 have been observed less frequently (Gonzalez et al., 1997; Muzeau et al., 1997).

Other tumor suppressor genes

Allelic loss is easily detected by PCR, which targets polymorphic DNA sequences, and which is therefore useful to screen for chromosome regions which might contain tumor suppressor genes. A few studies have investigated polymorphic markers at multiple loci on various chromosomal arms for loss of heterozygosity (LOH). Among the chromosome regions with highest rates of LOH, i.e. allelic loss in more than 60% of Barrett's carcinomas informative for this locus, were 3q, 4q, 5q, 6q, 9p, 12q, 17p, 17q and 18q (Gleeson et al., 1995a; Swift et al., 1995; Barrett et al., 1996a).

Among the chromosome regions which have been studied in more detail is 18q21.1. Allelic loss of this area in aneuploid cell populations has been seen in 45% of samples of Barrett's adenocarcinoma and 46% of premalignant epithelium without invasive carcinoma (Barrett et al., 1996b). Interestingly, in 3/4 samples containing only diploid cells, the same LOH was present which was detected in the corresponding carcinoma. These data suggest that allelic loss of 18q21.1 occurs as an early event in Barrett's epithelium and precedes the development of aneuploidy and carcinoma. A possible target gene in this region is the DPC4 gene (deleted in pancreatic carcinoma, locus 4) which is inactivated by homozygous deletion in approximately 30% of pancreatic adenocarcinomas (Barrett et al., 1996b; Hahn et al., 1996). DCC (deleted in colorectal cancer), located at 18q21, is another candidate tumor suppressor gene in Barrett's esophagus in this region (Barrett et al., 1996a,b).

Growth factors and receptors

The Fas/APO-1 (CD95) gene encodes for a transmembrane protein that is a member of the nerve growth factor family. Fas is involved in apoptosis and loss of its expression during carcinogenesis can result in interruption of the apoptotic pathway (Nagata and Golstein, 1995). Recently, Hughes et al. (1997) investigated the role of Fas in Barrett's carcinoma. By immunohistochemistry, adenocarcinomas had no or only faint staining for Fas, whereas high levels of Fas mRNA were detected by Northern blot analysis. Further studies using an esophageal adenocarcinoma cell line showed that wild-type Fas protein is usually expressed on the cell surface, but is retained in the cytoplasm in malignantly transformed cells. Presumably, malignant cells are able to evade Fas-mediated apoptosis by this mechanism.

One of the many peptides which are involved in differentiation and proliferation of cells is fibroblast growth factor (FGF). Immunostaining of esophageal resection specimens has revealed sequential accumulation of acidic fibroblast growth factor during the progression from Barrett's metaplasia to dysplasia and carcinoma. Therefore, antibodies to fibroblast growth factor may be useful in a panel of antibodies for diagnostic purposes in endoscopic biopsies (Soslow et al., 1997). The epidermal growth factor receptor (EGFR) has been reported to be expressed in 64% of Barrett's adenocarcinomas. EGFR gene amplification has been found in 31% of esophageal adenocarcinomas with co-amplification of c-erbB2 in 15% (al-Kasspooles et al., 1993) suggesting that there may be a form of synergy between these two factors. Correlation with patient survival with EGFR showed that it was a poor prognostic marker in stage II tumors. Expression of the transforming growth factor-alpha was not prognostically useful (Yacoub et al., 1997).

Cell adhesion molecules

Epithelial cells maintain their cell to cell contacts and polarity through cell adhesion molecules such as E-cadherin. Loss of E-cadherin expression correlates with invasion and metastatic potential in a variety of carcinomas (Behrens, 1994). The normal adhesive function of E-cadherin and its connection to the actin cytoskeleton depend on its interaction with three other proteins, i.e. alpha, beta and gamma-catenin. In one study of Barrett's esophagus, reduced expression of the cadherin/catenin complex was reported along the metaplasia-dysplasia-carcinoma sequence (Bailey et al., 1998). In this study, only the decrease of E-cadherin immunostaining was statistically significant, however.

In a study of invasive adenocarcinoma, Krishnadath et al. (1997) found a strong correlation between the expression of E-cadherin and catenins which were reduced in approximately 70% of the cases examined. These data suggest that in esophageal adenocarcinoma, decrease of the E-cadherin and catenin expression are related events. Moreover, the reduced expression of E-cadherin and beta-catenin were significant markers for survival prognosis, independent of disease stage. Down-regulation of the E-cadherin/catenin complex in tumors may be due to alterations affecting the function of these genes, e.g. mutations or transcription and translation abnormalities (Breen et al., 1993; Becker et al., 1994). Other possibilities are modulations by other proteins which interact with the E-cadherin complex, e.g. EGFR, c-erbB-2 or APC; proteins that are often involved in the development of advanced tumors (al-Kasspooles et al., 1993).

Proteases

Due to their important role in the basic processes of invasion and metastases, the serine protease family of enzymes has been studied in a variety of tumor types as potential prognostic markers. In a recent study of serine proteases in esophageal adenocarcinoma by ELISA, high expression of urokinase-like plasminogen activator (UPA) was found to be an independent predictor of poor survival in patients with complete surgical resection of their tumors (Nekarda et al., 1998). To date, no systematic study of serine protease expression by pre-cancerous (i.e. metaplastic or dysplastic) epithelium in Barrett's esophagus exists.

Proliferation markers

Ki-67 is a nuclear antigen which is expressed during the proliferative phase of the cell cycle (G1, S, G2 and M phases). Using the Mib-1 antibody for immunohistochemical identification of the Ki-67 antigen, a shift and expansion of the normal proliferative compartment towards the surface in Barrett's esophagus along the metaplasia-dysplasia-carcinoma sequence is seen. Specifically, there is an increase in the numbers of Mib-1-positive cells in Barrett's epithelium without dysplasia compared to normal gastric epithelium, but these cells are confined to the normal proliferative zone in the crypt base. In low-grade dysplasia proliferative cells begin to extend to the surface and in high-grade dysplasia, significantly greater numbers are found in the upper crypts compared with low-grade dysplasia (Hong et al., 1995). A few cases of low-grade dysplasia with a majority of proliferative cells in the upper crypt zones have been described, but these are associated with high-grade dysplasia in other areas which suggests that Mib-1 staining may useful for the identification of cases with a higher risk of development of high-grade dysplasia and carcinoma.

Membrane trafficking

Apart from the analysis of genes involved in cell proliferation and differentiation, there has been little progress in the definition of the molecular events by

which the dysplastic phenotype develops (Crawford, 1997). One recent study gives a possible explanation for the deranged morphology of the dysplastic cells in Barrett's esophagus. Ray et al. (1997) described the expression of Rab11 during neoplastic progression in Barrett's epithelium. Rab11 is a GTP-binding protein which is associated with intracellular vesicles and is involved in membrane trafficking to the apical border of the polarized cells (Goldenring et al., 1996).

In normal glandular mucosa as well as in intestinalized epithelia, there is only faint, perinuclear immunostaining for Rab11. In low grade dysplasia, which is characterized by derangement of cell polarity, Rab11 accumulates in a supra-nuclear region, which indicates a defect in apical membrane trafficking. In high-grade dysplasia and adenocarcinoma, these membrane pathways become completely deranged, and rab11 immunostaining decreases. The specificity of rab11 immunoreactivity for low-grade dysplasia could be diagnostically useful if included in panel of antibodies (Ray et al., 1997).

Conclusion

The ongoing characterization of the molecular events involved in carcinogenesis, as well as their correlation with the histopathological progression of Barrett's esophagus is steadily increasing our knowledge of the pathogenesis of Barrett's carcinoma. However, decision making in the clinical setting, i.e. the indication for esophagectomy, currently rests upon routine histopathology in endoscopic biopsies, a procedure which is cost-effective and will remain the gold standard in the near future. However, a few immunohistochemical markers have emerged which could be helpful for the differential diagnosis of low- and high-grade dysplasia. For this purpose, a panel of monoclonal antibodies including, for example, MIB-1, p53, rab11, fibroblast growth factor and E-cadherin might be particularly helpful for the pathologist. Markers which could predict the progression of premalignant Barrett's epithelium to carcinoma are still to be established. One candidate gene for such a marker could be p53, but molecular genetic analyses for mutation detection are required for accurate diagnosis. The potential application of such a test to the clinical setting would require careful histopathological definition of the area to be selected for molecular analysis.

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Accepted September 1, 1998