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Review

From Barrett metaplasia to esophageal adenocarcinoma: the molecular background

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Summary. The molecular landscape of Barrett's esophagus and Barrett-related neoplastic lesions is still far from being completely elucidated. Both *in vitro* and *in vivo* studies pinpointed the pathogenetic role of different morphogenic pathways (the para-homeobox, the Notch and the Sonic Hedgehog families in particular) implicated in the acquisition of the metaplastic phenotype of the esophageal mucosa. On the other hand, the most common genetic alterations observed during Barrett's carcinogenesis include disorders of major regulators of the cell cycle, as well as deregulation of the TGF-β/Smad and receptor tyrosine kinases signalling pathways. Recent comprehensive mutational profiling studies identified that the inactivation of the *TP53* and of the *SMAD4* tumour suppressor genes occurred in a stage-specific manner, confined to (high grade) dysplastic and neoplastic lesions, respectively. The next step will be the correlation of these findings into multidisciplinary diagnostic approaches integrating endoscopy, histology, molecular profiling and liquid biopsies. This will allow the introduction of innovative strategies for secondary prevention of esophageal adenocarcinoma based on biological rationales, and the implementation of potential novel therapeutic targets.

Key words: Barrett's esophagus, Molecular pathology, Biomarker, MicroRNA

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Introduction

In the last twenty years, esophageal adenocarcinoma (EAC) has become one of the most deadly neoplasms among Western populations (Rustgi and El-Serag, 2014). In fact, the dramatic increase observed for EAC incidence is coupled with a steady dismal prognosis with an overall 5-year survival rate of below 20% (Rustgi and El-Serag, 2014). This mainly depends on the fact that most patients present with locally advanced or widespread metastatic disease, where the current treatment is largely ineffective. Thus, an early diagnosis of EAC would significantly impact on patient overall survival.

Barrett's esophagus (BE) has been widely demonstrated as the most significant precursor lesion for EAC (Rugge et al., 2012b; Spechler and Souza, 2014). BE is the phenotypic outcome of longstanding exposure of the esophageal mucosa to gastro-esophageal reflux, resulting in the metaplastic replacement of the native squamous epithelia by columnar (intestinalized) cells (i.e. Barrett's mucosa; BM) (Fiocca et al., 2011). This cancer-prone epithelial population becomes the "cancerization field" in which intra-epithelial neoplasia (IEN, formerly called dysplasia) and Barrett-related EAC may develop (Fig. 1) (Rugge et al., 2000, 2015).

Several epidemiological studies confer to BE a 30-125 fold higher risk for EAC (Rugge et al., 2012a,b). However, only a small proportion of BE progresses to EAC, and routine BE endoscopic surveillance is recommended (Anaparthy and Sharma, 2014; Rustgi and El-Serag, 2014; Spechler and Souza, 2014). This secondary cancer prevention strategy is expensive,

invasive and limited by suboptimal adherence and access. Moreover, a histological diagnosis of dysplasia remains the only validated marker for identifying BE patients at risk of subsequent carcinoma, but timing of progression is not predictable and the molecular mechanisms behind Barrett's carcinogenesis are largely unknown (Genta and Rugge, 1999; Rugge et al., 2000; Fassan et al., 2013a). Hence, there is a great interest in identifying relatively inexpensive and effective (molecular) biomarkers for EAC secondary prevention.

This effort is currently limited by the complex genetic background of Barrett's carcinogenesis, which relies on multiple genetic (e.g. DNA mutations/polymorphisms, chromosomal instability, or aneuploidy), epigenetic (e.g. DNA methylation and chromatin modifications), and post-transcriptional (e.g. regulation of mRNA stability by miRNAs) alterations (Weaver et al., 2014). Moreover, the definition (and selection) of Barrett's related lesions (e.g. the categorization of metaplastic lesions, grading of intraepithelial neoplasia) is significantly affected by inter-observer variability (Rugge et al., 2015), and an adequate histological classification is achievable only with the use of formalin-fixed paraffin-embedded (FFPE) specimens. Of note, FFPE preparations are currently incompatible with many down-stream molecular biology techniques, which further affects the reliability of molecular studies on this particular topic. In fact, only recently, with the introduction of "FFPE-friendly" innovative technologies such as targeted next-generation sequencing, we are facing a feasible accurate and comprehensive molecular characterization of the stepwise progression of premalignant to malignant lesions in gastrointestinal carcinogenesis (Fassan et al., 2014b; Luchini et al., 2014).

This mini-review summarizes the current concepts and evidence on most important molecular mechanisms involved in the neoplastic transformation of the esophageal mucosa.

The molecular background of Barrett's mucosa onset

The acquisition of the metaplastic phenotype by the esophageal mucosa has been etiologically linked to chronic gastro-esophageal reflux disease (GERD). In particular, the pathogenetic effect of the two major noxious agents that characterise the reflux, acid and bile

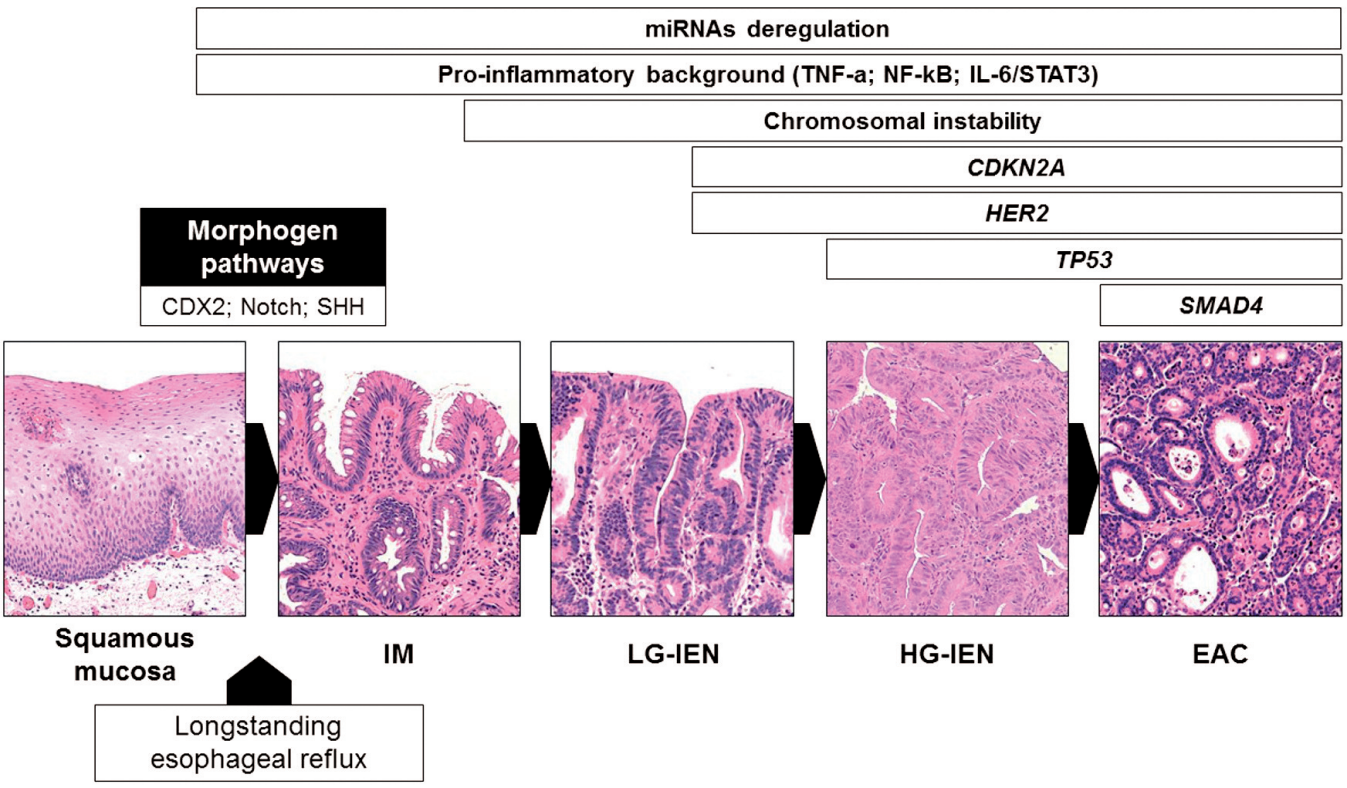

Fig. 1. Most deregulated molecular pathways during Barrett's carcinogenesis. IM: intestinal metaplasia; LG-IEN: low-grade intraepithelial neoplasia; HG-IEN: high-grade intraepithelial neoplasia; EAC: esophageal adenocarcinoma.

salts, on the squamous mucosa has been extensively investigated both *in vivo* and *in vitro* (Souza et al., 2008; Huo et al., 2010; Dall'Olmo et al., 2014).

The first molecular alteration observed after chronic exposure to gastro-esophageal reflux is a significant up-regulation of the *CDX* genes of the para-homeobox family in the squamous epithelia (Souza et al., 2008; di Pietro et al., 2012). These genes physiologically direct the formation of the simple columnar epithelium that characterizes the adult intestine. In fact, CDX2 is strongly expressed in both the small and large intestine, whereas the naive esophageal mucosa does not express this protein (Silberg et al., 2000; Gao et al., 2009).

In animal models of reflux esophagitis and BE, the reflux-injured esophageal squamous epithelium demonstrates increased CDX2 expression prior to the appearance of intestinal metaplasia in the esophagus (Kazumori et al., 2006; Ingravallo et al., 2009). Similarly, in humans CDX2 expression has been demonstrated in biopsy specimens of inflamed esophageal squamous epithelium, and has been shown to precede the expression of other intestinal markers like MUC2, sucrase-isomaltase, defensin-5, and alkaline phosphatase (Groisman et al., 2004; Vallbohmer et al., 2006). Overall, these studies implicate an early involvement of CDX2 over-expression in the phenotypic shift of the esophageal mucosa.

Several studies identified the activation of the hedgehog pathway as an additional significant early molecular event characterizing the esophageal mucosa after exposure to gastric and bile acids (Gibson et al., 2013). In particular, a significant up-regulation of Sonic hedgehog (SHH) has been described in Barrett's mucosa (BM) samples (Wang et al., 2010; Yamanaka et al., 2011). Interestingly, SHH is expressed in the embryonic esophagus, while it is lost in the squamous epithelium of the adult esophagus. In the inflamed esophageal mucosa, SHH overexpression determines a concomitant up-regulation of the bone morphogenetic protein 4 (BMP4) in the mesenchymal cells of the esophageal lamina propria. BMP4 is a downstream target of SHH and a member of the tumor growth factor β (TGFβ) signaling pathway (Milano et al., 2007). In turn, BMP4 signals back to the epithelial cells in a paracrine fashion, inducing the over-expression of the transcription factor SOX9 which drives the development of columnar epithelium. The activation of the SHH-BMP4-SOX9 axis in BM has been extensively validated both *in vitro and in vivo* (Wang et al., 2010; Yamanaka et al., 2011).

Notch signaling is another morphogenic pathway, which has been implicated in normal intestinal differentiation and in the acquisition of the intestinal phenotype in the case of BE (Quante et al., 2012; Tamagawa et al., 2012; Clemons et al., 2014). Moreover, Notch signaling regulates the differentiation of cardia progenitor cells and has been associated with malignant transformation of the esophagus.

The role of inflammation in the progression of Barrett's mucosa to advanced preneoplastic and neoplastic lesions

The carcinogenetic potential of longstanding inflammatory insults has been widely demonstrated in many fields of human pathology.

Chronic exposure to components of gastro-esophageal reflux triggers the release of inflammatory cytokines and chemokines by damaged esophageal cells and activates migration of inflammatory cells (Gibson et al., 2013). Subsequently, the cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the migrated inflammatory cells play a crucial role in EAC initiation (Dvorak et al., 2007; Mantovani, 2010).

Both ROS and RNS can contribute to DNA damage, which is a hallmark of Barrett's carcinogenesis. In fact, chromosomal instability and copy number alterations have been described as early events in BM and in its acquisition of the neoplastic phenotype (Paulson et al., 2009).

On the other hand, pro-inflammatory cytokines are involved in driving angiogenesis, tissue remodelling and tumor cell proliferation; the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IL-6/Signal Transducer and Activator of Transcription 3 (STAT3) pathways are the two best characterized inflammation-related signaling pathways associated with BE-related cancer progression (Dvorakova et al., 2004). NF-κB has been found to be up-regulated in BM and EAC samples (O'Riordan et al., 2005), where it regulates the expression of many pro-inflammatory and growth regulatory cytokines including tumour necrosis factor-α (TNF-α). Of note, TNF-α expression has been shown to increase along the metaplasia-dysplasia-carcinoma sequence leading to an increase in the proto-oncogene *MYC* via a β-catenin mediated pathway (Tselepis et al., 2002).

The IL-6/STAT3 pathway is activated in progression of BM to IEN and EAC. In particular, increased levels of IL-6 have been detected in BM compared to normal tissues, and even higher levels have been found in high-grade IEN lesions compared to nondysplastic BM samples. In addition, increased levels of phosphorylated STAT3 were detected in IEN and in EAC tissues (Dvorakova et al., 2004).

Key tumor suppressor genes and oncogenes in Barrett's carcinogenesis

Several studies have shown that the progression to EAC in patients with BE results from the accumulation of genetic or epigenetic alterations in tumor suppressor genes and oncogenes (Fassan et al., 2013a; Weaver et al., 2014).

The frequently observed early dysfunction in cell cycle regulation observed in BM is mainly due to the

inactivation of the two tumor suppressor genes *CDKN2A* (p16) and *TP53* (p53) (Zhang et al., 2010; Fassan et al., 2013a).

P16 regulates the G1-S cell cycle transition by inhibiting the cyclin D-cyclin-dependent kinases (CDK4/CDK6)-mediated phosphorylation of the retinoblastoma protein. While point mutations of the *CDKN2A* gene are rare (approximately 5%) in EAC, loss of heterozygosity (LOH) or promoter hyper-methylation (i.e. silencing) of *CDKN2A* have been identified in 80% of BE (Fassan et al., 2013a). Three independent studies demonstrated that CDKN2A promoter methylation (with or without *CDKN2A* LOH) is a common mechanism of *CDKN2A* inactivation during neoplastic progression of BM (Bian et al., 2002; Jin et al., 2009; Wang et al., 2009). Of note, in patients with non-dysplastic BM and low-grade IEN, *CDKN2A* methylation was demonstrated as an independent risk factor for progression to high-grade IEN and EAC (Schulmann et al., 2005). Moreover, p16 deregulation is associated with: i) a significant overexpression of cyclin D1, which is commonly observed during Barrett's carcinogenesis; ii) an increased risk of progression to EAC; and iii) correlates with the grading of IEN (Shi et al., 2008).

Another hallmark of esophageal mucosa transformation is the inactivation of the *TP53* tumor suppressor gene (Parenti et al., 1995). The most common combination of events leading to complete inactivation of *TP53* in the esophageal mucosa is the mutation of one allele with a concomitant deletion of the other allele (Bian et al., 2001; Weaver et al., 2014).

TP53 inactivation is a later event characterizing high-grade IEN lesions. In fact, several independent studies demonstrated that the percentage of patients with LOH of *TP53* gene locus (i.e. Chr 17p) significantly increased with the histological grade of IEN (Reid, 2001; Reid et al., 2001; Rygiel et al., 2007).

TP53 mutations may result in loss or stabilization of the mutant p53 protein, and the latter can be visualized by immunohistochemistry. Thus, p53 immunostaining (in combination with the MIB1 labeling index) is a useful diagnostic tool that is currently used in clinical practice to improve the histological evaluation of BE-related IEN lesions (Fassan et al., 2013a). In fact, the p53 staining pattern is changing during BE progression, with weak to moderate intensity in cases with low-grade IEN lesions and more intense diffuse staining throughout high-grade IEN and EAC (Moyes et al., 2012). However, because not all p53 mutations lead to mutant protein stabilization, immunohistochemistry is not as sensitive as DNA sequencing or LOH assays (Fitzgerald et al., 2014).

Also, transforming growth factor β (TGFβ) signaling plays a central role in the impairment of cell-cycle control in BE progression (Lao-Sirieix and Fitzgerald, 2010; Mendelson et al., 2011). In normal cells, TGFβ induces a reversible cell cycle arrest and many epithelial tumors are refractory to this response. In contrast, TGFβ is implicated in an epithelial to mesenchymal transition (EMT) in tumor cells, particularly at the invasive edges, where this change in phenotype is thought to aid invasion and formation of metastases. Both of these mechanisms have been implicated in the progression of BE to EAC (Mendelson et al., 2011). TGFβ expression is upregulated in EAC compared with normal esophageal squamous mucosa and BE and is associated with advanced stage of the cancer. An important mechanism of deregulation of TGFβ signalling in BM is the modulation of its downstream transcriptional mediators, particularly SMAD2 and 4. LOH at chromosome 18q, location of the *SMAD2* and *SMAD4* genes, is relatively frequent in EAC (Nancarrow et al., 2008). SMAD4 mRNA expression is progressively reduced in the metaplasia-dysplasia-adenocarcinoma sequence and SMAD4 promoter methylation is found in almost 70% of primary Barrett-related EACs (Onwuegbusi et al., 2006; Weaver et al., 2014).

Amplification/overexpression of several oncogenes, most notably of the epithelial growth factor receptor (EGFR) family members (e.g. EGFR and HER2) have been reported as a relatively frequent alteration in BE carcinogenesis. The expression of the EGFR protein is increased in up to two thirds of EAC and has been associated with a higher stage of disease (Cronin et al., 2011). *HER2* amplification (also known as *ERBB2* or neu gene) is observed only in IEN and EAC lesions, and its targeting (e.g. by the monoclonal antibody trastuzumab) has been successfully introduced in the treatment of advanced gastro-oesophageal cancers now representing a stage-dependent standard of care (Bang et al., 2010; Fassan et al., 2012a,b, 2013b; Grillo et al., 2013).

New biomarkers on the Barrett's scenario: the role of noncoding RNAs

Non-coding RNAs (ncRNAs) have generated a great deal of interest in oncological research (Fassan et al., 2011a; D'Angelo et al., 2015). Among several families, microRNAs (miRNAs) deregulation has been extensively characterized in human carcinogenesis.

MiRNAs have been suggested as an innovative class of biomarkers because of their small size and stability in biological samples, their capability to regulate hundreds of messenger RNAs (mRNAs), and their relatively small total number compared to mRNAs (Fassan and Baffa, 2013; Di Leva et al., 2014).

Several reports point to miRNAs as molecules involved in each step of Barrett's carcinogenesis by comprehensive miRNA expression profiling (Feber et al., 2008; Kan and Meltzer, 2009; Yang et al., 2009; Wijnhoven et al., 2010; Bansal et al., 2011; Fassan et al., 2011b, 2013c; Leidner et al., 2012; Revilla-Nuin et al., 2013; Saad et al., 2013; van Baal et al., 2013; Wu et al., 2013).

Among the most deregulated miRNAs, miR-21 has been found to be up-regulated during Barrett's

carcinogenesis in both high-grade IEN and EAC samples (Feber et al., 2008; Fassan et al., 2010; Wijnhoven et al., 2010). MiR-21 is considered a key oncomiR due to its targeting of several tumor suppressor genes such as *PTEN, PDCD4, RECK* and *TPM1* (Di Leva et al., 2014).

Two studies have reported a significant up-regulation of miR-196a during Barrett's carcinogenesis (Luthra et al., 2008; Maru et al., 2009). Of note, the expression of ANXA1, SPRR2C, and S100A9, which are targets of miR-196a, characteristically decreases or is even lost during the neoplastic transformation of the esophageal epithelium (Luthra et al., 2008; Maru et al., 2009).

Also, the miR-106b-25 polycistron on chromosome 7q22.1 (i.e., miR-25, miR-93 and miR-106b) has been found to be increasingly activated in successive stages of Barrett's carcinogenesis, with potentially proliferative, antiapoptotic, and cell cycle promoting effects *in vitro* and tumorigenic effects *in vivo* by targeting p21 and Bim (Kan et al., 2009).

More recently, a profile of four miRNAs (i.e., miR-192, miR-194, miR-196a, and miR-196b) has been demonstrated to adequately stratify BE patients according to their risk of disease progression over a course of 5 years (Revilla-Nuin et al., 2013).

Apart from miRNAs, another important class of ncRNAs is represented by the ultraconserved regions (UCRs) (Calin et al., 2007; Esteller, 2011). UCRs were discovered in 2004 as a result of bio-informatic comparisons drawn between mouse, rat, and human genomes and are absolutely conserved (100% identity with no insertions or deletions) among the three vertebrate species (Calin et al., 2007; Esteller, 2011). A large fraction of UCRs are transcribed (T-UCRs) in normal human tissues, and their deregulation has been demonstrated in human cancer (Calin et al., 2007).

Our group recently investigated T-UCRs expression profile during Barrett's carcinogenesis (Fassan et al., 2014a). We also observed that a similar T-UCRs expression profile was associated with similar histological lesions in humans and in two murine models of Barrett's carcinogenesis, supporting T-UCRs as a novel diagnostic as well as prognostic tool for the biological profiling of BE-associated lesions.

Concluding remarks

The mainstream carcinogenic process involved during Barrett's carcinogenesis is characterized by the phenotypic multistep progression cascade that eventually results in full-blown esophageal adenocarcinoma. In spite of the well-established understanding of the phenotypic lesions occurring in the shift from native esophageal squamous epithelia to invasive carcinoma, the molecular landscape of BE and Barrett-related neoplastic lesions is still far from being completely elucidated. This significantly affects the clinical introduction of strategies for secondary prevention of EAC based on a biological rationale. The implementation of innovative technologies (e.g. next-generation sequencing, high-throughput microarray analysis) coupled with the unexpected discovery of new classes of biomarkers (e.g. ncRNAs) will help us in the forthcoming accurate and comprehensive molecular characterization of premalignant and malignant lesions characterizing Barrett's associated carcinogenesis.

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