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REVIEW

Epigenetics in esophageal cancers

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Abstract Esophageal cancers are a challenging upper gastrointestinal tract tumor entity for interdisciplinary oncology. For the two main histotypes, namely esophageal squamous cell carcinomas and Barrett's adenocarcinomas, several genetic aberrations have been shown to contribute to carcinogenesis and progression as well as to represent potential novel targets for therapeutic intervention. This is paralleled by growing insight into epigenetic alterations of esophageal cancers. Studies involving the analyses of human tissue specimens predominantly describe altered patterns of miRNA expression, DNA methylation patterns, and histone marks levels. This review provides a critical update on this increasing knowledge of epigenetic alteration in esophageal cancers by specifically focusing on the translational aspects of epigenetic analyses from human tissue specimens.

Keywords Esophageal cancers · Epigenetics · miRNA · DNA Methylation · Histone marks

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Introduction

Esophageal cancers are upper gastrointestinal tract tumors of epithelial cell origin. The two major histotypes of esophageal cancer arise via distinct steps of carcinogenesis. Barrett's adenocarcinomas (BACs) evolve mainly in the lower esophagus as a consequence of chronic reflux disease via steps of trans-differentiation of normal squamous epithelial cells into columnar/intestinal epithelial cells (intestinal metaplasia), dysplasia, and malignant invasion. In contrast, esophageal squamous cell carcinomas (ESCCs) evolve in all parts of the esophagus via a mere progression from normal squamous epithelial cells to dysplasia and malignant invasion (Fig. 1).

Compared with lower gastrointestinal tract tumors, i.e. colorectal cancer, upper gastrointestinal tract tumors are generally less frequent but have a much higher mortality. With a rising incidence of esophageal cancers, particularly adenocarcinomas (Cook et al. 2009), a better understanding of esophageal cancer carcinogenesis and progression is urgently needed to improve patient risk stratification and therapeutic treatment options (Pennathur et al. 2013).

Several key genetic alterations have been identified in the carcinogenesis of esophageal carcinomas, such as mutations of key oncogenes and tumor-suppressor genes (e.g., *TP53*, *MYC*) or distinct patterns of chromosome/gene amplifications and deletions (e.g., *HER2*, *FHIT*) and tumor cell aneuploidy in general. Whereas some of these genetic alterations are

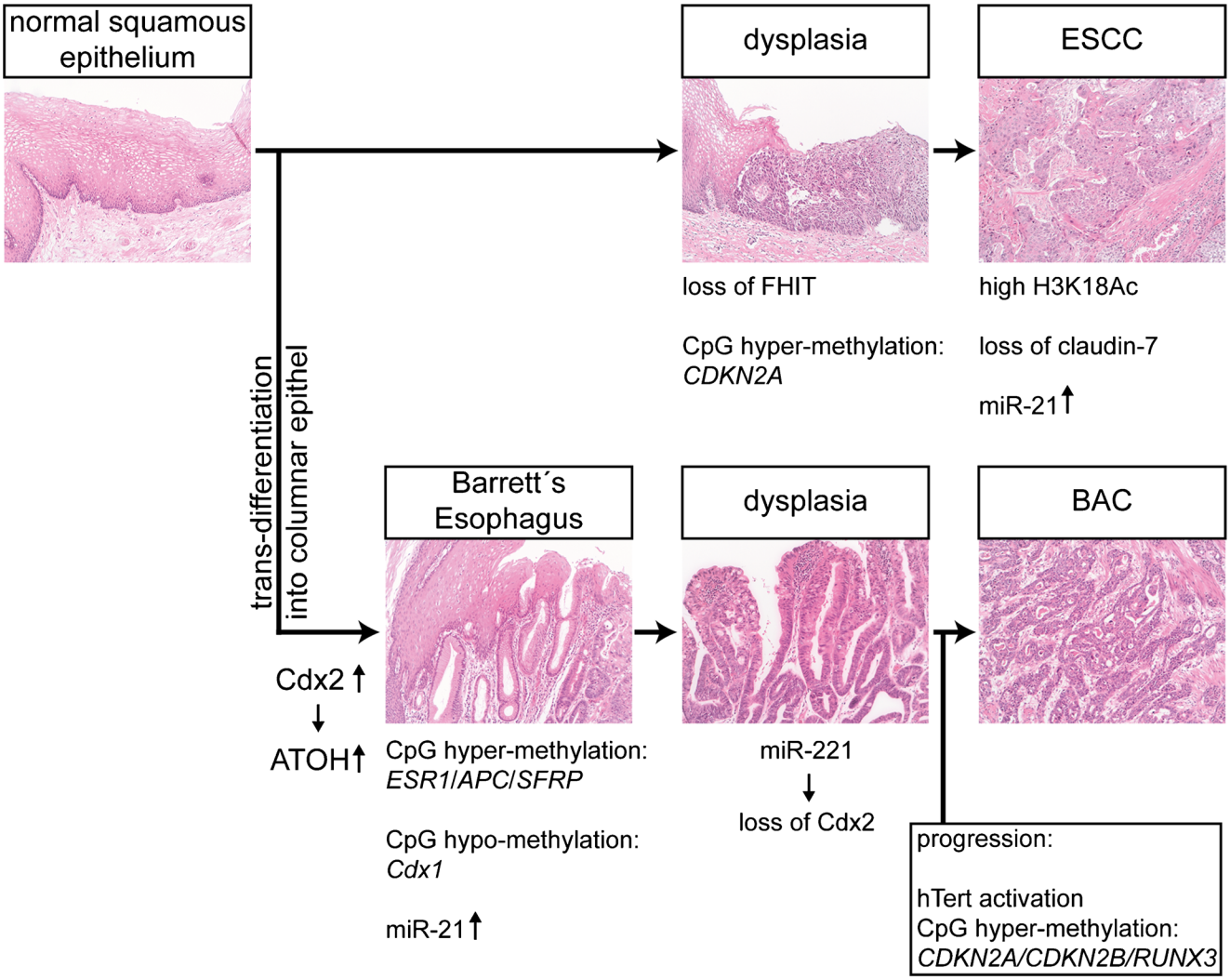


Fig. 1 Key epigenetic alterations involved at various stages of esophageal carcinogenesis. The major characteristic morphological features in the carcinogenesis of esophageal squamous cell carcinomas (*ESCC*) and Barrett's adenocarcinoma (*BAC*) are shown (hematoxylin and eosin staining). Selected key epigenetic events and respective genes/proteins regulated by epigenetic mechanisms in the distinct stages are indicated (see text, Tables 1, 2, 3, Supplementary Table S1, and references therein)

predominantly seen in ESCCs (e.g., *EGFR*; Lin et al. 2009), others are linked to the majority of BACs (e.g., *HER2*; Zhang et al. 2009). Importantly, such specific genetic alterations also provide the basis for targeted therapeutic intervention in esophageal cancers, with HER2-targeting inhibitors recently having been approved for first-line therapy of metastatic gastric and esophageal adenocarcinomas (Pennathur et al. 2013). In contrast to other epithelial tumors such as colorectal or lung adenocarcinomas, the treatment options and predictive markers are rather sparse in esophageal cancers. In addition, whether these key genetic alterations alone drive the distinct phenotypes of BAC or ESCC carcinogenesis and therefore also represent valid therapeutic targets for inhibiting esophageal cancer progression remains unclear.

In recent years, because of their role in normal tissue development and differentiation, epigenetic alterations have increasingly been appreciated to contribute to malignant transformation and tumor progression. For example, "environmental damage" imposed by tobacco or alcohol might contribute to the malignant transformation of normal squamous epithelium by the induction of genetic defects or possibly also by the induction of aberrant epigenetic modifications. Moreover, crosstalk appears to occur between (altered) oncogenic signaling pathways and epigenetic modulation (Mohammad and Baylin 2010). Therefore, the past few years have seen an increase in basic and applied research into the field of the epigenetics of esophageal cancer, with publications being registered, for example, via "*esophageal cancer, epigenetic, YEAR*" from 6 in 2003 to 30 in 2013 and, for example, via "*esophageal cancer*, *methylation*, *YEAR*" from 21 in 2003 to 53 in 2013 [http://www.ncbi.nlm.nih.gov/pubmed/]. True, these seemingly small numbers of

publications underline the lack of and necessity for further research into the epigenetics of esophageal cancers. However, (translational) research in esophageal cancer, especially with regard to translational clinical research combining in vitro and in situ analyses, is not as straightforward as in other epithelial tumors: validated cell line models are sparse, and tissue specimens are available from mainly the smallest of biopsies, particularly with respect to interest in precursor lesions and "primary tumors" without prior chemo- or radiotherapy. This rareness of "untouched" esophageal (cancer) tissues limits, in part, the investigational depth. In addition, whereas some methodologies such as microRNA (miRNA) expression and DNA methylation analyses are now readily performed on all kinds of samples, the analysis of histone modifications remains to be further standardized, especially when working with chromatin samples derived from routinely processed tissue specimens. Thus, epigenetic alterations in esophageal cancers are only beginning to be addressed at the level of basic and translational research, and they are still far from being implemented into routine clinico-pathological guidelines.

Irrespective of this, an interest is arising in the therapeutic targeting of epigenetic modifiers, including the inhibition of DNA methyltransferases (DNMTs; e.g., 5-Azacytidine, Decitabine) or histone deacetylases (HDACs; e.g., Vorinostat) in ongoing clinical trials involving patients with esophageal cancers (e.g., Azacytidine/NCT01386346, Depsipeptide/NCT00098644; http://www.cancer.gov/clinicaltrials). These largely build not only on promising studies of these inhibitors in other cancer entities, but also on the above-mentioned growing number of basic epigenetic research studies of esophageal cancers. Therefore, this review will reveal the current knowledge of epigenetic alterations in esophageal cancers from a translational view, by focusing on alterations of miRNAs, DNA methylation, and histone modifications investigated in patient tissue specimens of esophageal carcinomas and associated precursor lesions.

miRNAs in esophageal carcinogenesis

Interestingly, the topic of "microRNAs" (miRNAs or miRs) has been widely addressed in human tissue specimens of esophageal cancers, including candidate miR approaches or broad microarray-based screening approaches (see Table 1 for selected recent studies):

Individual studies, mainly in ESCCs, have pointed out (in part, contradictory) differences of individual miR expression levels in normal esophageal epithelial cells versus esophageal cancer cells (e.g., down-regulated in esophageal cancer: *miR-133*, *miR-145*, *miR-203*, *miR-205*, *miR-375*; up-regulated in esophageal cancer: *miR-21*, *miR-145*, *miR-223*). Low expression of selected miRs in esophageal cancer cells (mainly ESCC) has been found to be associated with more aggressive tumors and lymph node or distant metastasis (e.g., *let-7*/*let-7c*, *miR-375*) and/or with poor survival (e.g., *let-7*/*let-7c*, *miR-150*, *miR-375*). Similarly, other selected miRs showing high expression in esophageal cancer cells (mainly ESCC, some BAC) are associated with advanced tumor stages (e.g., *miR-21*, *miR-145*) and poor survival (e.g., *miR-103*/*107*, miR-200 family, *miR-223*). This is complemented by several studies that have measured miRs in the serum of ESCC patients (Supplementary Table S1). Indeed, the results of these independent analyses mirror the findings of human tissue specimen-based analyses only in a few aspects, e.g., the detection of high levels of *miR-21* or low levels of *miR-375* in the serum of ESCC patients. Since these studies have used different (mainly small) groups of patients with different stages of cancer and treatment histories and have involved different techniques and approaches for the measurement of miRs, some debate clearly remains concerning the functional role of the miRs in esophageal cancers. To illustrate this, the following will describe two selected miRs, namely *miR-21* and *miR-375*, in more detail, since several studies have been published for each of these two miRs.

miR-21 was one of the earlier discovered miRs, with a gene location on chromosome 17q23, i.e., a chromosomal region frequently altered by gene copy number gains, especially in BACs. The impact of altered *miR-21* expression is mainly seen on tumor suppressor genes, such as *PTEN*, as shown in other cancer entities.

Several studies have shown that *miR-21* is up-regulated in tumor cells of both ESCCs and BACs when compared with normal esophageal epithelium (Table 1; Feber et al. 2008; Akagi et al. 2011; Hamano et al. 2011; Garman et al. 2013; Wang et al. 2013). Feber et al. (2008) analyzed a small group of samples derived from fresh-frozen normal epithelium (n=9), ESCCs (n=10), and BACs (n=10) with associated precursor lesions (n=6) by microarray-based profiling and bioinformatics-clustering analyses. They detected an increase of up to five-fold of *miR-21* expression in tumor versus normal esophageal epithelial cells (Feber et al. 2008). In a second microarray-based study focusing on BAC cases (Garman et al. 2013), samples of normal epithelium (n=11), Barrett's esophagus (n=14), and esophageal adenocarcinoma (n=11) were first screened by microarray-based analysis. Selected candidate miRs (including *miR-21*) were then validated by quantitative reverse transcription plus the polymerase chain reaction (q-RT-PCR) in a second group of cases (n=18), but only for a comparison of normal squamous epithelium with BAC. Again, *miR-21* was one of the candidate miRs up-regulated in BACs. Three subsequent studies focused on ESCCs. Akagi et al. (2011) used q-RT-PCR to measure five selected miRs and found that *miR-21* was elevated in ESCCs, especially in those ESCCs with lymph node metastasis. In

Table 1 Overview of published studies addressing miRNAs in human tissue specimens of esophageal cancers. Selected studies are discussed in the text (*NE* normal epithelium, *ESCC* esophageal squamous cell carcinoma, *BAC* Barrett's adenocarcinoma and precursor lesions of Barrett's epithelium, *BE* Barrett's epithelium, *IEN* intraepithelial neoplasia

miRNA	Tumor	Findings	Reference
let-7/let-7c	ESCC	Reduced expression in ESCC tissues and correlation between low let-7 expression and lymph node metastasis	Liu et al. 2012
	ESCC	Low levels correlated with poor response to chemotherapy and poor prognosis	Sugimura et al. 2012
miR-10a	NE/IEN/ESCC	Downregulated in precursor lesions, but elevated in invasive carcinomas	Inoue et al. 2010
miR-21	NE/BE/BAC/ESCC	Higher expression compared with normal esophageal mucosa	Feber et al. 2008
	Stroma of ESCC	Increased levels in the surrounding stroma were associated with worse prognosis. Implication for possible association of miR-21 in the stromal environment.	Mathe et al. 2009
	ESCC/cell lines	Increased in ESCC patients/in vitro, miR-21 was shown to target PDCD4, regulating thereby proliferation and invasion	Hiyoshi et al. 2009
	NE/ESCC	Higher in ESCC tissues and associates with lymph node positivity	Akagi et al. 2011
	NE/ESCC	Higher expression in ESCC tissue than normal esophageal mucosa correlated with shortened survival	Hamano et al. 2011
	NE/BE/BAC	Higher expression compared in BE/BAC with normal esophageal mucosa	Garman et al. 2013
	NE/ESCC	Higher expression compared with normal esophageal mucosa	Wang et al. 2013
miR-23a	ESCC	Low expression in ESCC tissue correlated with high overall survival rate	Ogawa et al. 2009
miR-26a	ESCC	Low expression in ESCC tissue correlated with high overall survival rate	Ogawa et al. 2009
	BAC/cell lines	Downregulation in BAC tissues and metastasis/acquisition of anoikis-resistance in vitro	Zhang et al. 2013
miR-27b	ESCC	Low expression in ESCC tissue correlated with high overall survival rate	Ogawa et al. 2009
	NE/BE/BAC	Lower expression in BE/BAC compared with normal esophageal mucosa	Garman et al. 2013
miR-92a	ESCC/cell lines	Up-regulation significantly correlated with lymph node metastasis and TNM stage. In vitro suppression of E-cadherin by miR-92a was shown	Chen et al. 2011
miR-103/107	NE/ESCC	High expression correlated with poor survival	Guo et al. 2008
miR-129	ESCC	Low expression in ESCC tissue correlated with high overall survival rate. Overexpression of miR-129 was a significant and independent prognostic factor	Ogawa et al. 2009
miR-133a	ESCC/cell lines	Reduced expression compared with normal esophageal mucosa/in vitro direct targeting of FSCN1 was shown regulating cell growth and migration	Kano et al. 2010
	ESCC/cell lines	Reduced levels of miR-133a cancer tissues compared with adjacent non-cancerous tissues/miR-133a significantly inhibited tumorigenesis and growth in vivo via regulation of CD47	Suzuki et al. 2012
miR-145	ESCC	Reduced expression compared with normal esophageal mucosa	Kano et al. 2010
	NE/ESCC	Higher in ESCC tissues and associates with recurrence of metastasis	Akagi et al. 2011
	NE/ESCC	Higher expression in ESCC tissue than normal esophageal mucosa correlated with shortened survival	Hamano et al. 2011
miR-150	ESCC	Low expression of miR-150 in ESCC contributed to malignant potential: tumor depth, lymph node metastasis, lymphatic invasion, venous invasion, clinical staging, and poor prognosis/in vitro targeting of ZEB1 with subsequent mesenchymal to epithelial transition was shown	Yokobori et al. 2013
miR-192	NE/BE	Overexpressed in BE lesions	Fassan et al. 2013
	BE	Significantly higher in BE cases with progression to BAC	Revilla-Nuin et al. 2013
miR-194	BE	Significantly higher in BE cases with progression to BAC	Revilla-Nuin et al. 2013
miR-196a	BE	Significantly higher in BE cases with progression to BAC	Revilla-Nuin et al. 2013
miR-200 family	Gastric/BE/BAC	miR-200 members were downregulated in BAC	Smith et al. 2011
	NE/ESCC	Higher miR-200c levels correlated with shortened survival and inversely correlated with response to chemotherapy	Hamano et al. 2011
miR-203	NE/BE/BAC/ESCC	Lower expression compared with normal esophageal mucosa	Feber et al. 2008
	ESCC (stage I–IV)	Reduced expression compared with normal esophageal mucosa	Kano et al. 2010
	ESCC and cell lines	Decreased miR-203 compared with normal esophageal mucosa inversely correlated with LASP1 mRNA levels and correlated with relapse-free survival	Takeshita et al. 2012
	BE	Downregulated in BE cases	Fassan et al. 2013
miR-205	NE/BE/BAC/ESCC	Lower expression compared with normal esophageal mucosa	Feber et al. 2008

Table 1 (continued)

miRNA	Tumor	Findings	Reference
	ESCC and cell lines	Validated MiR-205 in cell line models, but differentially expression in human ESCC tissues failed	Matsushima et al. 2011
	BE	Downregulated in BE	Fassan et al. 2013
miR-221/222	BE/BAC/cell lines	Increased levels during progression from BE to BAC, promoting degradation of CDX2 in vitro	Matsuzaki et al. 2013
miR-223	ESCC	Significantly higher in ESCC tissues than in the corresponding normal tissues and patients with high miR-223 expression demonstrated a significantly poorer prognosis	Kurashige et al. 2012
	Cardiac tissue/BE/BAC	Stepwise increase during BAC carcinogenesis	Streppel et al. 2013
miR-375	BAC	Low levels correlated with worse prognosis	Mathe et al. 2009
	ESCC	Reduced expression compared with normal esophageal mucosa	Mathe et al. 2009
	ESCC	miR-375 is downregulated by hyper-methylation of the promoter	Li et al. 2011
	ESCC	Downregulation was frequently detected in ESCC tissues and significantly correlated with advanced stage/distant metastasis/poor overall survival and disease-free survival/in vitro targeting of the IGFR1 was shown and in situ IGFR1 levels negatively correlated with miR-375 levels	Kong et al. 2012
	NE/BE/BAC	Marked downregulation exclusively in BAC tissues	Leidner et al. 2012
	NE/BE/BAC	Exclusively downregulated in BAC	Wu et al. 2013
	NE/ESCC	Downregulation in tumor tissue was associated with advanced stage, metastasis and poor outcome	Li et al. 2013

addition, Hamano et al. (2011) examined selected miRs by q-RT-PCR, but this time in ESCCs with previous neoadjuvant chemotherapy (n=98). In this group of patients, *miR-21* expression was also higher in tumor than in normal esophageal epithelial cells. Moreover, *miR-21* expression was marginally associated with poor survival. Similarly, Wang et al. (2013) detected overexpression of *miR-21*, again by q-RT-PCR, in 16 cases.

Thus, the above studies indicate that the up-regulation of *miR-21* occurs in both histotypes of esophageal cancers. This fits well with the known effect of *miR-21* on tumor suppressors. However, interestingly, one large study has also detected *miR-21* in ESCC "associated stroma" (Mathe et al. 2009). In their study, Mathe and colleagues investigated 100 BACs and 70 ESCCs, derived from various countries, by microarray-based expression profiling (smaller discovery case set of a total of n=76) and validation in a separate case set (n=94) by q-RT-PCR. The data support the finding that miR-21 is generally up-regulated in ESCCs and BACs compared with normal epithelium. However, in ESCCs, the data raised the issue that *miR-21* expression in the normal esophageal epithelium was associated with poor patient prognosis; the authors here comment on a potential contribution of the "stroma" to *miR-21* function. Indeed, *miR-21* is also associated with the immune system (Tili et al. 2013). Since both ESCCs and BACs are composed not only of tumor cells, but also of, for example, various proportions of infiltrating lymphocytes, the specificity of the *miR-21* measurements in all of the above studies needs to be reconsidered in terms of the degree of precision with which the tumor cells were selected for miR isolation and down-stream analyses. Without prior microdissection to enrich for pure cell populations of normal esophageal epithelial and invasive ESCC and BAC cells, some vagueness remains as to whether the measured *miR-21* levels are tumor-associated or additionally driven by the level of the inflammatory component of the lesions.

As seen for *miR-21*, several studies have addressed *miR-375* in ESCCs and BACs (Table 1). The gene of *miR-375* is located on chromosome 2 and has been associated with the regulation of oncogenes such as *MYC* and *TP53*. In agreement with this, *miR-375* expression has been found to be down-regulated in ESCCs and BACs, possibly thereby reflecting/supporting the activity of the tumor cells (Mathe et al. 2009; Li et al. 2011; Kong et al. 2012; Leidner et al. 2012; Wu et al. 2013). Kong et al. (2012) not only detected *miR-375* down-regulation in ESCCs and its correlation to advanced stage ESCCs and to poor survival, but also showed that *miR-375* could interact with the 3'-untranslated region of the *insulin-like growth factor receptor 1* (*IGFR*) mRNA. This interaction led to the down-regulation of *IGFR* in in vitro model systems. Moreover, *miR-375* and *IGFR* expression in ESCCs was inversely correlated. In the same study, the expression of *miR-375* was shown to be regulated by promoter methylation (Kong et al. 2012), thereby also supporting a higher level of epigenetic complexity with the concept that DNA methylation and miRNA regulation are able to cross-talk (Lujambio et al. 2008; Suzuki et al. 2013).

DNA methylation in esophageal carcinogenesis and esophageal cancer therapy

As seen for other epithelial tumors, DNA methylation has been quite widely characterized by analysis of specific CpG-island promoter regions of selected candidate genes in DNA obtained from human tissue specimens of esophageal carcinomas and/or associated precursor lesions. The reader is referred to two recent excellent reviews of DNA methylation in ESSCs (Baba et al. 2013; Chen et al. 2013). An overview of studies for ESCCs, BACs, and associated pre-cursor lesions is provided in Table 2.

In ESCCs, hyper-methylation of genes associated with DNA replication (e.g., *MGMT*, *FHIT*), DNA repair genes (e.g., *MLH1*, *MSH2*), and genes involved in cell cycle progression, oncogenic signaling, cell differentiation, or motility (e.g., *p16*/*CDKN2A*, *IGF2*, *E-Cadherin*/*CDH1*, *claudins*) have all been associated with a poor prognosis (see references listed in Table 2). Of interest, the intestinal/columnar differentiation gene *caudal-related homeobox gene* (*CDX2*) was found to be silenced by DNA methylation in ESCCs (Guo et al. 2007), thus underlining the importance of CDX2 in the various mechanisms of carcinogenesis and diverse histology of ESCCs and BACs (see below).

Only a few studies have addressed or detected DNA hypomethylation in ESCCs (Table 2), including a recent study regarding the global hypo-methylation of *GADD45a*, which is associated with poor tumor differentiation and prognosis (Wang et al. 2012). Moreover, the loss of imprinting of *IGF2* has been recently described in ESCC, and this loss of methylation is associated with a shorter length of survival (Murata et al. 2013). In addition, LINE-1 element hypo-methylation is associated with poor prognosis (Iwagami et al. 2013) and is apparently detectable even in "normal" esophageal epithelium of patients with a strong smoking history (Shigaki et al. 2012).

In BACs, CpG promoter DNA methylation patterns of selected genes have been investigated, including the detection of DNA hyper-methylation in genes involved in cell cycle progression (*p16*/*CDKN2A*), in apoptosis (e.g., *SFRP1*), in the WNT/β-catenin pathway (e.g., *APC*), and in protease inhibitors (e.g., TIMPs; see references listed in Table 2). The use of candidate gene approaches has demonstrated DNA hypomethylation in genes involved in intestinal/columnar differentiation (e.g., *CDX1*; Wong et al. 2005).

However, the most recent and broad data on DNA hypomethylation in ESCCs and BACs (including precursor lesions) have evolved from two recent studies on microarray-based or genome-wide DNA methylation patterns (Alvarez et al. 2011; Lima et al. 2011). Lima and colleagues (2011) selected 10 cases of ESCCs and analyzed DNA methylation patterns of samples derived from fresh-frozen tissue specimens of invasive tumor cells, "tissue surrounding the tumor" (not further specified), and normal squamous epithelium. Samples were analyzed first by the Illumina GoldenGate methylation assay, focusing on 807 cancer-related genes, and candidates were then further confirmed by pyrosequencing in a larger cohort of about 100 ESCCs and 27 non-case-matched normal esophageal epithelial controls. The data revealed an increase of general DNA methylation from samples of normal epithelium via "surrounding tissue" to tumor, including methylation of genes observed previously in ESSCs (e.g., *p16*/*CDKN2A*, *MGMT*). Hyper-methylated genes that were specifically emphasized in the study in terms of tumor evolvement were *BCL3*, belonging to the inhibitory κB (IκB)-family and regulating NFκB activity, and *TFF1*, a trefoil factor family member (Lima et al. 2011). The functional consequences of altered *BCL3* or *TTF1* DNA methylation and protein expression remain to be elucidated. Moreover, details of the cell populations (mixtures) present in the samples denoted as "surrounding tumor" were lacking (Lima et al. 2011). In the second genome-wide DNA methylation study in BACs, Alvarez and colleagues (2011) used a small group of histologically proven fresh-frozen tissue samples of BACs and associated precursor lesions for parallel analysis of (1) genome-wide cytosine methylation, (2) RNA profiling, and (3) array-based comparative genomic hybridization (aCGH). DNA methylation analysis was based on the "HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) assay", and candidate loci were validated by mass spectrometry-based high-throughput quantitative methylation PCR analyses (Sequenome). Of note, this study revealed a predominance of DNA hypo-methylation rather than DNA hyper-methylation at early stages of BAC carcinogenesis (Alvarez et al. 2011). Moreover, the authors of this study detected DNA hypomethylation in a series of genes associated with the immune system, such as chemokines (e.g., CXCL1, CXCL3). Another novel finding of Alvarez et al. (2011) was that, in BAC carcinogenesis, DNA methylation also occurred in genomic regions outside of CpG-islands. Furthermore, by combining DNA methylation, RNA profiling, aCGH analyses, and functional in vitro experiments, the study by Alvarez et al. (2011) nicely supports the fact that single candidate loci and/or DNA methylation analysis alone is insufficient to unravel the complex interaction between epigenetic, genetic, and functional consequences.

In this context, the cross-talk of DNA methylation and miR expression mentioned above (Lujambio et al. 2008; Suzuki et al. 2013) is further underlined by a recent study in ESSCs, showing by bisulfite-sequencing PCR (BSP) and methylation-specific PCR (MSP) that the low expression of several miRs (*miR-34a*, *miR-34b*/*c*, *miR-129-2*) is attributable to DNA methylation (Lujambio et al. 2008). Moreover, this can be reversed by treatment with the DNA methyltransferase (DNMT) inhibitor 5-aza-2′-deoxycytidine/decitabine (DAC).

Together, the above studies rise the question as to how DNA methylation itself is regulated by DNMTs, especially

Table 2 Overview of published studies addressing DNA methylation in human tissue specimens of esophageal cancers. Selected studies are discussed in the text (*NE* normal epithelium, *ESCC* esophageal squamous cell carcinoma, *BAC* Barrett's adenocarcinoma and precursor lesions of Barrett's epithelium, *BE* Barrett's epithelium, *IEN* intraepithelial neoplasia

Gene	Tumor	Findings	Reference
Hyper-methylated genes			
APC	Barrett/BAC	Possible serum biomarker for BAC	Kawakami et al. 2000
	Barrett/BAC	Methylation occurs in majority of all cells suggesting a clonal expansion	Eads et al. 2000
	Barrett/BAC	Higher methylation in tumors	Sarbia et al. 2004
	NE/BE/BAC	More frequently methylation in patients with progression from BE to BAC	Clement et al. 2006
BCL3	ESCC	May represent an early event in ESCC carcinogenesis	Lima et al. 2011
CDH1	BAC (stage I–IV)	Commonly inactivated by methylation during carcinogenesis	Corn et al. 2001
	ESCC	Expression was induced by 5-aza-20-deoxycytidine in cell lines	Si et al. 2001
	ESCC	Correlation with SNAIL overexpression	Takeno et al. 2004
	ESCC	For stage I cancers, CDH1 methylation was associated with a high risk of recurrence and a poor recurrence-free survival after surgery	Lee et al. 2008
CDKN2A/p16	BAC	Usually found in a large contiguous field	Eads et al. 2000
	NE/BE/BAC	*CDKN2A* methylation is an early event and the predominant mechanism for p16 downregulation	Bian et al. 2002
	Barrett/BAC	Complete loss of p16 in 45 % of all tumors/associated with CDKN2A promoter methylation	Sarbia et al. 2004
	NE/BE/BAC	*CDKN2A* hypermethylation was independently associated with an increased risk of progression from BE to BAC	Schulmann et al. 2005
	IEN (ESCC)	Increased methylation during ESCC carcinogenesis and tended to be higher in patients with p53 mutation	Ishii et al. 2007
	ESCC	Correlation between loss of p16 expression (by methylation) and poorer prognosis	Fujiwara et al. 2008
	ESCC	Hyper-methylated in ESCC	Lima et al. 2011
CDKN2B	Barrett/progression	Hyper-methylated during BAC carcinogenesis	Alvarez et al. 2011
CDX2	ESCC	Inactivated in ESCCs by methylation/marker to discriminate between ESCC (negative) and BAC (positive)	Guo et al. 2007
Claudin-4	ESCC	Low claudin-4 levels were associated with histological differentiation, invasion depth, and lymph node metastasis and was an independent predictor of poor overall survival	Sung et al. 2011
DAPK	Barrett	Early event during BAC carcinogenesis	Schildhaus et al. 2005
	Barrett/BAC	Loss of DAPK associated with invasion depth and advanced stages	Kuester et al. 2007
	IEN (ESCC)	Tended to be higher in patients with p53 mutation	Ishii et al. 2007
DCC	IEN (ESCC)	Tended to be higher in patients with p53 mutation	Ishii et al. 2007
ESR1	Barrett/BAC	With high frequency in reflux esophagitis and all subsequent stages	Eads et al. 2000
FHIT	ESCC (stage I–IV)	Associated with a poor prognosis in cases of stage 1–2 cancer, independent of recurrence	Lee et al. 2006
	Dysplasia/ESCC	Patients with high tobacco and/or alcohol consumption showed higher frequency of loss	Mori et al. 2000
	ESCC (stage I/II)	May play an important role in the early stage of ESCC carcinogenesis	Kuroki et al. 2003
	ESCC (stage I–IV)	Positive correlation with stage, T status, and N status/81.7 % concordance rate with reduced expression of acetylated histone H4	Tzao et al. 2006
	Barrett	Hyper-methylation was also found in adjacent normal tissue, constitute an early epigenetic precursor	Schildhaus et al. 2005
HIN-1	ESCC	*HIN-1* silencing by dense promoter methylation/may be an early event during dysplastic transformation	Guo et al. 2008
hMLH1	ESCC (stage I–IV)	83.3 % concordance between hMLH1 expression and promoter methylation	Tzao et al. 2005
HPP1	Barrett	Associated with progression to BAC	Schulmann et al. 2005
MSH2	ESCC (+serum)	High MSH2 methylation was associated with lower disease-free survival and methylated MSH2 DNA was detectable in serum samples	Ling et al. 2012
integrin α4	ESCC	Association with an increased risk of recurrence and a poor recurrence-free survival for stage II cancers	Lee et al. 2008
MGMT	NE/BAC	Suggested as biomarker for therapy with alkylating agents	Schildhaus et al. 2005
	Barrett	Promoter methylation correlates with protein expression, but not with patient outcome	Baumann et al. 2006

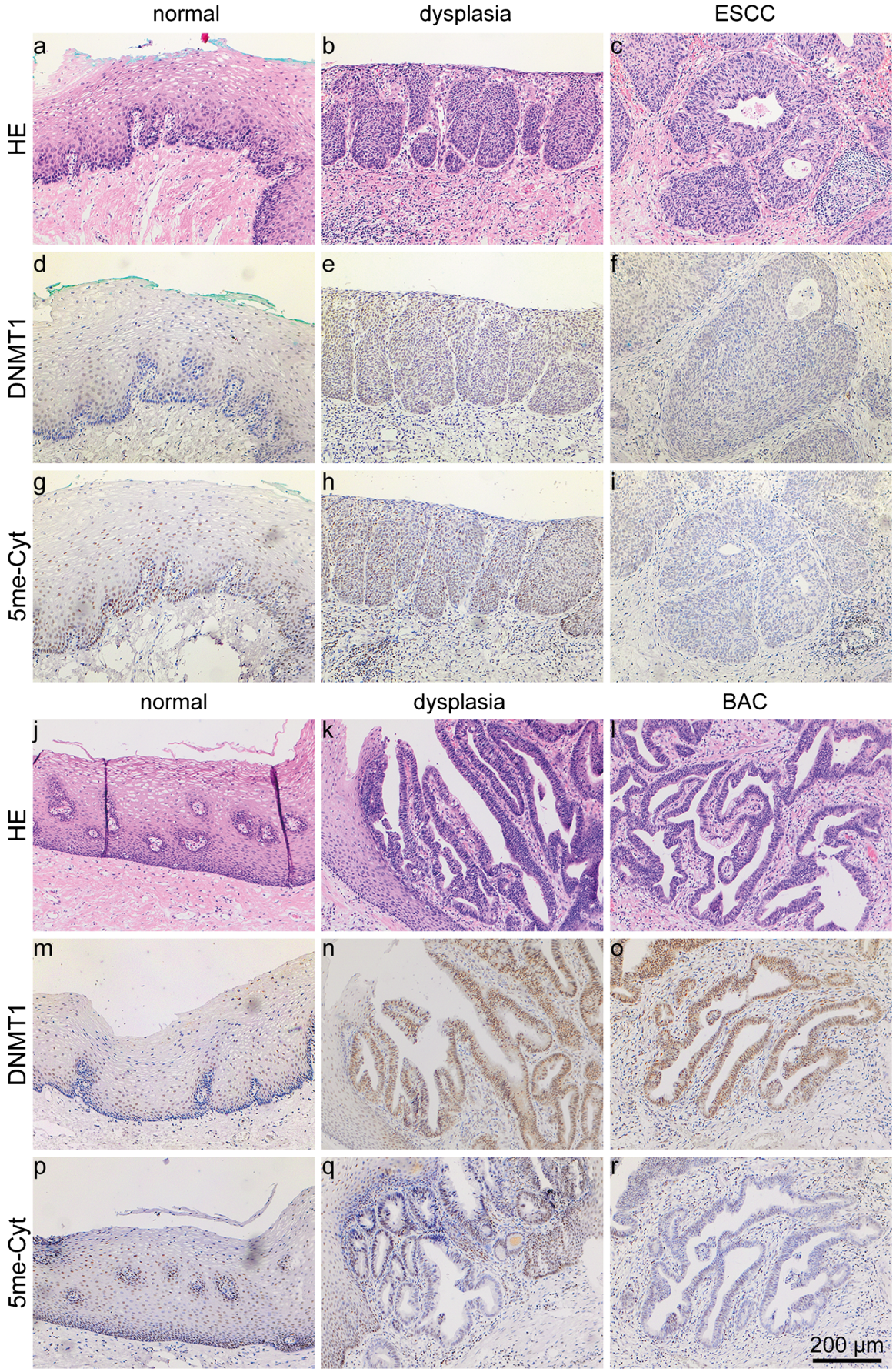
Table 2 (continued)

Gene	Tumor	Findings	Reference
	NE/ESCC	38.7 % of patients showed hyper-methylation, no association with p53 mutations	Zhang et al. 2003
	NE/IEN/ESCC	Gradually increase during carcinogenesis	Fang et al. 2005
	NE/ESCC	Association with lymph node metastasis and correlation with MTHFR polymorphism	Xue et al. 2008
	IEN (ESCC)	Higher methylation during carcinogenesis	Ishii et al. 2007
	ESCC	Hyper-methylated in ESCC	Lima et al. 2011
MINT1	IEN (ESCC)	Higher methylation during carcinogenesis	Ishii et al. 2007
MINT31	IEN (ESCC)	Higher methylation during carcinogenesis	Ishii et al. 2007
MYOD1	Barrett	Significant methylation difference compared with NE	Eads et al. 2001
p14ARF	IEN (ESCC)	Higher methylation during carcinogenesis	Ishii et al. 2007
RAR-β	ESCC (stage I and II)	Might play an important role in the early stage of ESCC carcinogenesis	Kuroki et al. 2003
RUNX3	Barrett	Associated with progression to BAC	Schulmann et al. 2005
	ESCC (stage I-IV)	Correlation with clinical pathological stages was observed	Long et al. 2007
SFRP1	IEN (ESCC)	Higher methylation during carcinogenesis	Ishii et al. 2007
	NE/BE/BAC	Methylated in 91 % of BACs, compared with 17 % of NEs	Clement et al. 2006
	Barrett/BAC	Methylated in 93 % of BACs and expression was restored by 5-aza-2′deoxycytidine in vitro	Zou et al. 2005
TERT	NE/BE/BAC	More frequently methylation in patients with progression from BE to BAC	Clement et al. 2006
TFF1	ESCC	Potential marker for early ESCC carcinogenesis	Lima et al. 2011
TIMP3	Barrett	Significant difference in methylation frequency between NE and BE tissues	Eads et al. 2001
	NE/BE/BAC	Associated with progression to BAC	Clement et al. 2006
Hypo-methylated genes			
CDKN1C	Cell lines	Indirectly regulated by loss of methylation in DMR-LIT1	Soejima et al. 2004
CDX1	Barrett's metaplasia	Silenced in NE by methylation	Wong et al. 2005
CXCL1	Barrett/BAC	Additionally amplified	Alvarez et al. 2011
CXCL3	Barrett/BAC	Additionally amplified	Alvarez et al. 2011
DMBT1	Barrett/BAC	Upregulated during BAC carcinogenesis	Alvarez et al. 2011
GADD45α	ESCC (stage I-III)	Promotes global DNA de-methylation/expression was associated with poor differentiation and lymph node metastasis	Wang et al. 2012
GATA6	Barrett/BAC	Upregulated during BAC carcinogenesis	Alvarez et al. 2011
IGF2	ESCC	*IGF2* DMR0 hypo-methylation was associated with shorter survival time and hence might be a prognostic biomarker	Murata et al. 2013
IL6	ESCC	Hypo-methylated in tumors compared with surrounding tissue	Lima et al. 2011

since (clinical) inhibitors to DNMTs are available (Christman 2002). Few studies have addressed DNMT expression or alterations in esophageal cancers (Kassis et al. 2006; Fan et al. 2010). In our own studies (unpublished data), we find frequent loss or down-regulation of DNMT1 in invasive tumor cells of ESSC (Fig. 2), which is accompanied by the reduction of 5me-Cytosine labeling. This effect is also seen in BACs, but here it is not as prominent (Fig. 2). Moreover, as seen by DNMT1 knockdown experiments in the above-mentioned study by Kassis et al. (2006), our recent data show that the inhibition of DNMTs by azacytidine in esophageal cancer cells induces the loss of cell viability, particularly in ESCCs (unpublished data).

In conclusion, although numerous individual genes regulated by DNA methylation have been identified, comprehensive in situ and functional analysis of DNA methylation and associated biological effects in ESSCs and BACs are urgently needed for a better understanding of their carcinogenesis from a molecular pathological view and for potential future work into the action of drugs targeting DNA methylation.

Fig. 2 DNA methylation in human tissue specimens of esophageal squamous cell carcinoma (*ESCC*) and Barrett's adenocarcinoma (*BAC*). Serial analysis of DNA methyltransferase (*DNMT1*) expression and levels of 5-methyl-Cytosine (*5me-Cyt*) by immunohistochemistry of case-matched tissue specimens of ESCCs (**a–i**) and BACs (**j–r**), including normal esophageal epithelium (*normal*) and precursor lesions (*dysplasia*). Hematoxylin and eosin (*HE*) staining is shown in **a–c** for ESCC and in **j–l** for BAC. Note the loss of DNMT1 (**f**) and detectable 5me-Cytosine levels (**i**) in ESCC. Note also DNMT1 expression (**o**) and low 5me-Cytosine levels (**r**) in BAC. *Bar* 200 μm


normal
dysplasia
ESCC
HE
DNMT1
5me-Cyt
a
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normal
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200 μm

Histone modifications in esophageal carcinogenesis and esophageal cancer therapy

The investigation of histone modifications in human tissue specimens of esophageal cancers has been restricted to a series of studies exclusively on ESSCs and has involved the analysis of specific histone marks by immunohistochemical staining (Table 3). Two more comprehensive studies combining several histone marks in ESCCs have been performed. Tzao et al. (2009) have examined histone 3 lysine 18 (H3K18ac), acetylated histone 4 lysine 12 (H4K12ac), dimethylated histone 4 arginine 3 (H4R3me2), dimethylated histone 3 lysine 4 (H3K4me2), and trimethylated histone 3 lysine 27 (H3K27me3). In their study, correlations of histone marks have been detected with tumor differentiation (H3K18ac, H4K3me2, H3K27me3), with tumor stage and lymph node status (H3K27me3), and with improved prognosis (low H3K18ac, low H3K27me3). In the study by I and colleagues (2010), the levels of H3K18ac and H4R3me2 have been found to be of prognostic value. The genes and associated cellular functions regulated by the above-detected "aberrant" histone marks remain unknown, as chromatin immunoprecipitation (ChIP) from formalin-fixed and paraffin-embedded tissue specimens derived from diagnostic procedures is not yet routinely established (Fanelli et al. 2010).

In view of their potential for improved esophageal cancer treatment, HDACs are starting to play a more prominent role, since several inhibitors (with different specificities) are available (Bolden et al. 2013). Interestingly, a pubmed-based literature research retrieved only two studies that examined HDAC expression in esophageal cancers. In ESCCs, Toh and colleagues (2003) described the down-regulation of HDAC1 in ESCC as compared with normal squamous epithelium. In a far more comprehensive study on BACs, Langer and colleagues (2010) investigated about 180 BACs, with 2/3 being primary resected tumors and 1/3 having received prior chemotherapy treatment. In general, reduced HDAC1 and HDAC2 expression was observed in up to 50 % and 30 % of BACs, respectively. Only HDAC2 was associated with pathological parameters (tumor differentiation, lymph node stage), and neither HDAC1 nor HDAC2 showed a prognostic impact (Langer et al. 2010). In our recent study of ESCCs and BACs, including precursor lesions (unpublished data), we have detected generally prominent and strong HDAC1 and HDAC2 expression in basal cells/glands of normal esophageal epithelium, dysplastic lesions, and invasive tumor cells (Fig. 3). Tumor-cell-specific reduction of HDAC1 and HDAC2 is only seen in 10–20 % of ESCC or BAC cases. Interestingly, serial analysis of pan H3 acetylation reveals a similarly strong level of this mark in dysplasia and BAC (Fig. 3). In contrast, ESCC cells show a decrease of pan H3 acetylation, suggesting that HDAC activity, but not mere expression, is differentially altered (especially in ESCCs). Inhibition of HDACs might hence be a potential future tool for the therapeutic targeting of esophageal cancer. Indeed, preliminary evidence has been presented showing that the inhibition of HDAC1 and HDAC2 by the specific HDAC inhibitor depsipeptide/FK228 has an antitumor effect on ESCC cells in vitro and in xenograft models in vivo (Hoshino et al. 2005).

Thus, very little is still known about the role of histone modifications in esophageal cancer, clearly supporting further investigations.

Table 3 Overview of published studies addressing histone marks in human tissue specimens of esophageal cancers. Selected studies are discussed in the text (*NE* normal epithelium, *ESCC* esophageal squamous cell carcinoma, *BAC* Barrett's adenocarcinoma and precursor lesions of Barrett's epithelium, *BE* Barrett's epithelium, *IEN* intraepithelial neoplasia)

Histone mark	Tumor	Findings	Reference
H3K18ac	ESCC (stage I–IV)	Better patient survival with low expression in early stages (T1-T2)/positive correlation with tumor differentiation	Tzao et al. 2009
	ESCC (stage I–IV)	High global levels were associated with poor recurrence free survival (only in stage III cases)	I et al. 2010
H3K27me3	ESCC (stage I–IV)	Better patient survival with low expression in early stages (T1-T2)/positive correlation with nodal status, stage and tumor differentiation/considered as survival predictor	Tzao et al. 2009
H3K9me	ESCC (in vitro)	Low CDKN1C expression with loss of H3K9 methylation	Soejima et al. 2004
H4R3me2	ESCC (stage I–IV)	Positive correlation with tumor differentiation	Tzao et al. 2009
H4ac	ESCC	Histone H4 was hyper-acetylated in early stages, but changed to a hypo-acetylated state during progression	Toh et al. 2003
	ESCC	High H4 acetylation levels correlated with better prognosis/H4Ac inversely correlated to stage and invasion depth	Toh et al. 2004
	ESCC (stage I–IV)	Reduced expression was associated with tumor stage, N status and M status. High concordance with promoter methylation of the FHIT gene	Tzao et al. 2006
Combination of H3K18ac/H4R3me2	ESCC (stage I–IV)	High global levels were associated with poor recurrence free survival (only in stage IIB and III cases)	I et al. 2010

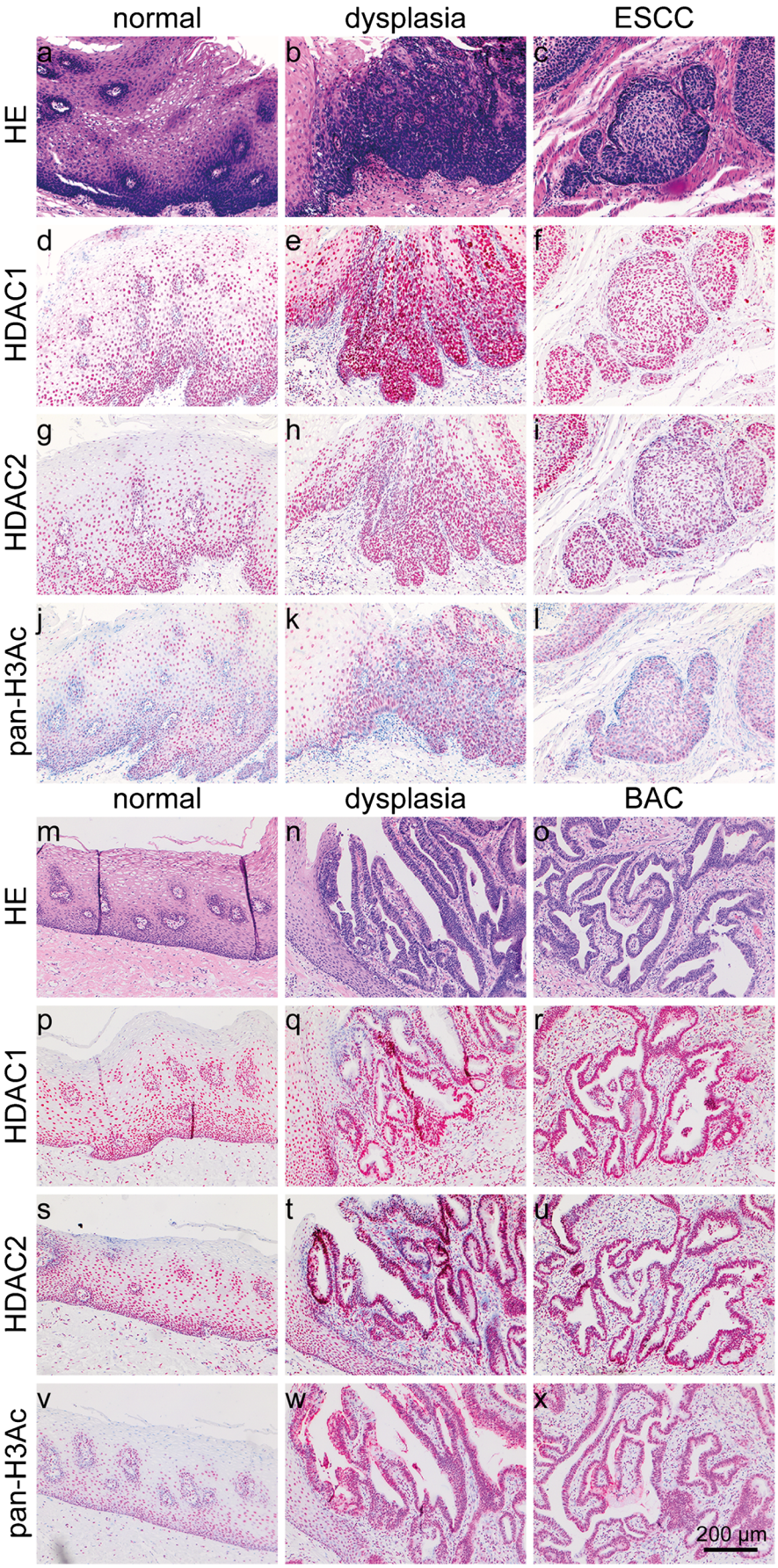

Fig. 3 Histone alterations in human tissue specimens of esophageal squamous cell carcinoma (*ESCC*) and Barrett's adenocarcinoma (*BAC*). Serial analysis of histone deacetylase 1 (*HDAC1*), histone deacetylase 2 (*HDAC2*) and pan histone H3 acetylation (*pan-H3Ac*) by immunohistochemistry of case-matched tissue specimens of ESCCs (**a–l**) and BACs (**m–x**), including normal esophageal epithelium and precursor lesions. Hematoxylin and eosin (*HE*) staining is shown in **a–c** for ESCC and **m–o** for BAC. Note the general strong expression of HDAC1, HDAC2, and strong levels of pan-H3Ac in BACs and weaker in ESCCs. *Bar* 200 µm

Concluding remarks

Esophageal cancers still represent a major challenge of interdisciplinary oncology. Numerous translational approaches have yielded better insight into the genetic aberrations driving esophageal cancer carcinogenesis and progression, and the identification of novel (genetic) targets for therapeutic intervention is advancing. Recently, these aspects have been paralleled by a growing interest in epigenetics of esophageal cancers, with several exciting studies reporting the alterations of miRNAs, DNA methylation, and histone modifications in patient tissue specimens of esophageal carcinomas and associated precursor lesions. Increases in our knowledge of epigenetic regulation of esophageal cancers will clearly contribute potential further biomarkers and treatment options in future. The gained insight into the epigenetics and genetics of esophageal cancer might open a wide window for improved patient care.

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