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Cancer Epidemiology, Biomarkers & Prevention

Review

Early Diagnostic Biomarkers for Esophageal Adenocarcinoma—The Current State of Play

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Abstract

Esophageal adenocarcinoma (EAC) is one of the two most common types of esophageal cancer with alarming increase in incidence and very poor prognosis. Aiming to detect EAC early, currently high-risk patients are monitored using an endoscopic-biopsy approach. However, this approach is prone to sampling error and interobserver variability. Diagnostic tissue biomarkers related to genomic and cell-cycle abnormalities have shown promising results, although with current technology these tests are difficult to implement in the screening of high-risk patients for early neoplastic changes. Differential miRNA profiles and aberrant protein glycosylation in tissue samples have been reported to improve performance of existing tissue-based diagnostic biomarkers. In contrast to tissue biomarkers, circulating biomarkers are more amenable to population-screening strategies, due to the ease and low cost of testing. Studies have already shown altered circulating glycans and DNA methylation in BE/EAC, whereas disease-associated changes in circulating miRNA remain to be determined. Future research should focus on identification and validation of these circulating biomarkers in large-scale trials to develop *in vitro* diagnostic tools to screen population at risk for EAC development. *Cancer Epidemiol Biomarkers Prev; 22(7); 1185–209. ©2013 AACR.*

Introduction

After heart disease, cancer is the second leading cause of death globally. Four major cancer sites account for half of the cancer-related mortalities: lung, colorectal, prostate in men, and breast in women. In past 2 decades, a steady decrease in deaths of these 4 major site malignancies led to an overall decrease in cancer-related death rates in men and women (1). In contrast, the incidence of esophageal adenocarcinoma (EAC) is increasing faster than any other cancer type. EAC together with esophageal squamous cell carcinoma (ESCC) is the eighth most-common cancer by prevalence and sixth most-common cause of cancer-related death globally (2). In 1970s, the incidence of EAC represented less than 5% of total esophageal cancer, and a majority of esophageal cancer cases diagnosed were ESCC. Over a period of 3 decades, EAC incidences have been increasing continuously, especially in western countries among Caucasians. Now almost half of the esophageal malignancy cases diagnosed are EAC (3, 4). EAC and ESCC show marked differences in their geographic spread. EAC is more common in developed countries such as the United Kingdom (8 in 100,000 individuals; ref. 5), Australia, and the United States. Within Europe, southern Europe has the highest EAC incidence (5). On the other side, ESCC is the most common type of esophageal cancer among developing Asian countries (6). Racial disparity also occurs between the 2 types of esophageal cancer. ESCC is more prevalent among Blacks, whereas EAC is at least twice as common in Whites as compared with other ethnic groups (7, 8). Once diagnosed, Black patients showed poorer overall survival than Whites (9, 10). Taken together, strong genetic and environmental factors relating to ethnicity and geographic distribution seem to be playing critical roles in the incidence of esophageal cancer. Studies also suggest possible links between socioeconomic status and the prevalence of esophageal cancer phenotype (6).

Risk Factors

In the majority of cases, EAC is diagnosed at a late stage, leading to a poor 5-year survival of less than 15% (11). Hence, recent research for EAC has focused on understanding risk factors and the identification of early diagnostic biomarkers.

Esophageal cancer is unlikely to develop in individuals younger than 40 years of age; however, after that the incidence increases significantly with each decade of life (9). Changing lifestyle and food habits are primarily responsible for the dramatic epidemiologic changes in EAC as described in recent reviews (11–13). Known EAC risk factors include accumulation of visceral fat in the abdomen (14), male gender, high intake of dietary fat and cholesterol with low intake of fruits and vegetables (15), tobacco smoking (16), reduction in *Helicobacter pylori*

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infections (17), and Barrett's esophagus (BE), a metaplastic change to the esophageal lining. Individuals with Barrett's esophagus carry almost 30 to 125 times more risk for EAC development, and 0.5% to 1% of patients with Barrett's esophagus are estimated to develop EAC each year (18). Barrett's esophagus is characterized by replacement of normal stratified squamous epithelium with metaplastic columnar epithelium and is considered to be a successful adaptation of the distal esophagus in response to chronic gastroesophageal reflux disorder (GERD; ref. 19).

GERD is a very common condition in the western population with around 20% reporting weekly symptoms of heartburn and acid regurgitation (20). Refluxate-containing bile acid, along with gastric acid, is considered to be more harmful, leading to inflammation, ulceration, Barrett's esophagus, and ultimately EAC. Development of Barrett's esophagus is a slow process and distinctive mucus-secreting goblet cell formation can take 5 to 10 years (21, 22). Typically, EAC develops through metaplasia–dysplasia–carcinoma sequence involving genetic and epigenetic modifications, leading to uncontrolled cell proliferation, and is characterized by the presence of intestinal metaplasia with low-grade (LGD) to high-grade dysplasia (HGD), which eventually may progress to invasive carcinoma (20).

Current Diagnosis Scenario

To detect pathologic changes leading to EAC development before onset of disease, current clinical practice involves endoscopic screening of patients with high-risk GERD and to characterize the degree of dysplasia in biopsy samples collected during endoscopy (23, 24). Enrollment of patients into an endoscopic screening program may be facilitated by a patient questionnaire of self-evaluated symptoms/complications (25, 26). Once enrolled into the screening program, a patient undergoes endoscopy-biopsy every 3 months to 2 years depending on the degree of dysplasia, during which 4 quadrant biopsy samples are taken every 1 to 2 cm and evaluated for histologic changes by expert pathologists (23, 24). As a significant number of patients histologically diagnosed with HGD develop EAC, endoscopic mucosal ablation or esophageal resection (esophagectomy) are options to stop further disease progression in those high-risk patients (27, 28). Significantly improved survival is observed in patients diagnosed at an early stage during surveillance endoscopy program as compared with symptomatically diagnosed EAC (29–32).

Although current screening methodology shows promise, outcome of endoscopy-biopsy in many cases is nonreproducible due to interobserver variability and sampling error (28, 33). Furthermore, histologic dysplastic changes may be patchy and present heterogeneously in the tissue sample. This makes the diagnosis challenging, especially in the early stages of transition to LGD (28, 34). In up to 40% of patients, invasive cancer has been found in resected tissue despite negative endoscopic examination for the malignancy (35). Moreover, false-positive results also occur, meaning despite intramucosal carcinoma in a biopsy, the subsequently resected tissue has no signs of carcinoma (28). These evidence suggest dysplasia grading is an imperfect measure of cancer risk.

Despite extensive screening with currently available techniques, more than 80% of EACs are diagnosed without any prior diagnosis of Barrett's esophagus or GERD (36, 37). According to an estimate, more than 80% of Barrett's esophagus cases are undiagnosed and therefore are not getting the benefit of the screening program (38). On the other hand, a large proportion of patients undergoing routine biopsy screening do not progress to EAC (13). These suggest inability of current methodologies in screening population to detect high-risk patients and to distinguish between disease progressors from nonprogressors. In addition, the screening procedure is not very cost-effective (39). To overcome these challenges, adjunct use of biomarker has been proposed to stratify the risk associated with EAC development.

Biomarkers in EAC

According to United States' NIH, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (40)."

In transit from intestinal metaplasia to LGD to HGD to EAC, cells acquire abilities to become self-sufficient for growth, evade apoptosis, proliferate uncontrollably, promote angiogenesis, invade underlined epithelium, and start to metastasize. These changes are accompanied with histologic changes in tissue architecture, genomic instability, development of tumor microenvironment, modulation of immune response, and are therefore reflected in body fluids (serum/plasma/mucus/urine) or tissue samples and differentiate in terms of their genome/proteome/metabolome profile (41). Thus, a biomarker can be from any of these sources and reflect underlying pathologic or homeostatic changes. Table 1 summarizes different classes of biomarkers proposed for BE/EAC.

National Cancer Institute Early Detection Research Network (EDRN) guidelines outline biomarker discovery and development to a 5-phase process summarized below (42) and depicted in Fig. 1.

Phase I—Preclinical exploratory study: it compares normal versus cancer samples (body fluids/tissue) using technologies such as genomics, microarray expression, proteomics, immunohistochemistry, or immunoblotting to detect significant changes in proteins/genes/metabolites between the groups.

Phase II—Clinical assay development and validation: it is aimed at developing a clinical assay using a minimally invasive sample collection method. The assay is meant to be robust, reproducible, and suitable for stored clinical samples to be used in later phases of development. At the end of this phase, one should

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Table 1. Comprehensive summary of different classes of BE/EAC biomarkers

Biomarker class	Ref.
Tissue biomarkers	
Genomic abnormalities (ploidy and LOH)	(47–51)
DNA methylation	Refer to Table 2
SNPs/expression array studies	Refer to Table 3
Inflammatory markers	
COX-2	(69, 72–77)
NF-κB	(78–81)
Cytokines	(67, 79, 81–86)
MMPs	(87–93)
Cell-cycle abnormalities	(94, 95, 101)
miRNA	Refer to Table 4
Glycosylation changes	(121, 123–125)
Circulatory biomarkers	
DNA methylation changes	(130–132)
Glycan alterations	(135–138)
Metabolic profiling	(142–145)

expect high specificity and sensitivity for the assay. However, it remains to be determined how early the biomarker can predict the disease.

Phase III—Retrospective longitudinal repository studies: the assay is applied on prospectively collected stored samples to determine the ability of the biomarker to detect the disease before clinical presentation. If so, then criteria for positive screening is determined for future use.

Phase IV—Prospective screening: the test is prospectively applied to real population to detect the extent and characteristic of disease detected by the biomarker. This phase gives positive predictive value for the test and gives an idea about feasibility for last phase of control trials.

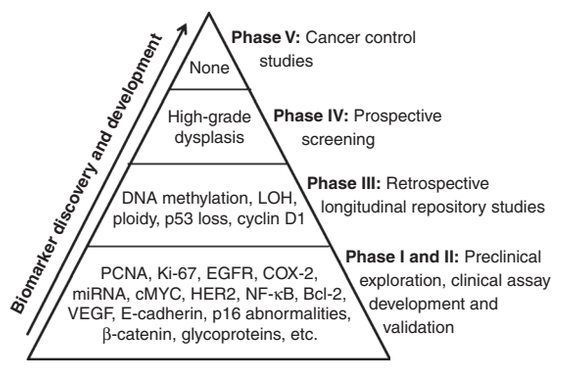

Figure 1. Summary of current BE/EAC Biomarkers with respect to EDRN clinical phase of development.

Phase V—Cancer control studies: it comprises large-scale clinical trial to determine the impact of new screening process on the disease burden in the community.

With respect to EAC, none of the biomarkers, including high-grade dysplasia, have been evaluated in phase V, whereas very few are evaluated in phase III and IV. Figure 1 summarizes proposed EAC biomarkers and how well they are characterized in the process of biomarker discovery. The following sections will discuss some of the classes of BE/EAC biomarkers.

Genomic Instability

Many groups have studied genomic instability induced by aneuploidy, tetraploidy, DNA methylation, allelic loss and shown some predictive power for these changes. A role for hypermethylation in the promoter regions of tumor-suppressor genes during the development of EAC has also been well established. Table 2 summarizes DNA methylation changes associated with metaplasia–dysplasia–carcinoma development. In the majority of patients, methylation changes are acquired very early during EAC development, hence these alterations could be used as an early diagnostic biomarker. Apart from discriminating patients at different stages of EAC development, DNA methylation signatures may be useful as predictors for progression from Barrett's esophagus to EAC (43, 44) and for response to chemotherapy and survival in patients with EAC (45, 46).

Although the individual genomic abnormality has the potential to diagnose disease at different stages, best results are obtained when they are used in combination (47–49). LOH at chromosome 9p and 17p locus are considered to be early events during Barrett's esophagus pathogenesis (50). If present with other chromosomal alterations such as aneuploidy and tetraploidy, it increases the 10-year risk for development of EAC from 12% to approximately 80% (51). However, with the current flow cytometry technology, it is technically very challenging for clinical laboratories to assess these genomic biomarkers in the samples, which limits widespread use of these biomarkers in the clinic.

Alternatively, genomic alterations can be detected at the protein level using immunohistochemistry. One of the most common and earliest genomic abnormality occurs at chromosome 17p, which codes for tumor-suppressor p53 protein. Loss of p53 protein expression in tissue samples correlates with disease progression (52). However, as p53 expression only reflects alterations at one particular gene, it has lower predictive value as compared with techniques monitoring multiple genomic abnormalities. Furthermore, sensitivity drops as mutations or deletions at genomic level may not necessarily be detected at the protein level (53).

In line with the genomic abnormalities described earlier, single-nucleotide polymorphism (SNP)–based genotyping can also stratify cancer risk in patients with Barrett's esophagus. As summarized in Table 3, in the past

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Table 2. Summary of hypermethylated genes during BE/EAC development

Gene	Location	Function	Method	Number (%) of samples showing hypermethylation or study findings					Ref.
				Normal	BE	LGD	HGD	EAC	
p16 (or *CDKN2A* or *INK4A*)	*9p21*	Cyclin-dependent kinase inhibitor	Methylation-specific PCR	5/9 (56%)	14/18 (77%)	—	—	18/21 (85%)	(146)
			Methylation-sensitive single-strand conformation analysis	0/10 (0%)	4/12 (33%)	3/11 (27%)	3/10 (30%)	18/22 (82%)	(147)
			Methylation-specific PCR	0/17 (0%)	14/47 (30%)	9/27 (32%)	10/18 (56%)	22/41 (54%)	(148)
			Methylation-specific PCR	2/64 (3%)	14/93 (15%)	—	—	34/76 (45%)	(149)
			Methylation-specific PCR	—	3/10 (30%)	—	—	5/11 (45%)	(150)
			Methylation-specific PCR	—	27/41(66%)	21/45 (47%)	17/21 (81%)	65/107 (61%)	(151)
			Methylation-specific PCR	0%	1/15 (7%)	4/20 (20%)	12/20 (60%)	8/15 (53%)	(152)
			Methylation-specific PCR	Separately determined exon 1 and 2 methylation. Five of 16 (31%) exon-1, 8/16 (50%) exon 2 in EAC patient samples showed hypermethylation. Exon 2 methylation correlates with stage of the tumor ($P = 0.01$)					(153)
O^6-*Methylguanine-DNA Methyltransferase* (or *MGMT*)	*10q26*	DNA repair	Methylight technique	2/10 (20%)	8/13 (62%)	—	—	84/132 (64%)	(154)
			Methylation-specific PCR	6/29 (21%)	24/27 (89%)	13/13 (100%)		37/47 (79%)	(155)
APC	*5q21-q22*	Wnt/β-catenin signaling	Methylation-specific PCR	0/17 (0%)	24/48 (50%)	14/28 (50%)	14/18 (78%)	20/32 (63%)	(148)
			Methylation-sensitive single-strand conformation analysis and methylation-sensitive dot blot assay	0/16 (0%)	11/11 (100%)	—	—	20/21 (95%)	(156)
				Eight of 14 histologically normal gastric mucosa adjacent to EAC showed significantly different methylation of *APC* promoter.					(157)
GSTM2	*1p13.3*	Antioxidants and protection against DNA damage	Bisulfite pyrosequencing (sample size: EAC-100, BE-11, dysplasia-11, normal esophageal/gastric mucosa-37)	<10%	∼50%	∼55%		69%	(158)
GSTM3				<10%	∼13%	∼37%		15%	(158)
GPX7	*1p32*			<10%	∼18%	∼80%		67%	(158)
GPX3	*5q23*			<10%	∼90%	∼88%		62%	(158)
			Methylation-specific PCR	2/12 (17%)	13/21(62%)	9/11 (82%)		30/34 (88%)	(159)
TIMP-3	*22q12.3*	MMP inhibitor	Methylight technique	1/8 (13%)	6/12 (50%)	—	—	9/13 (69%)	(160)
Death-associated protein kinase (*DAPK*)	*DAPK1: 9q21.33 DAPK2: 15q22.31 DAPK3: 19p13.3*	Tumor-suppressor and mediator of apoptosis	Methylation-specific PCR	4/20 (20%)	14/28 (50%)	11/21 (53%)		21/35 (60%)	(161)

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Table 2. Summary of hypermethylated genes during BE/EAC development (Cont'd)

Gene	Location	Function	Method	Number (%) of samples showing hypermethylation or study findings					Ref.
				Normal	BE	LGD	HGD	EAC	
Tachykinin-1 (*TAC1*)	*7q21-22*	Smooth muscle contractility, epithelial ion transport, vascular permeability and immune function	Methylation-specific PCR	5/67 (7.5%)	38/60 (63.3%)	12/19 (63.2%)	11/21 (52.4%)	41/67 (61.2%)	(162)
Reprimo	*2q23*	Regulates p53-mediated cell-cycle arrest in G_2-phase	Methylation-specific PCR	0/19 (0%)	9/25 (36%)	—	7/11 (64%)	47/75 (63%)	(163)
E-Cadherin	*16q22.1*	Ca^{+2}-dependent intercellular adhesion and maintains normal tissue architecture	Methylation-specific PCR	0/4 (0%)	—	—	—	26/31 (84%)	(164)
SOCS-3	*17q25.3*	Inhibits cytokine signaling	Methylation-specific PCR	0%	4/30 (13%)	6/27 (22%)	20/29 (69%)	14/19 (74%)	(165)
SOCS-1	*16p13.13*			0%	0/30 (0%)	1/27 (4%)	6/29 (21%)	8/19 (42%)	
Secreted frizzled-related proteins (*SFRP*)									
SFRP1	*8p11.21*	Wnt antagonist	Methylation-specific PCR	7/28 (25%)	30/37 (81%)	—	—	37/40 (93%)	(166)
SFRP2	*4q31.3*			18/28 (64%)	33/37 (89%)	—	—	33/40 (83%)	
SFRP1	*8p11.21*		Methylation-sensitive single-strand conformation analysis and methylation-sensitive dot blot assay	1/12 (8%)	6/6 (100%)	—	—	23/24 (96%)	(156)
SFRP2	*4q31.3*			11/15 (73%)	6/6 (100%)	—	—	19/25 (76%)	
SFRP4	*7p14.1*		Methylation-specific PCR	9/28 (32%)	29/37 (78%)	—	—	29/40 (73%)	(166)
SFRP5	*10q24.1*			6/28 (21%)	27/37 (73%)	—	—	34/40 (85%)	
Plakophilin-1 (*PKP1*)	*1q32*	Cell adhesion and intracellular signaling	Methylation-specific PCR	5/55 (9.1%)	5/39 (12.8%)	—	1/4 (25%)	20/60 (33.3)	(167)
GATA-4	*8p23.1-p22*	Transcription factor and regulate cell differentiation	Methylation-specific PCR	0/17 (0%)	—	—	—	31/44 (71%)	(168)
GATA-5	*20q13.33*			0/17 (0%)	—	—	—	24/44 (55%)	
CDH13 (or *H-cadherin* or *T-cadherin*)	*16q24*	Cell adhesion	Methylation-specific PCR	0/66 (0%)	42/60 (70%)	15/19 (78.9%)	16/21 (76.2)	51/67 (76.1%)	(169)

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Table 2. Summary of hypermethylated genes during BE/EAC development (Cont'd)

Gene	Location	Function	Method	Number (%) of samples showing hypermethylation or study findings					Ref.
				Normal	BE	LGD	HGD	EAC	
NELL-1 (*nel-like 1*)	*11p15*	Tumor suppressor	Methylation-specific PCR	0/66 (0%)	28/60 (46.7%)	8/19 (42.1%)	13/21 (61.9%)	32/67 (47.8%)	(170)
Eyes Absent 4	*6q23*	Apoptosis modulator	Methylation-specific PCR	2/58 (3%)	27/35 (77%)	—	—	33/40 (83%)	(171)
A-kinase anchoring protein 12 (or *Gravin* or *AKAP12*)	*6q24-25.2*	Cell-signaling, adhesion, mitogenesis, and differentiation	Methylation-specific PCR	0/66 (0%)	29/60 (48.3%)	10/19 (52.6%)	11/21 (52.4%)	35/67 (52.2)	(172)
Vimentin	*10p13*	Cytoskeleton protein	Methylation-specific PCR	0/9 (0%)	10/11 (91%)	—	5/5 (100%)	21/26 (81%)	(173)
RUNX3	*1p36*	Transcription factor	Methylation-specific PCR	1/63 (2%)	23/93 (25%)	—	—	37/77 (48%)	(149)
HPP1	*19pter-p13.1*	Tumor suppressor		2/64 (3%)	41/93 (44%)	—	—	55/77 (71%)	
3-OST-2	*16p12*	Sulfotransferase enzyme		1/57 (2%)	47/60 (78%)	—	—	28/73 (38%)	
Wnt inhibitory factor-1 (*WIF-1*)	*12q14.3*	Wnt antagonist	Methylation-specific PCR	81% of patients with Barrett's esophagus suffering from EAC showed hypermethylated WIF-1 as compared with 20% of patients with Barrett's esophagus without EAC					(174)
CHFR (*checkpoint with forkhead associated and ring finger*)	*12q24*	Mitosis check point protein	Bisulfite pyrosequencing	EAC samples 31% (18/58) showed significantly higher *CHFR* promoter methylation as compared with normal samples ($P = 0.01$).					(175)
Metallothionein 3 (or *MT3*)	*16q13*	Metal homeostasis and protection against DNA damage	Bisulfite pyrosequencing (sample size: normal-33, BE-5, EAC-78)	Identified 2 regions (R2 and R3) of CpG nucleotides, which showed significantly higher methylation in EAC as compared with normal epithelium (FDR < 0.001). Increased DNA methylation of MT3 promoter R2 correlates with advanced tumor stage ($P = 0.005$) and lymph node metastasis ($P = 0.03$). DNA methylation of MT3 promoter R3 correlates with tumor staging ($P = 0.03$) but not with lymph node status ($P = 0.4$).					(176)

Methylation marker panel

Sample size	Method	Findings	Ref.
EAC-35 undergoing chemoradiotherapy	Methylation-specific PCR	Combined mean of promoter methylation of *p16*, *Reprimo*, *p57*, *p73*, *RUNX-3*, *CHFR*, *MGMT*, *TIMP-3*, and *HPP1* was lower in patients who responded to chemoradiotherapy (13/35) as compared with patients who did not respond (22/35; $P = 0.003$).	(46)
BE-62 (28 patients with Barrett's esophagus progressed to EAC and remaining 34 patients with Barrett's esophagus were nonprogressors)	Methylation-specific PCR	Three-tiered stratification model was developed using methylation index (*p16*, *HPP1*, and *RUNX3*), Barrett's esophagus length and pathology. Combined model based on 2- (ROC: 0.8386) and 4-year (ROC: 0.7910) prediction was able to categorize patients with Barrett's esophagus into low-risk, intermediate-risk, and high-risk groups for EAC development.	(44)

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Table 2. Summary of hypermethylated genes during BE/EAC development (Cont'd)

Methylation marker panel

Sample size	Method	Findings	Ref.
BE-195 (145 patients with Barrett's esophagus progressed to EAC and remaining 50 patients with Barrett's esophagus were nonprogressors)	Methylation-specific PCR	*HPP1* ($P = 0.0025$), *p16* ($P = 0.0066$), and *RUNX3* ($P = 0.0002$) were significantly hypermethylated in progressors as compared with nonprogressors. In combination, panel of 8 methylation markers (*p16*, *HPP1*, *RUNX3*, *CDH13*, *TAC1*, *NELL1*, *AKAP12*, and *SST*) showed sensitivities of 0.443 and 0.629 at specificity of 0.9 and 0.8 for EAC progression in patients with Barrett's esophagus using combined model designed on the basis of 2 and 4 years of follow-up.	(43)
EAC-41 (adjacent normal samples as control)	Methylation-specific PCR	Patients having more than 50% of their genes methylated (*APC*, *E-cadherin*, *MGMT*, *ER*, *p16*, *DAP-kinase*, and *TIMP3*) showed significantly poor 2-year survival ($P = 0.04$) and 2-year relapse-free survival ($P = 0.03$) as compared with the patients having less than 50% methylation.	(45)
BE-18, EAC-38 (multiple biopsies were taken and classified into normal, Barrett's esophagus, HGD, and EAC)	Bisulfite-modified DNA with PCR	The methylation frequencies of 9 genes (*APC*, *CDKN2A*, *ID4*, *MGMT*, *RBP1*, *RUNX3*, *SFRP1*, *TIMP3*, and *TMEFF2*) found to be 95%, 59%, 76%, 57%, 70%, 73%, 95%, 74%, and 83%, respectively, in EAC samples, whereas 95%, 28%, 78%, 48%, 58%, 48%, 93%, 88%, and 75%, respectively, in Barrett's esophagus samples, which was significantly higher as compared with normal squamous epithelium. The methylation frequency for *CDKN2A* and *RUNX3* was significantly higher for EAC as compared with Barrett's esophagus biopsy samples.	(177)
Normal-30, BE-29, HGD-8, EAC-29	Illumina GoldenGate methylation bead array	Overall median methylation at the total 706 numbers of most informative CpG sites gradually increased from normal-BE-HGD/EAC ($P < 0.001$). The authors differentiated between EAC vs. normal, HGD vs. normal, Barrett's esophagus vs. normal, EAC vs. Barrett's esophagus, and HGD vs. Barrett's esophagus based on 422, 225, 195, 17, and 3 numbers of CpG sites, which is showing differential methylation between respective groups.	(178)
Identification phase (BE-22, EAC-24); retrospective validation phase (BE-60, LGD/HGD-36, EAC-90); prospective validation phase (98 patients under surveillance).	Identification phase: Illumina Infinium assay; retrospective/prospective validation phase: pyrosequencing	On the basis of initial identification phase, 7 genes (*SLC22A18*, *ATP2B4*, *PIGR*, *GJA12*, *RIN2*, *RGN*, and *TCEAL7*) showing most prominent methylation changes were selected for validation. Combination of 4 genes (ROC 0.988) *SLC22A18*, *PIGR*, *GJA12*, and *RIN2* showed sensitivity of 94% and specificity of 97%. This panel of 4 genes showing differential methylation, stratified patients into low-, intermediate-, and high-risk groups for EAC development in prospective validation.	(179)
Nondysplastic Barrett's esophagus (not progressed to EAC)-16, Barrett's esophagus mucosa from patients progressed to EAC-12	Methylation-sensitive single-strand conformation analysis and methylation-sensitive dot blot assay	Barrett's esophagus samples collected from patients who progressed to EAC in 12 months time period showed 100%, 91%, and 92% hypermethylation of *APC*, *TIMP-3*, and *TERT*, respectively, as compared with 36%, 23%, and 17% in Barrett's esophagus mucosa collected from patients who did not progress to EAC.	(180)

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Table 3. Summary of gene expression profiling studies for BE/EAC

Sample size	Array description	Outcome	Findings	External validation	Ref.
BE-21 (paired normal esophageal and gastric samples as control)	Serial analysis of gene expression, PCR and immunoblotting	Disease progression	Of note, 534 tags were significantly differentially expressed between normal esophageal squamous epithelium and Barrett's esophagus. The most upregulated genes in Barrett's esophagus as compared with normal epithelium were identified to be trefoil factors, annexin A10 and galectin-4 with each different type of tissue showed an unique cytokeratin expression.	No	(181)
Barrett's esophagus and HGD-11 (matched biopsy samples)	cDNA microarray	Disease progression	Using 2.5-fold cutoff, identified 131 upregulated and 16 downregulated genes in HGD. Twenty-four of 28 most significantly different genes showed similar changes during validation.	Real-time PCR	(182)
EAC-91	Oligo-microarray	Disease progression	A 4-gene panel consists of deoxycytidine kinase, 3′-phosphoadenosine 5′-phosphosulfate synthase 2, sirtuin-2, and tripartite motif-containing 44 predicted 5-year survival.	Immunohistochemistry	(183)
Twenty-three paired Barrett's esophagus and normal epithelium samples	Transcriptional profiling and proteomics	Disease progression	Identified 2,822 genes to be differentially expressed between Barrett's esophagus and normal epithelium. Significantly overexpressed genes during Barrett's esophagus belonged to cytokines and growth factors, constituents of extracellular matrix, basement membrane and tight junctions, proteins involved in prostaglandin and phosphoinositol metabolism, nitric oxide production and bioenergetics. While genes encoding HSP and various kinases were downregulated.	No	(184)
Lymph node metastatic ($n = 55$) and nonmetastatic ($n = 22$) EAC samples	Oligo-microarray	Disease progression	Lymph node–positive samples showed significant downregulation of argininosuccinate synthetase as compared with lymph node nonmetastatic samples ($P = 0.048$).	No	(185)
EAC-6 and gastric cardia cancer-8	aCGH	Disease progression	Identified *HGF* (45%) and *BCAS1* (27%) to be most frequently overexpressed genes respectively at 7q21 and 20q13 locus.	No	(186)
Eleven matched sample sets (healthy-BE-EAC matched-6, normal-BE matched-4 and normal-EAC matched-1)	SNP microarray	Disease progression	60% of Barrett's esophagus and 57% of EAC samples contained at least one of the genomic alterations in the form of deletions, duplications, amplifications, copy number changes, and neutral LOH.	No	(187)

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Table 3. Summary of gene expression profiling studies for BE/EAC (Cont'd)

Sample size	Array description	Outcome	Findings	External validation	Ref.
Normal-39, BE-25, EAC-38, and ESCC-26	cDNA microarray	Disease progression	Clustering showed the separation of samples into 4 distinct groups. Of note, 2,158 clones were differentially expressed between normal and Barrett's esophagus samples, whereas 1,306 between Barrett's esophagus and EAC. BE/EAC samples showed differential expression of hydrolases, lysozyme, fucosidase, transcription factors, mucins, and the trefoil factors.	No	(188)
BE-20, LGD-19, HGD-20 and EAC-42	SNP microarray	Disease progression	Increasing numbers of SNPs and loss of chromosomes with disease progression. Chromosomal disruption was identified in the *FHIT*, *WWOX*, *RUNX1*, *KIF26B*, *MGC48628*, *PDE4D*, *C20orf133*, *GMDS*, *DMD*, and *PARK2* genes in EAC.	No	(189)
EAC-75 specimens from 64 patients, adjacent paired normal tissue from patients with EAC-28	DNA microarray	Disease progression	Identified *AKR1B10*, *CD93*, *CSPG2*, *DKK3*, *LUM*, *MMP1*, *SOX21*, *SPP1*, *SPARC*, and *TWIST1* genes as biomarker based on transcriptomics data. Quantitative real-time PCR identified *SPARC* and *SPP1* genes to be associated with EAC patient survival ($P < 0.024$).	Real-time PCR	(190)
EAC-8, gastric cardia cancer-3	aCGH and cDNA microarray	Disease progression	Transcriptomics data identified 11 genes to be differentially expressed (*ELF3*, *SLC45A3*, *CLDN12*, *CDK6*, *SMURF1*, *ARPC1B*, *ZKSCAN1*, *MCM7*, *COPS6*, *FDFT1*, and *CTSB*). IHC analysis revealed significant overexpression of *CDK6* a cell-cycle regulator in tumor samples.	No	(191)
BE-20	aCGH arrays and high density SNP genotyping	Disease progression	Copy number losses were detected at FRA3B (81%), FRA9A/C (71.4%), FRA5E (52.4%), and FRA 4D (52.4%) sites in early Barrett's esophagus. Validation study confirmed loss of FRA3B and FRA16D in early Barrett's esophagus samples.	Real-time PCR and pyrosequencing	(192)
BE-11, gastroesophageal junction (GEJ) adenocarcinoma-11	aCGH with a whole chromosome 8q contig array	Disease progression	Overexpression of *MYC* and *EXT1*, while downregulation of *MTSS1*, *FAM84B*, and *C8orf17* is significantly associated with GEJ adenocarcinoma.		(193)
BE-14, EAC-5, ESCC-3	cDNA microarray	Disease progression	Identified 160 genes that can differentiate between Barrett's esophagus and esophageal cancer.	No	(194)
Twenty-four paired samples of normal, Barrett's esophagus, and EAC phenotype	cDNA microarray	Disease progression	Of note, 214 differentially regulated genes could differentiate between normal, Barrett's esophagus, and EAC phenotype. Genes involved in epidermal	No	(195)

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Table 3. Summary of gene expression profiling studies for BE/EAC (Cont'd)

Sample size	Array description	Outcome	Findings	External validation	Ref.
			differentiation are underexpressed in EAC as compared with Barrett's esophagus. Expression ratio of *GATA6* to *SPRR3* can differentiate between 3 phenotypes studied.		
Pooled biospy samples from Barrett's esophagus, esophageal squamous, gastric, and duodenum	Oligo-microarray	Disease progression	Differentiate different tissue clusters based on gene expression profile. Identified 38 genes that are upregulated in Barrett's esophagus tissue cluster, which belong to cell cycle (P1cdc47, PCM-1), cell migration (urokinase-type plasminogen receptor, LUCA-1/HYAL1), growth regulation (TGF-β superfamily protein, amphiregulin, Cyr61), stress responses (calcyclin, ATF3, TR3 orphan receptor), epithelial cell surface antigens (epsilon-BP, ESA, integrin β4, mesothelin CAK-1 antigen precursor), and 4 mucins.	No	(196)
Normal-24, BE-18, EAC-9	cDNA microarray	Disease progression	Identified 457, 295, and 36 differentially expressed genes, respectively, between normal-EAC, normal-Barrett's esophagus, and BE–EAC groups.	No	(197)
89-EAC	cDNA-mediated annealing, selection, extension, and ligation assay with 502 known cancer-related genes	Disease progression	Identified differential gene expression between early stages of EAC (T1 and T2) vs. late (T3 and T4). Gene expression profile revealed *ERBB4*, *ETV1*, *TNFSF6*, *MPL* genes to be common between advanced tumor stage and lymph node metastasis.	No	(198)
Normal esophageal mucosa-9, esophagitis-6, BE-10, EAC-5, GEJ adenocarcinoma-9, stomach samples-32 (normal mucosa-11, IM-9, intestinal-type adenocarcinoma-7, and diffuse carcinoma-5)	cDNA microarray	Disease progression	On the basis of the expression profile, genes associated with the lipid metabolism and cytokine nodule are found to be significantly altered between EAC and other groups.	No	(199)
Seventeen paired samples of normal, BE/EAC	cDNA microarray	Disease progression	Each tissue type expresses distinct set of genes, which can differentiate between their phenotypes. Barrett's esophagus and EAC expresses similar set of stromal genes that are different from normal epithelium.	No	(200)
BE-19, EAC-20 (98 tissue specimens were collected and categorized into different groups)	On the basis of previous microarray studies 23 genes were validated using real-time PCR	Disease progression	Out of 23 genes, panel of 3 genes (*BFT*, *TSPAN*, and *TP*) was able to discriminate between Barrett's esophagus and EAC in internal validation with 0% classification error.	N.A.	(201)

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Table 3. Summary of gene expression profiling studies for BE/EAC (Cont'd)

Sample size	Array description	Outcome	Findings	External validation	Ref.
Normal-30, BE-31, gastric mucosa-34, duodenum-18	Biomarkers for Barrett's esophagus were identified using 3 publicly available microarray datasets and validated using real-time PCR and immunohistochemistry.	Disease progression	Out of 14 genes identified, dopa decarboxylase (*DDC*) and Trefoil factor 3 (*TFF3*) were validated to be upregulated in Barrett's esophagus.	N.A.	(202)
EAC-56	Oligonucleotide microarray and aCGH	Disease progression	Identified 4 new genes (*EGFR*, *WT1*, *NEIL2*, and *MTMR9*) to be overexpressed in 10% to 25% EAC. Expression levels of these 4 genes differentiated patients with EAC into 3 groups namely good, average, and poor depending upon their prognosis ($P < 0.008$)	Immunohistochemistry	(203)
BE/LGD-72, HGD-11, EAC-15	Bacterial artificial chromosome aCGH	Disease progression	Copy number changes were more common and larger as disease progress to later stages. Patients having copy number alterations involving more than 70 Mbp were at increased risk of progression to EAC ($P = 0.0047$)	No	(60)
EAC-30, BE-6, LGD-9, HGD-10	Genome-wide CGH	Disease progression	Loss of 7q33-q35 was found in HGD as compared with precursor LGD ($P = 0.01$). Loss of 16q21-q22 and gain of 20q11.2-q13.1 was significantly different between HGD and EAC ($P = 0.02$ and 0.03, respectively).	No	(56)
EAC-30, lymph node metastasis-8, HGD-11, LGD-8, and BE-6 from 30 EAC patient biopsy samples	CGH	Disease progression	Identified regions undergoing copy number loss and amplification during each stage of transition. Average number of chromosomal imbalance sequentially increased from BE–LGD–HGD–EAC–lymph node metastasis.	No	(54)
Forty-two patients represent different stages of disease	SNP array	Disease progression	SNP abnormalities increases from 2% to more than 30% as the disease progress from Barrett's esophagus to EAC. Total number of SNP alterations in tissue samples is tightly correlated with DNA abnormalities such as aneuploidy and LOH.	No	(57)
EAC-27 and matched normal-14	SNP array	Disease progression	Confirmed previously described genomic alterations such as amplification on 8q and 20q13 or deletion/LOH on 3p and 9p. Also identified alterations in several novel genes and DNA regions in EAC samples.	No	(58)

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Table 3. Summary of gene expression profiling studies for BE/EAC (Cont'd)

Sample size	Array description	Outcome	Findings	External validation	Ref.
EAC-26	SNP array	Disease progression	Confirmed previously reported frequent changes to *FHIT*, *CDKN2A*, *TP53*, and *MYC* genes in EAC. Identified *PDE4D* and *MGC48628* as tumor-suppressor genes.	No	(59)
EAC-35	cDNA microarray	Response to chemotherapy	Identified 165 differentially expressed genes between poor ($n = 17$) and good outcome ($n = 18$) patient groups. Top functional pathway based on differential gene expression was identified to be Toll-receptor signaling.	No	(204)
EAC-47 (locally advanced tumor)	cDNA microarray	Response to chemotherapy	Identified 86 genes showing at least 2-fold difference between chemotherapy responders ($n = 28$) and nonresponders ($n = 19$). Ephrin B3 receptor, which showed highest difference between the groups, showed strong membrane staining in chemotherapy responding tumors using immunohistochemistry.	No	(205)
Patients with EAC-19 undergoing chemoradiotherapy	Oligo-microarray	Response to chemoradiotherapy	Reduced expression of *IVL*, *CRNN*, *NICE-1*, *S100A2*, and *SPPR3* genes correlated with poor survival and nonresponse to chemotherapy.	No	(206)
19 patients (EAC-16, ESCC-2 and adenosquamous carcinoma-1) undergoing chemoradiotherapy	Oligo-microarray	Response to chemoradiotherapy	Lower expression for panel of genes *PERP*, *S100A2*, and *SPRR3* was associated with nonresponse to therapy. Pathway analysis identified downregulation of apoptosis in nonresponders.	No	(207)
EAC-174, ESCC-36	SNPs associated with the chemotherapy drug action pathway	Response to chemoradiotherapy	Identified association between genetic polymorphisms and response to preoperative chemotherapy (fluorouracil and platinum compounds) and radiotherapy.	No	(208)

NOTE: Majority of studies described in this table include validation of results in the same patient cohort as used in discovery phase. Only studies that included validation using independent patient cohort are described as external validation.

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decade, several studies conducted using advanced genomic techniques such as array-comparative genomic hybridization (aCGH) and SNP arrays confirmed previously reported copy number alterations and identified novel genomic loci undergoing changes during process of metaplasia–dysplasia–carcinoma development (54–60). It has been shown that as the disease progresses from early to late stages, SNP abnormalities increase from approximately 2% to 30% (54, 57). The total number of SNP alterations in tissue samples is tightly correlated with previously reported DNA abnormalities such as aneuploidy, copy number alterations, and LOH highlighting the application of SNP-based genotyping to assess genomic abnormalities (54–60). Thus, SNP-based genotyping provides an alternative way to assess genomic abnormalities during EAC pathogenesis.

Studies on gene expression changes in EAC have been propelled by recent progress in genomic technologies, each identifying unique sets of gene expression profile, which can be used as a biomarker panel for disease diagnosis, prognosis, or to predict response to therapy (Table 3). Moreover, determination of the gene expression changes has been extremely helpful to understand detailed pathogenesis and will form basis for developing future therapies. However, future validation using independent sample cohorts will be necessary for the majority of these potential biomarkers.

Apart from genomic abnormalities associated with the disease progression, inheriting genetic factors are also implicated for EAC development. Risk for BE/EAC and GERD is increased by 2- to 4-fold when a first-degree relative is already affected by any of these conditions (61). Recently, a study conducted by The Esophageal Adenocarcinoma Genetics Consortium and The Wellcome Trust Case Control Consortium identified link between SNPs at the MHC locus and chromosome 16q24.1 with risk for Barrett's esophagus (62). They also identified SNPs associated with body weight measures that were present with more than expected frequency in Barrett's esophagus samples supporting epidemiologic findings about obesity as a risk factor for Barrett's esophagus and EAC (62). Wu and colleagues examined the relationship between presence of risk genotypes and the onset of EAC. They identified 10 SNPs associated with the age of EAC onset. Genes associated with 5 of 10 SNPs identified were known to be involved in apoptosis (63).

Recently, published cancer genome–sequencing studies have given deeper insights into the genomic abnormalities associated with the EAC pathogenesis. The comparative genomic analysis between EAC and ESCC reported by Agrawal and colleagues (64) confirmed previously very well-described association of *p53* gene mutations with esophageal cancer development. The authors also conducted comparative genome-wide analysis between matched Barrett's esophagus and EAC patient tissue samples and concluded that the majority of genomic changes occur early during EAC development, at the stage of Barrett's esophagus (64). Similar conclusions were made by next-generation sequencing of biopsy samples obtained from the same patient at the stage of Barrett's esophagus and EAC (65). The authors also identified *ARID1A* as novel tumor-suppressor gene and around 15% of patients with EAC showed loss of ARID1A protein in tissue samples. *In vitro* studies suggested it to be associated with cell growth, proliferation, and invasion (65). Very recently published high-resolution methylome analysis has provided first evidence for methylation changes at genomic regions that encode noncoding RNAs. The authors identified long noncoding RNA, AFAP1-AS1, to be severely hypomethylated in Barrett's esophagus and EAC tissue samples, silencing of which significantly reduced aggressiveness of EAC cell lines OE33 and SKGT4 (66).

Taken together, genomic abnormalities play key roles during each stage of transformation from normal squamous epithelium to EAC.

Cancer-Related Inflammation

Gastric and bile acid exposure in the esophageal epithelium leads to the development of chronic inflammatory conditions mainly driven by elevated levels of proinflammatory cytokines. Chronic inflammatory responses induce cell survival and increase cell proliferation, hence play key roles in the development of EAC (67, 68). Expressions of various inflammatory molecules such as COX-2, NF-κB, interleukin (IL)-6, IL-8, and matrix metalloproteinases (MMP) have been evaluated as prognostic biomarkers for BE/EAC development.

Exposure to gastric/bile acid and cytokines leads to increased COX-2 expression (69). COX-2 is a rate-limiting enzyme that regulates synthesis of prostaglandins from arachidonic acid. COX-2 directly increases cell proliferation and promotes tumor invasion (69), and COX-2–mediated increase in prostaglandin synthesis could result in tumor growth and angiogenesis (70). COX-2 expression has been detected in disease-free esophageal tissue homogenates using immunoblotting (69). In comparison with GERD, patients suffering from erosive reflux show slightly higher gene expressions of this enzyme in tissue samples (71). Several studies have shown significantly increased COX-2 expression correlating with the disease progression from Barrett's esophagus to dysplasia and EAC (69, 72–75). Furthermore, expression levels of COX-2 have been shown to have a prognostic value in EAC with higher levels associated with poor survival and increased chances of tumor relapse (76, 77).

Another well-studied inflammatory biomarker NF-κB is activated in response to exposure with bile acid and elevated NF-κB expression levels are found during Barrett's esophagus, dysplasia, and adenocarcinoma (78–80). Activated NF-κB translocates from cytoplasm to nucleus and upregulates transcription of the genes involved in inflammatory processes. Moreover, nuclear NF-κB expression has been shown to be correlated with the patient response to chemoradiotherapy. All of the patients who showed complete response to chemoradiotherapy

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had elevated NF-κB levels pretreatment and showed lack of active NF-κB posttreatment (81).

In line with NF-κB and COX-2, expression of individual or combinations of proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α is significantly increased in Barrett's esophagus and EAC as compared with squamous epithelium (82–84). IL-1β and IL-8 expression levels also correlate with the stage of EAC (79). Patients who responded to neoadjuvant chemotherapy treatment showed significantly reduced expressions of IL-8 and IL-1β in postchemotherapy esophageal tissue sections (81). IL-6 is activated in response to reflux and the IL-6/STAT3 antiapoptotic pathway may underlie the development of dysplasia and tumor (85). Serum IL-6 levels were reported to provide 87% sensitivity and 92% specificity for EAC diagnosis in a recent retrospective study (86). However, the study only compared between healthy and EAC groups. It would be interesting to see how early it can diagnose EAC during the process of metaplasia–dysplasia. Combination of cytokines IFN-γ, IL-1α, IL-8, IL-21, and IL-23 along with platelet proteoglycan and miRNA-375 expression profiling has been shown to build an inflammatory risk model, which has clinical use to determine prognosis for patients with EAC (67).

MMPs are a family of proteolytic enzymes involved in the degradation of extracellular matrix components. MMPs play a role in both inflammation and tumor metastasis. Immunohistochemical staining for MMP-1, MMP-2, MMP-7, and MMP-9 has been reported to be significantly higher in EAC as compared with healthy individuals (87, 88). Higher level of MMP-1 expression has been associated with the lymph node metastases and possibly poor patient survival (89). Expression of MMP-9 is shown to be an early event during the EAC transformation and its expression levels are correlated with the progression of the disease (90–92). Activity of MMPs is inhibited by a family of proteins called tissue inhibitors of metalloproteinases (TIMP). Specifically, *TIMP-3* gene is methylated in EAC development and its reduced expression is associated with stage of the tumor and patient survival (93). On contrary, Salmela and colleagues described elevated TIMP-1 and TIMP-3 expression in EAC tumor samples (88).

Although the underlying tissue inflammation is very closely associated with EAC development and several inflammation-related biomarkers have been identified, these remain to be validated in large-scale biomarker studies.

Cell Cycle–Related Abnormalities

To compensate for the tissue damage induced by gastric/bile acid, the underlying epithelium starts to proliferate rapidly and become uncontrolled resulting in neoplasia. To meet the proliferation requirements, the cells have to overcome cell-cycle checkpoints. Cyclin D1 overexpression is one such means by which cells overcome G_1–S checkpoint, and cyclin D1 immunohistochemical staining has been proposed to identify patients with Barrett's esophagus with an increased risk for EAC (94). In contrast to cyclin D1, expression of p16 protein results in cell-cycle arrest in G_1 phase as it has been shown to inhibit cyclin-dependent kinase–induced phosphorylation of retinoblastoma protein. Early genomic abnormalities during EAC development significantly affect p16 protein expression, which can be determined using immunostaining and implemented as a potential biomarker (95). Further large-scale trials are required to confirm cell-cycle abnormalities during EAC development to implement them as a biomarker.

Bottom of the pyramid in Fig. 1 represents list of biomarkers in the initial stages of development. Tumors harboring overexpression of growth factor receptors [EGF receptor (EGFR) and HER-2] are associated with poor patient survival (96, 97), whereas those overexpressing apoptosis regulator Bcl-2 protein showed prolonged survival (98). Incipient angiogenesis is a marked feature of Barrett's esophagus and underlining tissue expresses angiogenesis markers VEGF and its receptors (99). Neovascularization continues as the disease progresses from Barrett's esophagus to EAC. Measuring the degree of neovascularization correlated with histopathologic grade of the tumor and associated with the patient survival (100). Expression of 2 prominent cell proliferation markers, PCNA and Ki-67, has been described to be altered during BE–EAC development (101).

miRNA

miRNA was first discovered in *Caenorhabditis elegans* (102) and since then it has been widely studied in a variety of biologic phenomena. These short stretches of approximately 21 nucleotides do not code for protein but play important roles in gene regulation by either suppressing protein synthesis or causing mRNA cleavage. Unlike siRNA, miRNA can target multiple genes on remote loci and therefore control diverse group of proteins. Several key properties of carcinogenesis have been shown to be regulated via miRNA, for example, angiogenesis and metastasis (103).

With increased biologic understanding of miRNAs and their role in cancer, they have been proposed in several different clinical applications including cancer diagnosis and tumor prognosis, tumor classification, and also as a therapeutic target for disease intervention. Differential tissue miRNA expression has been observed in several different malignancies and these changes can be used for diagnosis and classification of the tumors (103). miRNA bioarrays were first used to show differential miRNA expression in healthy, Barrett's esophagus, and EAC tissue samples (104). Since then, a number of different studies have identified miRNA changes associated with the development of the BE–EAC. Table 4 summarizes primary findings of miRNA expression profiling studies along with statistical significance and fold-change values. Biologic significance for some of the miRNA-related changes is discussed later.

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Smith and colleagues identified reduced expression of miR-200 and miR-141 in Barrett's esophagus and EAC tissue samples. They conducted bioinformatics analysis and correlated these miRNA expression changes with cellular processes such as cell cycle, cell proliferation, apoptosis, and cell migration (105). miR-196a, which is described as a marker of progression from Barrett's esophagus to EAC, can increase cell proliferation and anchorage-independent growth and inhibit apoptosis in EAC cell lines *in vitro* (106). The downstream targets for miR-196a are verified to be Annexin A1, S100 calcium-binding protein A9, small proline-rich protein 2C, and Keratin 5, which showed reduced expression in EAC patient tissue samples as compared with normal epithelium (106, 107). Several studies described in Table 4 report overexpression of miR-192 during EAC carcinogenesis. miR-192 has been reported to be a target of p53 and has been able to suppress cancer progression in osteosarcoma and colon cancer cell lines through p21 accumulation and cell-cycle arrest (108). As shown in Table 4, miR-21 is overexpressed during BE/EAC and it can function as an oncogene as shown in tumors of breast, brain, lung, prostate, pancreas, colon, liver, and chronic lymphocytic leukemia. It negatively regulates tumor- and metastasis-suppressor genes *PTEN*, *TPM1*, *PDCD4*, and *Sprouty2* (109–112). miR-194 expression is regulated by hepatocyte nuclear factor (HNF)-1α transcription factor, which is induced during BE/EAC and may lead to upregulation of miR-194 (109). Higher expression of miR-194 is also observed in metastatic pancreatic cell lines (113). Among miRNAs found to be downregulated during EAC development, let-7 family of miRNAs is tumor-suppressive and negatively regulates *Ras* oncogene. Fassan and colleagues confirmed upregulation of *HMGA2*, which is one of the target of let-7 miRNA, using immunohistochemistry in tissue samples (110, 112, 114). Further studies in the regards of miRNA and miRNA target genes will improve the biologic understanding of EAC pathogenesis and may also provide novel molecular targets for disease intervention.

Notably, miRNAs are found to be stable in serum encapsulated in microvesicles and can be accessed easily (115). In fact, circulating miRNA profiling has shown distinct expression patterns in a number of cancers, other than EAC (116). This opens up new avenues for circulating miRNA changes as a potential biomarker for EAC.

Glycoproteins

Protein glycosylation is a common posttranslational modification with almost half of the proteins synthesized undergoing 1 of the 2 major types either N-linked or O-linked glycan modifications. The biosynthetic process of glycosylation is regulated by the expression and localization of glycosyltransferases/glycosidases and the availability of substrate glycans (117).

Aberrant glycosylation changes have previously been reported in several different cancers namely breast cancer, prostate cancer, melanoma, pancreatic cancer, ovarian cancer, etc. (118, 119). These changes include truncated forms of O-glycans, increased degree of branching in N-glycans, and elevated sialylation, sulfation, and fucosylation with a range of other possible variations (119). The differential glycosylation can alter protein interactions, stability, trafficking, immunogenicity, and function (118). Tumor-specific glycosylation changes are actively involved in neoplastic progression, namely metastasis, as glycoproteins are found abundantly on cell surfaces and extracellular matrices and therefore play a vital role in cellular interactions.

Lectins are a family of glycan-binding proteins extensively used in glycobiology due to preferential binding of each lectin to recognize specific glycan structures (119, 120). The first effort to identify differential glycosylation in the progression to Barrett's esophagus and EAC was made in 1987 by Shimamoto and colleagues using differential binding pattern to 5 lectins in tissue specimens (121). The glycoconjugate expression profile in Barrett's esophagus was found to be significantly different from normal esophageal epithelium. Interestingly, glycoconjugate expression between Barrett's esophagus and normal duodenum was quite similar. There were minimal glycoconjugate expression changes between Barrett's esophagus and LGD. However, EAC tissue samples showed significantly different lectin-binding pattern than BE/LGD (121). Using rabbit esophageal epithelium, Poorkhalkali and colleagues showed differential lectin binding in response to acid/pepsin exposure suggesting acid exposure can induce cell surface glycosylation changes (122). In 2008, Neumann and colleagues used 4 different lectins to identify pathologic mucosal changes (123). They observed 2 distinct lectin-binding patterns. One was associated with the GERD, whereas the other pattern was characteristic for Barrett's esophagus mucosa. Specifically, UEA (*Ulex europaeus*) lectin binding was upregulated in Barrett's esophagus tissue sections, which suggests possible increase in fucosylation during the disease progress (123). A recently published study has concluded that dysplasia can alter glycan expression and lectin binding to the tissue samples. Fluorescently labeled WGA (wheat germ agglutinin) lectin-binding intensity was found to be inversely related to the degree of dysplasia (124). Furthermore, the authors used fluorescent-capable endoscope *ex vivo* in the study and followed all the protocols in a manner that exactly mimics a clinical study *in vivo*. Followed by topical fluorescein-labeled WGA spray, the authors measured fluorescence in the tissue samples. Measurement of lectin fluorescence was a more sensitive approach to identify dysplastic lesions as compared with white light endoscopic technique. Their data show clinical use of such a lectin-based endoscopic technique if developed further (124). In a phase III biomarker clinical trial study, Bird-Liberman and colleagues combined 3 different abnormalities to predict EAC progression in patients with Barrett's esophagus. Along with using conventional LGD and DNA content abnormalities they used AOL (*Aspergillus oryzae)* lectin binding to the tissue samples, which detects presence of α1-6 fucose on the cell surface

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Table 4. Summary of literature describing miRNA expression changes in BE/EAC

Sample size	Upregulated in BE/EAC	Downregulated in BE/EAC	Ref.
71 (BE-12, Barrett's esophagus without dysplasia-20, LGD-27, EAC/HGD-12)	miR-192 ($P < 0.00001$), miR-196a ($P < 0.05$): upregulated in Barrett's esophagus as compared with healthy tissue.miR-196a expression is correlated with progression from IM-LGD-HGD-EAC ($P < 0.005$).	miR203 ($P < 0.00001$): downregulation in Barrett's esophagus as compared with healthy tissue.	(209)
22 (Barrett's esophagus without dysplasia-11, Barrett's esophagus with dysplasia-11)	miR-15b (3.3-fold; $P < 0.05$), miR-203 (5.7-fold; $P < 0.05$): upregulated in dysplasia as compared with nondysplastic Barrett's esophagus.	miR-486-5p (4.8-fold; $P < 0.05$), miR-let-7a (3.3-fold; $P < 0.05$): downregulated in dysplasia as compared with nondysplastic Barrett's esophagus.	(110)
100 (EAC-100, adjacent normal tissue as control)	miR-21 (~3-fold; $P < 0.05$), miR-223 (~2-fold; $P < 0.05$), miR-192 (~3.5-fold; $P < 0.05$), and miR-194 (~3.5-fold; $P < 0.05$): upregulated in EAC as compared with adjacent normal tissue.	miR-203 (~3-fold; $P < 0.05$): downregulated in EAC as compared with adjacent normal tissue.	(111)
25 (Healthy-9, BE-5, HGD-1, EAC-10)	miR-192 (1.7-fold; FDR < 1 e−07), miR-194 (2-fold; FDR < 1e−07), miR-21 (3.7-fold; FDR = 0.0003), miR-200c (1.9-fold; FDR = 0.0015), miR-93 (1.3-fold; FDR = 0.0108): upregulated in EAC as compared with Barrett's esophagus.	miR-27b (1.43-fold; FDR = 0.0003), miR-342 (1.25-fold; FDR = 0.0015), miR-125b (2-fold; FDR = 0.0108), miR-100 (1.25-fold; FDR = 0.011): downregulated in EAC as compared with Barrett's esophagus.	(104)
75 (Healthy-15, BE-15, LGD-15, HGD-15, EAC-15)	miR-215 (62.8-fold; $P <$ 1e−07), miR-192 (6.34-fold; $P <$ 1e−07): upregulated in Barrett's esophagus in comparison with normal tissue and remained at similar levels with disease progress.	miR-205 (10-fold; $P =$ 1.39e−0.5), let-7c (2.04-fold; $P =$ 3.11e−05), miR-203 (6.67-fold; $P =$ 3.2e−0.5): downregulated in Barrett's esophagus in comparison with normal tissue and remained at similar levels as disease progresses.	(114)
91 (LGD-31, HGD-29, EAC-31, In all cases adjacent normal tissue used as a control)	miR-200a (13.5-fold; $P = 0.02$), miR-513 (1.58-fold; $P = 0.03$), miR-125b (9.2-fold; $P = 0.04$), miR-101 (1.83-fold; $P = 0.04$), miR-197 (1.61-fold; $P = 0.04$): upregulated in LGD to HGD transition.	miR-23b (1.45-fold; $P = 0.007$), miR-20b (1.56-fold; $P = 0.01$), miR-181b (2.22-fold; $P = 0.03$), miR-203 (1.49-fold; $P = 0.03$), miR-193b (2.70-fold; $P = 0.04$), miR-636 (4.17-fold; $P = 0.04$): downregulated in LGD to HGD transition. let-7a (1.75-fold; $P = 0.01$), let-7b (1.59-fold; $P = 0.009$), let-7c (1.69-fold; $P = 0.03$), let-7f (1.69-fold; $P = 0.03$), miR-345 (2-fold; $P = 0.02$), miR-494 (1.72-fold; $P = 0.03$), miR-193a (2.27-fold; $P = 0.05$): downregulated in HGD-EAC development process.	(112)
48 (BE-19, EAC-29)	miR-21 (~2.8-fold; $P < 0.05$), miR-143 (~11.3-fold; $P < 0.05$), miR-145 (~3.4-fold; $P < 0.05$), miR-194 (~126-fold; $P < 0.05$), miR-215 (~18-fold; $P < 0.05$): upregulated in Barrett's esophagus as compared with adjacent normal tissue.	miR-203 (~17-fold; $P < 0.05$), miR-205 (~175-fold; $P < 0.05$): downregulated in Barrett's esophagus as compared with adjacent normal tissue.miR-143 (~3-fold; $P < 0.05$), miR-145 (~1.8-fold; $P < 0.05$), miR-215 (~3.1-fold; $P < 0.05$): Lower expression in EAC as compared with Barrett's esophagus.	(109)
49 (IM-15, HGD-14, and EAC-20, adjacent normal tissue)	—	miR-31 (>4-fold; $P < 0.02$), miR-375 (>4-fold; $P < 0.001$): downregulated in transition from Barrett's esophagus to EAC.	(210)
37 (BE-17, EAC-20, 9 adjacent normal tissue samples)	—	miR-141 (~2-fold; $P = 0.0126$), miR-200a (~2.5-fold; $P = 0.0001$), miR-200b (~2.1-fold; $P < 0.0001$), miR-200c (~1.9-fold; $P = 0.0014$), miR-429 (~1.8-fold; $P = 0.0031$): underexpressed in EAC as compared with Barrett's esophagus.	(105)

(Continued on the following page)

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Table 4. Summary of literature describing miRNA expression changes in BE/EAC (Cont'd)

Sample size	Upregulated in BE/EAC	Downregulated in BE/EAC	Ref.
11 (EAC-11, different lesions were collected from these patients and classified into Barrett's esophagus, LGD, HGD, and EAC)	miR-196a is overexpressed in early EAC (151-fold) > HGD (62.2-fold; $P = 0.00002$) > LGD (31.1-fold; $P = 0.0005$) > Barrett's esophagus (28.9-fold; $P = 0.00001$). Fold changes are calculated as compared with normal epithelium.	—	(107)
45 (patients with EAC undergoing surgery)	miR-143 ($P = 0.0148$), miR-199a_3p ($P = 0.0009$), miR-199a_5p ($P = 0.0129$), miR-100 ($P = 0.0022$) and miR-145 ($P = 0.1176$) expression predicted a worse survival followed by esophagectomy. Overexpression of miR-199a_3p/_5p and miR-99b was associated with lymphnode metastasis.	Downregulation of miR-143 ($P = 0.0049$) and miR-145 ($P = 0.0069$) in EAC as compared with adjacent normal tissue.	(211)
24 (BE-24, progression to EAC-7, not progressed to EAC-17 in at least 5-y follow-up)	miR-192 (ROC AUC = 0.61), 194 (ROC AUC = 0.70), 196a (ROC AUC = 0.80), and 196b (ROC AUC = 0.74) showed significantly higher expression in Barrett's esophagus samples from patients who progressed to EAC as compared with those who did not progress to EAC.	—	(212)
5 (patients with EAC undergoing surgery. Adjacent benign tissue as a control)	miR-296 is overexpressed ~2-fold in EAC as compared with adjacent benign tissue.	—	(213)
22 patients with locally advanced EAC tumor undergoing surgery	Negative association between miR-148a expression and tumor differentiation ($P < 0.001$). Significantly higher expression of miR-148a in tumors located in the lower esophagus as compared with tumors in the middle esophagus ($P = 0.021$).	—	(214)
99 EAC patient tissue samples undergoing surgery	miR-30e ($P = 0.002$) and miR-200a ($P = 0.044$) expression were associated with poor overall survival. miR-16-2 ($P = 0.027$) and miR-30e ($P = 0.002$) expression were associated with poor disease-free survival.	—	(215)

NOTE: Wherever needed, fold-change values are calculated/adapted from the expression/fold-change values described in the original article to have uniform format for the purpose of this review.

(125). Thus, monitoring tissue glycan changes can be combined with existing biomarkers to improve the predictive power of the currently used biomarkers.

A potential mechanism responsible for these changes is considered to be bile acid exposure-induced gene expression and secretory pathway changes in esophageal epithelium. Using carbohydrate-specific lectins that detect N- and O-linked glycosylation and core fucosylation, Byrne and colleagues have shown differential lectin binding to the cell surface and differential intracellular localization when normal squamous and Barrett's metaplastic cell lines were treated with deoxycholic acid (126). Nancarrow and colleagues profiled whole-genome expression in normal squamous esophageal epithelium, Barrett's esophagus, and EAC and concluded that Barrett's esophagus is a tissue with enhanced glycoprotein synthesis machinery to provide strong mucosal defense against acid exposure (127).

Outlook—Circulating Biomarkers

Last 3 decades showed continuously increased EAC incidences and similar trend is expected in future because of rising incidences of obesity and GERD in the population. Current endoscopic screening program might benefit the highest risk population to monitor disease progression. Monitoring dysplasia in the tissue samples has not provided fruitful outcome for early diagnosis; however, inclusion of the genomic and cell-cycle biomarkers has shown definite improvement in the predictive power over currently used histologic technique. Any biomarker

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requiring tissue samples is going to be difficult to implement for population screening and will not be economically viable. An alternative to tissue-based techniques is to investigate changes in circulating biomarkers. Blood is relatively easy to access and can be monitored frequently, ultimately increasing the possibility of detecting early dysplastic changes.

Circulating tumor cells could be one source of biomarkers. Although readily found in the blood, technological advancements are required for sensitive early detection of the low number of tumor cells present in the circulation (128, 129). Alternative to the detection of circulating tumor cells, Zhai and colleagues applied genome-wide DNA methylation profiling approach to cell-free circulating DNA. They found that cell-free circulating DNA methylation profile is a replica of methylation profile found in matched tumor tissue samples and can discriminate between healthy, Barrett's esophagus, and EAC conditions (130). Kawakami and colleagues (131) studied methylation of *APC* gene in matched tumor samples and plasma. Unlike tumor samples that showed hypermethylation of *APC* DNA early during the EAC development, matched plasma samples from patients suffering from Barrett's esophagus and gastritis were found to be negative for *APC* methylation changes. Moreover, as compared with 92% (48 of 52) of EAC tissue samples, only 25% (13 of 52) of plasma samples were positive for circulatory *APC* methylation changes. However, there was a strong correlation between stage of the tumor and plasma positivity for methylated *APC* (131). In combination with *DAPK* methylation, measurement of preoperative *APC* methylation in peripheral blood was able to discriminate between long (>2.5 years) and short survivors with a sensitivity of 99.9% and specificity of 57.1% (132). Taken together, tracking circulatory DNA methylation changes during EAC development may be an alternative approach to predict early EAC.

Tumor cell moulds the microenvironment to support oncogenesis by releasing soluble and vesicular components, including enzymes, microvesicles, proteoglycans, chemokines, and cytokines (133). The tumor microenvironment components are shed into the circulation and may be extremely useful as an early diagnostic biomarker. This concept was recently showed by Pitteri and colleagues using an inducible HER2/neu mouse model (134). They showed that plasma proteome profiling has the ability to detect cancer before it actually develops. Furthermore, a linear correlation was shown for plasma levels of candidate biomarker proteins with the tumor progression, which were reversed upon tumor regression (134).

Both encapsulated miRNAs and secreted glycoproteins are prime candidates for circulating biomarkers released by the tumor microenvironment. Circulating miRNA is secreted in nanometer-sized vesicles called exosomes or microvesicles. An advantage of circulating miRNA over protein biomarkers is the ability for amplication, increasing the sensitivity of detection. Comparative analysis of circulating miRNA can be conducted using miRNA microarray and quantitative real-time PCR (116). Future studies should aim to discover and validate circulating miRNA changes associated with EAC development and progression.

Glycan Profiling

For Barrett's esophagus and EAC, serum glycan profiling using mass spectrometry has identified differential expression of glycan structures in different disease states. Mechref and colleagues analyzed N-linked glycan diversity present in 84 patient serum samples (Healthy-18, BE-5, HGD-11, and EAC-50; ref. 135). They identified 98 glycan features with different intensities in disease onsets and 26 of them correspond to known glycan structures. They showed statistically significant glycan changes between 4 different conditions (Healthy/BE/HGD/EAC) with 3 of the known potential N-glycan biomarkers predicting EAC with 94% sensitivity and 60% specificity (135). Another study used microchip electrophoresis with laser-induced fluorescence detection for N-glycan profiling and were able to differentiate between the healthy, Barrett's esophagus, HGD, and EAC conditions (136). Similar to abovementioned N-glycan profiling studies, very recently, Gaye and colleagues showed that ion mobility-mass spectrometric analysis of serum N-glycan can also distinguish between normal and EAC phenotype (137). All of these studies unanimously suggest circulatory N-linked glycan changes during EAC pathogenesis. Mann and colleagues enriched fucosylated serum glycoproteins using lectins and then used shot gun proteomics to identify protein in different physiologic states, including healthy samples, Barrett's esophagus, and EAC (138). Although the study showed promising trends, the statistical power was not achieved because of the very low number of samples. To improve the throughput of glycoproteomics studies, we developed lectin magnetic bead array-mass spectrometry (LeMBA-MS), a high-throughput platform where a panel of lectins individually immobilized on magnetic beads is used to capture glycoproteins followed by on-bead trypsin digest and liquid chromatography–tandem mass spectrometry for protein identification (139, 140). Parallel screening of a panel of lectins may be helpful to identify differentially glycosylated circulating proteins during EAC pathogenesis.

Metabolic Profiling

In recent past, efforts have been made to profile metabolic changes associated with EAC pathogenesis. Metabolic profiling studies have identified changes associated with nucleoside metabolism, tricarboxylic acid cycle, fatty acid, and amino acid metabolism during EAC development in tissue samples and more importantly using easily accessible biofluids, blood and urine. Early metabolic changes in the histologically normal epithelium were observed, particularly for phosphocholine, glutamate, myo-inositol, adenosine-containing compounds, uridine-containing compounds, and inosine (141). Djukovic and colleagues used targeted approach to profile 8

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different serum nucleosides between healthy subjects ($n = 12$) and patients with EAC ($n = 14$) using high-performance liquid chromatography coupled with triple quadrupole mass spectrometer. Among 8 nucleosides they profiled, 5 were significantly different between the 2 groups. Three of 5 significantly different nucleosides, 1-methyladenosine, N^2,N^2-dimethylguanosine, and N^2-methylguanosine, were methylated nucleosides indicating increased tRNA methylation, similar to DNA hypermethylation in EAC condition (142). Zhang and colleagues studied serum metabolomic changes using nuclear magnetic resonance (NMR) alone and NMR in combination with liquid chromatography/mass spectrometry (LC/MS) in EAC ($n = 67$), HGD ($n = 9$), Barrett's esophagus ($n = 3$), and healthy volunteers ($n = 34$). Their model based on Partial Least Square Discrimination Analysis was able to distinguish between different phenotypes by achieving area under receiver operating characteristics curve (AUROC) as high as 0.95. On the basis of candidate metabolites, they identified altered pathways associated with EAC development to be energy metabolism, fatty acid metabolism, and amino acid metabolism (143, 144). Urine metabolomics could also distinguish between healthy, Barrett's esophagus, and EAC phenotypes. Davis and colleagues generated urine metabolic signatures, which were able to discriminate between healthy, Barrett's esophagus, and EAC phenotypes, as well as distinguish EAC from pancreatic cancer (145). These metabolic profiling studies open up new avenues to detect early EAC using circulatory biomarkers.

Improved biologic understanding, in combination with technical advancements in the field of genomics, proteomics, glycomics, and metabolomics, has played key roles in the identification and validation of circulatory biomarkers for EAC. Development of an assay platform, which can be clinically used for these circulatory biomarkers, will help to conduct the large scale multicentered trials and transform the circulatory biomarkers into clinical use.

Summary and Future Perspectives

Clinical advancements in endoscopy and new tissue sampling techniques such as brush cytology can improve the endoscopic-biopsy management of BE/EAC in near future. Genomic abnormalities and cell-cycle biomarkers have already shown their potential use to diagnose early pathologic changes using tissue samples. However, wider clinical application will depend on the technical ability of individual clinical pathology laboratories. As these changes are detected in the tissue samples, it would be difficult to implement them in large-scale high-risk population screening to identify early neoplastic changes. Recent advancements in RNA sequencing, circulatory DNA methylation profiling, metabolic profiling, and glycoproteomics may provide ways for the development of noninvasive *in vitro* diagnostic biomarker for routine monitoring and identification of patients with nonsymptomatic BE/EAC. Future studies should focus to combine different classes of circulatory biomarkers in large-scale trials to improve the predictive power of the individual marker. Development of novel cost-effective assay platforms that can transform discoveries from research laboratories to the clinics require equal emphasis for the widespread benefit from the circulatory biomarkers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.K. Shah, N.A. Saunders, M.M. Hill
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. Shah
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. Shah
Writing, review, and/or revision of the manuscript: A.K. Shah, N.A. Saunders, A. Barbour, M.M. Hill

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