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Review Article

Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma

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Abstract: Barrett's esophagus, a pre-malignant condition that can lead to esophageal adenocarcinoma, is characterized by histological changes in the normal squamous epithelium of the esophagus. Numerous molecular changes occur during the multistage conversion of Barrett's metaplasia to dysplasia and frank adenocarcinoma. Epigenetic changes, especially changes in DNA methylation are widespread during this process. Aberrant DNA methylation has been shown to occur at promoters of tumor suppressor genes, adhesion molecules and DNA repair genes during Barrett's esophagus. These epigenetic alterations can be used as molecular biomarkers for risk stratification and early detection of esophageal adenocarcinoma. We also show that genome wide analysis of methylation surprisingly reveals that global hypomethylation and not hypermethylation is the dominant change during Barrett's metaplasia. The transformation of Barrett's esophagus to frank adenocarcinoma is in turn characterized by much smaller wave of selective promoter hypermethylation. These studies reveal many novel, potential targets for new therapies and illustrate the utility of incorporating these epigenetic changes as biomarkers during endoscopic surveillance interval for patients with Barrett's esophagus.

Keywords: Barrett's esophagus, DNA methylation, esophageal adenocarcinoma, global hypomethylation

Introduction

Epigenetics is defined as the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence [1]. DNA methylation, histone modifications and short non coding micro RNAs are the three major ways by which epigenetic changes are regulated. Recent evidence shows that alterations in these epigenetic regulators can disrupt normal gene expression homeostasis and can lead to the development of cancer [2].

Barrett's esophagus is a premalignant condition in which the normal squamous epithelium that normally lines the distal esophagus is replaced by a specialized columnar epithelium in response to chronic inflammation secondary to prolonged acid reflux exposure [3].

The role of epigenetic alterations in the development of Barrett's esophagus and its progression to dysplasia-neoplasia stages has been the focus of attention by many researchers during the past several years. DNA methylation has been the widely studied epigenetic alteration during esophageal carcinogenesis and review of literature reveals numerous genes that are affected by aberrant DNA methylation during this process. A detailed review of these data and their impact on diagnosis, prognosis and treatment of Barrett's esophagus is presented.

DNA methylation is a stable epigenetic change that regulates gene expression

The inheritance of information based on gene expression levels is known as epigenetics, as opposed to genetics, which refers to information transmitted on the basis of gene sequence [4]. Therefore, epigenetic events are those heritable changes in gene expression that do not involve any changes in DNA sequence [5].

Epigenetics can be divided into three major

categories: modifications of the DNA itself, changes in its packaging (histone modifications) and by short non coding micro RNAs [5, 6].

Among epigenetic events, DNA methylation is the most well studied modification in eukaryotic genomes, being so far the only one that directly targets DNA and therefore, known to have a profound effect on gene expression [7, 8]. Under normal circumstances, it is involved in the regulation of imprinted gene expression and X-chromosome inactivation during normal development. However, aberrant DNA methylation has shown to play an important role in carcinogenesis.

DNA methylation occurs almost exclusively on a cytosine in a CpG dinucleotide, a process that is achieved by enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionine to the 5 position of a cytosine ring [5, 9, 10].

Through evolution, most CpG sites have been lost from mammalian genomes and only around 1% of human DNA consists of short areas where CpG sites have escaped deamination and depletion. These CpG sites are asymmetrically distributed into CpG poor regions and dense regions throughout the genome. Areas of GpG dense regions are known as CpG islands and are frequently located in regulatory regions of genes called promoters [4, 5, 9, 10].

Two mechanisms have been proposed to account for transcriptional repression via DNA methylation. First, it has been shown that methylated DNA attracts DNA binding proteins that form a spatial obstacle to restrict binding of transcription factors (TF) to promoter regions. Secondly they recruit repressor complexes containing histone deacetylases resulting in inactive chromatin [7, 11, 12]. Aberrant methylation can consequently lead to the silencing of important genes thus triggering neoplastic transformation.

Barrett's esophagus (BE) is a premalignant condition that leads to esophageal adenocarcinoma

The distal esophagus is normally lined by squamous epithelium. When there is prolonged exposure to acid reflux, a columnar-lined esophagus with intestinal metaplasia accompanied by goblet cells develops. This condition is known as Barrett's esophagus and represents the most important risk factor for developing esophageal adenocarcinoma [13-29].

Three subtypes of Barrett's esophagus have been described: the most commonly found is specialized type epithelium that resembles intestinal mucosa, with villi and crypts. It is characterized by the presence of goblet and columnar cells. The other two are the fundic type, which has mucus-secreting cells and few parietal or chief cells and the junctional type or cardiac mucosa which is histologically indistinguishable from normal gastric cardiac mucosa. Among these, it is the specialized intestinal type which has clearly been associated with malignant transformation [17, 30].

The abnormal columnar epithelium that characterizes Barrett's esophagus is an incomplete form of intestinal metaplasia and represents the first step in the transformation to esophageal adenocarcinoma (EAC) [19, 21]. EAC is thought to develop through a multistage process in which Barrett's metaplasia progresses through low-grade and high-grade dysplasia (also known as intraepithelial neoplasia) to invasive cancer [15, 21]. The molecular mechanism of this carcinogenic sequence has not been fully understood. It is believed that long term inflammation due to gastro-esophageal reflux may cause genetic and epigenetic alterations and that accumulation of these would lead to the acquisition of malignant characteristics in the Barrett's cells [3].

Although many risks factors have been shown to predispose to Barrett's metaplasia such as obesity, male gender, white race and to a lesser extent, tobacco exposure, alcohol, and nitrosamine intake, there is compelling evidence that gastroesophageal reflux disease (GERD) is the most important risk factor for this transformation [32]. Interestingly, only approximately 5-10 percent of patients with reflux develop metaplasia, demonstrating the influence of host genetic factors for the acquisition of Barrett's esophagus [19, 32, 33]. At the same time, not all patients with intestinal metaplasia develop cancer, being the risk of approximately 0.4-0.5 percent per year [26, 33]. Thus a study of molecular events that result in neoplastic transformation will potentially uncover the patients that have a higher chance of transforming to cancer.

Esophageal adenocarcinoma is rapidly rising in the US and needs newer biomarkers for surveillance and prognostic risk assessment

The risk of esophageal adenocarcinoma (EAC) in patients with Barrett's esophagus appears to be approximately 30 to 125- fold greater than in the general population with an estimated incidence of 1 in 180-patient years [3, 17]. Because of this increased risk, patients with BE usually undergo endoscopic surveillance at regular intervals. However, because the incidence of EAC in Barrett's esophagus is uncommon, most of these surveillance endoscopies in BE patients do not detect cancer. Therefore, surveillance would benefit from effective markers to stratify patient according to their level of cancer progression risk [3]. So far, dysplasia is the currently accepted marker for cancer risk, with high grade dysplasia being considered more predictive than low-grade dysplasia. Whereas low-grade dysplasia (LGD) has no histologic abnormalities specific for neoplasia given its similarity to those changes occurring in normal tissue in response to injury, by the time high grade dysplasia (HGD) is evident, approximately one third of patients will have already invasive cancer.

In contrast to HGD, the predictive value of LGD for cancer risk assessment is controversial. Therefore, more accurate tissue-based biomarkers capable of predicting the risk of progression to EAC would be of significant use [3]. Moreover, serum biomarkers such as freely circulating plasma DNA abnormalities could be of potential use in predicting level of aggressiveness and behavior of this type of malignancy, as demonstrated in the study performed by Kawakami *et al.*, where high plasma levels of hypermethylated APC DNA were correlated with reduced survival in patients with EAC [34]. At the same time, the study found higher quantity of methylated DNA in esophageal adenocarcinoma than in squamous cell carcinoma, suggesting that quantitative differences in degree of methylation exist between different tumor types.

Epigenetic alterations are seen in Barrett's esophagus

The role of epigenetic alterations in pathogenesis of Barrett's esophagus has been studied thoroughly by many researchers over the past couple of decades. Inactivation of tumor suppression genes (TSGs) by promoter hypermethylation has been considered as a potentially important mechanism involved in the development of Barrett's [15, 27]. Also, methylation of multiple genes in EAC has been found to be an independent and strong predictor for disease-specific recurrence and survival of patients. Furthermore, multivariate analyses have confirmed that epigenetic tumor profiling by methylation status was a more powerful biomarker of risk prediction in esophageal adenocarcinoma than the classic clinico-pathological features of stage and age [31].

Most studies have studied changes in DNA methylation at single loci and have shown that hypermethylation of selected loci is an early epigenetic alteration in the multistep progression of EAC [16, 33]. Furthermore, given that not all patients with Barrett's esophagus develop EAC, it has been proposed that unique epigenetic alterations may serve as markers for the distinction between progressive and non-progressive BE [3, 28, 29]. This was illustrated a study where APC, TIMP3 and TERT promoters were found to be hypermethylated in 100%, 91% and 92% of cases of progressors; whereas in BE mucosa from patients who had not progressed to adenocarcinoma, methylation was found only in 36%, 23% and 17% of these gene promoters respectively. These early findings are promising for the development of new methods which could identify these molecular alterations at the earliest stages of the disease and therefore prevent the progression of this condition to its most lethal form, adenocarcinoma, which is known for having an extremely poor prognosis, with an overall 5-year survival of approximately 3-25 percent [35, 36]

Overall, these single locus studies have shown that there is a general increase in the frequency and in the quantitative level of CpG island hypermethylation at progressively advanced stages of the disease. However, the propensity for aberrant methylation of the genes is not uniform since they differ both in their frequency and in their levels of hypermethylation among the various tissues. Eads *et al.* screened 104 tissue samples from 51 patients with different stages of Barrett's esophagus and or associated adenocarcinoma. It was found that 20 genes segregated into classes of similar epigenetic behavior, with each class undergoing unique epigenetic changes at different steps of disease

progression of EAC suggesting a step-wise loss of multiple protective barriers against CpG island hypermethylation. In this study, the percentage of fully methylated molecules at a specific locus (PMR) was calculated. Samples were divided as follows: less than 4 PMR, 4-20 PMR, 21-50 PMR and > 50 PMR. Classes A, B and C are methylated at higher frequency in intestinal metaplasia tissue than in normal esophageal mucosa. In the same way, the transition from IM to dysplasia or malignancy was associated with an increase in class a methylation. It was noticed that there is clearly a tissue-specific and tumor-specific propensity for particular genes to become hypermethylated; as an example, the APC gene was hypermethylated in normal stomach but not in the normal esophageal mucosa [15], whereas its promoter hypermethylation occurs in 83 to 92 percent of HGD and adenocarcinomas in Barrett's esophagus and in 40 to 50 percent of patients with metaplasia without dysplasia [20].

Interestingly, it has been seen that in some tumors such as colorectal carcinoma, aberrant CpG island methylation consistently involves certain group of genes [16, 28, 37]. From this finding, the concept of "CpG island methylation phenotype" (CIMP) was developed. CIMP tumors are a distinct group of tumors that are defined by a high degree of concordant CpG island hypermethylation of genes exclusively methylated in cancer (named as type C genes). Studies of these genes in EAC have not shown clear evidence of a separate group of CIMP tumors in esophageal tumors [16].

Overall, several genes have been shown to be epigenetically altered from early stages of Barrett's esophagus and through its progression to the more advance stages of dysplasia and adenocarcinoma. We have tabulated these changes (**Table 1**) and further discuss the genes that are most frequently involved by epigenetic alterations in BE and EAC.

Various genes frequently hypermethylated in Barrett's esophagus

AKAP12

The A-Kinase anchoring protein 12 (AKAP12) is a multivalent anchoring protein and an important regulator of the beta2-adrenergic receptor complex [39]. The AKAP12 gene maps to chromosome 6q24-25.2 [38], a locus that is frequently deleted in human cancers. It also controls cell signaling, cell adhesion, mitogenesis and differentiation and possesses tumor suppressor activity [39]. In the study by Jin *et al.* using MSP in 259 esophageal tissues, AKAP12 hypermethylation frequency was not seen in normal esophagus but increased to 38.9% in Barrett's mucosa derived from patients with Barrett's alone, 48.3 % in all Barrett's tissue derived from Barrett's and EAC, 52.5% in dysplastic tissue, and 52.2% in EAC. In contrast only 2 (7.7%) of 26 esophageal squamous cell carcinoma samples exhibited AKAP12 methylation. AKAP12 methylation also correlated with decreased mRNA levels. Treatment of BIC and OE33 EAC cells with 5-aza-2'-deoxycytidine reduced AKAP12 methylation and increased AKAP12 mRNA expression The above study also found a significant correlation between AKAP12 hypermethylation and BE segment length [39].

APC

The adenomatous polyposis coli (APC) gene maps to 5q21 [40], is a tumor suppressor gene and is involved in cell adhesion through its interaction with beta catenin-cadherin proteins. Furthermore, the APC protein binds to the ends of microtubules that form the spindle apparatus during chromosome segregation, and so APC defects may mediate chromosomal instability during metaphase [19].

APC methylation has been examined in multiple studies [16, 26, 28, 31, 41, 42]. The rate of methylation of APC promoter has varied from 0 to 25% in normal tissue. Higher levels of promoter methylation have been found in normal appearing mucosa derived from EAC specimens [41]. APC methylation in metaplastic tissue ranges from 50 to 95% and goes up to 61 to 100% in dysplastic tissues. Wang *et al.* [42] also demonstrated in a nested case control study that APC and p16 hypermethylation is predictive of progression to cancer. In fact, another study Brock *et al.* [31] demonstrated that hypermethylation of APC promoter was a more powerful predictor of survival and tumor recurrence than age or stage.

CDH13

CDH13 (also known as H-cadherin and T-cadherin), is a member of the cadherin gene

Table 1. Comprehensive table of genes aberrantly methylated in BE and EAC

Genes	Location	Method*	Normal			Metaplastic			Dysplastic			Carcinoma			Source
			N	Meth	%	N	Meth	%	N	Meth	%	N	Meth	%	
3OST2/HS3ST2	16p12	MSP	57	1	1.8	60	47	78.3	nd	nd	nd	73	28	38.4	[26]
AKAP12	6q24-25	MSP	66	0	0.0	60	29	48.3	40	21	52.5	67	35	52.2	[39]
APC	5q21-q22	MS-SSCA and MS-DBA	16	0	0.0	25	16	64.0	nd	nd	nd	27	25	92.6	[28]
APC	5q21-q22	MSP	17	0	0.0	48	24	50.0	46	28	60.9	32	20	62.5	[42]
APC	5q21-q22	MSP	64	9	14.1	92	75	81.5	nd	nd	nd	77	54	70.1	[26]
APC	5q21-q22	MSP	41	3	7.3	nd	nd	nd	nd	nd	nd	41	28	68.3	[31]
APC	5q21-q22	Mc-MSP	56	14	25.0	40	38	95.0	7	7	100.0	37	35	94.6	[41]
APC	5q21-q22	MethyLight	31	3	9.7	10	8	80.0	9	8	88.9	22	15	68.2	[16]
CALCA	11p15.2	MethyLight	31	4	12.9	10	4	40.0	9	5	55.6	22	11	50.0	[16]
CDH13	16q24	MSP	66	0	0.0	60	42	70.0	40	31	77.5	67	51	76.1	[44]
CRBP1/RBP1	3q23	MSP	64	1	1.6	93	31	33.3	nd	nd	nd	77	30	39.0	[26]
DAPK1	9q34.1	MSP	20	4	20.0	28	14	50.0	21	11	52.4	35	21	60.0	[33]
DAPK1	9q34.1	MSP	41	2	4.9	nd	nd	nd	nd	nd	nd	41	8	19.5	[31]
ESR1	6q25.1	MSP	41	5	12.2	nd	nd	nd	nd	nd	nd	41	21	51.2	[31]
CHD1 / E-Cadherin	16q22.1	MSP	41	5	12.2	nd	nd	nd	nd	nd	nd	41	27	65.9	[31]
ESR 1	6q25.1	MethyLight	31	0	0.0	10	4	40.0	9	8	88.9	22	19	86.4	[16]
GPX3	5q23	MSP	12	2	16.7	21	13	61.9	11	9	81.8	34	30	88.2	[25]
GPX3	5q23	Quantitative bisulphite pyrosequencing	37	3	8.1	10	9	90.0	8	7	87.5	100	62	62.0	[46]
GPX7	1p32	Quantitative bisulphite pyrosequencing	37	0	0.0	11	2	18.2	10	8	80.0	100	67	67.0	[46]
GSTM2	1p13.3	Quantitative bisulphite pyrosequencing	37	2	5.4	10	5	50.0	9	5	55.6	97	67	69.1	[46]
GSTM3	1p13.3	Quantitative bisulphite pyrosequencing	36	0	0.0	8	1	12.5	8	3	37.5	100	15	15.0	[46]
HPP1/TMEF2	2q32.3	MSP	64	2	3.1	93	41	44.1	20	15	75.0	77	55	71.4	[26]
ID4	6p22.3	Mc-MSP	56	12	21.4	40	31	77.5	7	6	85.7	37	28	75.7	[41]
MGMT	10q26	MSP	28	6	21.4	27	24	88.9	13	13	100.0	47	37	78.7	[48]
MGMT	10q26	MethyLight	10	2	20.0	13	8	61.5	nd	nd	nd	132	84	63.6	[47]
MGMT	10q26	MSP	63	11	17.5	60	15	25.0	nd	nd	nd	77	18	23.4	[26]
MGMT	10q26	MethyLight	31	17	54.8	10	6	60.0	9	8	88.9	22	16	72.7	[16]
MGMT	10q26	Mc-MSP	56	1	1.8	40	19	47.5	7	5	71.4	37	21	56.8	[41]
MGMT	10q26	MSP	41	10	24.4	nd	nd	nd	nd	nd	nd	41	24	58.5	[31]
MYOD1	11p15.4	MethyLight	31	0	0.0	10	4	40.0	9	6	66.7	22	10	45.5	[16]
NELL1	11p15.1	MSP	66	0	0.0	60	28	46.7	40	21	52.5	67	32	47.8	[36]

p16/CDKN2A	9p21	MSP	17	0	0.0	47	14	29.8	45	19	42.2	41	22	53.7	[42]
p16/CDKN2A	9p21	MSP	41	10	24.4	nd	nd	nd	nd	nd	nd	41	16	39.0	[31]
p16/CDKN2A	9p21	MSP	64	2	3.1	93	14	15.1	20	11	55.0	76	34	44.7	[26]
p16/CDKN2A	9p21	MS-SSCA and MS-DBA	16	0	0.0	27	2	7.4	nd	nd	nd	27	13	48.1	[28]
p16/CDKN2A	9p21	MethyLight	31	0	0.0	10	2	20.0	9	2	22.2	22	9	40.9	[16]
p16/CDKN2A	9p21	Mc-MSP	56	5	8.9	40	11	27.5	7	3	42.9	37	22	59.5	[41]
CRBP1/RBP1	3q23	Mc-MSP	56	10	17.9	40	23	57.5	7	4	57.1	37	26	70.3	[41]
RPRM reprimo	2q23	MSP	19	0	0.0	25	9	36.0	11	7	63.6	75	47	62.7	[35]
RIZ1/PRDM2	1p36.21	MSP	63	2	3.2	69	41	59.4	nd	nd	nd	77	45	58.4	[26]
RUNX3	1p36	Mc-MSP	56	3	5.4	40	19	47.5	7	4	57.1	37	27	73.0	[41]
RUNX3	1p36	MSP	63	1	1.6	93	23	24.7	20	12	60.0	77	37	48.1	[26]
SFRP1	8p11.21	Mc-MSP	56	7	12.5	40	37	92.5	7	7	100.0	37	35	94.6	[41]
SFRP1	8p11.21	MS-SSCA and MS-DBA	12	2	16.7	21	21	100.0	nd	nd	nd	23	21	91.3	[28]
SFRP1	8p11.21	MSP	58	10	17.2	37	30	81.1	nd	nd	nd	40	37	92.5	[27]
SFRP2	4q31.3	MSP	58	38	65.5	37	33	89.2	nd	nd	nd	40	33	82.5	[27]
SFRP4	7p14.1	MSP	58	9	15.5	37	29	78.4	nd	nd	nd	40	29	72.5.	[27]
SFRP5	10q24.1	MSP	58	10	17.2	37	27	73.0	nd	nd	nd	40	34	85	[27]
SOCS1	16p13.13	MSP	20	0	0.0	30	nd	nd	56	7	12.5	19	8	42.1	[58]
SOCS3	17q25.3	MSP	20	0	0.0	30	4	13.3	56	26	46.4	19	14	73.7	[58]
SST	3q28	MSP	67	6	9.0	60	44	73.3	40	27	67.5	67	48	71.6	[60]
TAC1	7q21-22	MSP	67	5	7.5	60	38	63.3	40	23	57.5	67	41	61.2	[63]
TERT	5p15.33	MS-SSCA and MS-DBA	16	0	0.0	24	13	54.2	nd	nd	nd	25	16	64.0	[28]
TIMP3	22q12.1-q13.2; 22q12.33	MethyLight	31	6	19.4	10	6	60.0	9	7	77.8	22	19	86.4	[16]
TIMP3	22q12.1-q13.2; 22q12.33	MSP	64	3	4.7	93	55	59.1	nd	nd	nd	77	43	55.8	[26]
TIMP3	22q12.1-q13.2; 22q12.33	MS-SSCA and MS-DBA	16	0	0.0	24	13	54.2	nd	nd	nd	26	17	65.4	[28]
TIMP3	22q12.1-q13.2; 22q12.33	Mc-MSP	56	1	1.8	40	35	87.5	7	5	71.4	35	26	74.3	[41]
TIMP3	22q12.1-q13.2; 22q12.33	MSP	41	0	0.0	nd	nd	nd	nd	nd	nd	41	8	19.5	[31]
HPP1/TMEFF2	2q32.3	Mc-MSP	56	2	3.6	40	30	75.0	7	7	100.0	36	30	83.3	[41]
WIF-1	12q14.3	MS-SSCA	4	0	0.0	31	16	51.6	nd	nd	nd	12	10	83.3	[29]
WIF-1	12q14.3	MSP	17	4	23.5	nd	nd	nd	nd	nd	nd	18	11	61.1	[29]

* MSP = Methylation-specific PCR; Mc-MSP = Melt curve Methylation-specific PCR; MS-SSCA = Methylation sensitive single strand conformation analysis; MS-DBA = Methylation sensitive dot blot assay. Note: In the metaplastic, dysplastic and carcinoma columns, nd = tissues not studied for methylation.

superfamily and maps to 16q24 [43], a locus that frequently undergoes deletion in human cancers including esophageal carcinoma [44]. Hypermethylation of the CDH13 promoter was studied by Jin *et al.* [44] in 259 esophageal biopsy specimens by MSP. CDH13 hypermethylation frequency was 0% in normal esophagus but increased during neoplastic progression, rising to 70% in BE, 77.5% in dysplasia and 76.1% in EAC. In contrast, only 5 (19.2%) of 26 esophageal squamous cell carcinoma samples exhibited CDH13 hypermethylation. Notably, in the above study, CDH13 showed a strong relationship to BE segment length.

DAPK1

Death-associated protein kinase1 (DAPK1) maps to the chr9q34.1 locus [45]. DAPK1 encodes a structurally unique 160-kD calmodulin dependent serine-threonine kinase that is a positive mediator of gamma-interferon induced programmed cell death and therefore is a tumor suppressor [33]. In a study done by Kuester *et al.* [33] hypermethylation of the DAPK promoter was detected in 20% of normal mucosa, 50% of Barrett's metaplasia, 53% of dysplasia and 60% of adenocarcinomas and resulted in marked decrease in protein expression. In this study, the loss of this protein was significantly associated with advanced depth of tumor invasion and advanced tumor staging. The severity of the reflux esophagitis also correlated significantly with the DAPK promoter hypermethylation.

GPX and GST

Since one of the most clinical risk factors for development of Barrett's is reflux, 2 authors studied the effect on glutathione *S*-transferase superfamily (GST) and glutathione peroxidase (GPX) on the development of EAC [25, 46]. Normal cells can handle the oxidative stress through intact antioxidative systems. Glutathione *S*-transferase superfamily (GST) and glutathione peroxidase (GPX) are major cellular antioxidants. GSTs catalyze the conjugation of the tripeptide glutathione to a wide variety of exogenous and endogenous chemicals with electrophilic functional groups thereby neutralizing their electrophilic sites and rendering the products more water soluble. GPXs catalyze the reduction of H_2O_2, organic hydroperoxide and lipid peroxides by reduced glutathione, thereby protecting cells against oxidative damage [46].

Peng *et al.* [46] systematically analyzed the promoter regions of 23 genes of the glutathione *S*-transferase (GST) and glutathione peroxidase (GPX) families. Out of the 14 genes that had CPG islands in their promoter regions, promoter DNA hypermethylation and mRNA downregulation was found for *GPX3*, *GPX7*, *GSTM2*, *GSTM3* and *GSTM5*. They then analyzed 159 primary human samples (37 normal, 11 Barrett's, 11 dysplasia and 100 adenocarcinoms samples using quantitative bisulphite pyrosequencing. GSTM5 showed higher level of methylation in normal tissues and therefore is not presented in the table. The range of methylation for normal tissues ranged from 0% to 8.1%, for metaplastic tissues from 12.5 to 90%, dysplastic tissues from 37.5% to 87.5% and carcinoma from 15% to 69.1%. A significant inverse correlation between DNA methylation and mRNA expression level was shown for *GPX3* ($p<0.001$), *GPX7* ($p=0.002$), *GSTM2* ($p<0.001$) and *GSTM5* ($p<0.01$). Treatment of esophageal cancer cell lines with 5-aza-2′-deoxycytidine led to reversal of the methylation pattern and re-expression of these genes at the mRNA and protein levels. Immunohistochemical analysis of GPX3, GPX7 and GSTM2 on a tissue microarray that contained 75 BACs with normal squamous esophageal samples demonstrated absent to weak staining in tumors (52% for GPX3, 57% for GPX7 and 45% for GSTM2) and a moderate to strong immunostaining in normal samples.

Lee *et al.* had earlier studied Glutathione Peroxidase-*3* (GPx3) alone by using MSP. They detected GPx3 promoter hypermethylation was detected in 16.7% of normal tissues, 81.1% of Barrett's metaplasia, 82.2% of dysplasia and 88% of carcinoma samples [25].

MGMT

O-Methylguanine-DNA Methytransferase (MGMT) maps to chromosome 10q26 [49] and codes for a DNA repair enzyme that removes methyl or alkyl groups from guanine after chemical modification and therefore protects cells from G to A mutations. This gene has been studied by multiple authors [16, 26, 31, 41, 47, 48]. Methylation of normal tissues ranged from 1.8% to 54.8%, metaplastic tissues from 25% to 88.9%, dysplastic tissues from 71.4% to 100% and EAC from 23.4% to 78.7%. Kuester *et al.* also demonstrated MGMT hypermethylation correlated with down expression of protein and

mRNA and with progressed stage of disease [48]. Baumann *et al.* found that MGMT hypermethylation correlated with MGMT protein expression but not p53 status or with patient outcomes [47].

NELL1

The nel-like*1* (NELL1) has been mapped to chromosome 11p15 [50], a locus that frequently shows loss of heterozygosity (LOH) in human cancers including colon cancer and EAC [50]. The NELL1 gene encodes a protein kinase C-binding protein that contains six EGF-like domains and belongs to a new class of signaling molecules controlling cell growth and differentiation. The precise roles of NELL1 in physiology and pathophysiology remain unclear although it has been reported that overexpression of NELL1 promotes apoptosis in osteoblasts and this apoptotic activity may be associated with Fas signaling pathway [51]

NELL1 hypermethylation was examined by methylation specific PCR in 259 human esophageal tissues by Jin *et al 2007*. NELL1 hypermethylation frequency was 0% in normal esophagus but increased early during neoplastic progression, to 41.7% in BE alone, 46.7 in all tissues with Barrett's, 52.5% in dysplastic tissues and 47.8% in EAC. Treatment of these cells with 5-Aza-dC led to concomitant increases in mRNA expression and reduction in NELL1 methylation levels. Nell1 hypermethylation showed a strong direct relationship with Barrett's segment length and hypermethylation of NELL1 was significantly associated with shortened survival in stages I-II EAC patients [36]

REPRIMO/RPRM

Reprimo (RPRM) encodes a cytoplasmic protein that belongs to a family of molecules controlled by p53 and is a candidate tumor suppressor gene involved in regulation of p53 mediated cell cycle arrest at the G2 phase of the cell cycle [52]. The Reprimo gene maps to chromosome 2q23 a locus that commonly shows allelic imbalance in human cancers. In the study done by Hamilton *et al.*, Reprimo methylation was found to 0% in normal tissues, 36% in metaplastic tissues, 63.6% in dysplastic tissues and 62.6% in EAC. In addition, within the set of patients with Barrett's, those with long-segment BE had significantly more Reprimo promoter methylation than those with short-segment disease [35]. They also treated OE33 cell lines with 5-aza and found reduced methylation and increased expression of gene with increased mRNA levels.

P16/CDKN2A

Inactivation of CDKN2A/P16 tumor suppressor gene is one of the most common genetic abnormalities in human neoplasia [53]. The CDKN2A gene located on 9p21, encodes a cell cycle regulatory protein CDK1 that inhibits cyclin-dependant kinases 4 and 6, preventing the phosphorylation of pRb protein and release of transcription factor E2F. This blocks cell cycle progression from G1 to S phase. Alterations of the CKN2A gene lead to its inactivation, resulting in deregulation of cell proliferation and consequent genomic instability [54] Mutations, loss of heterozygosity and hypermethylation are the most frequent modes of inactivation of the gene. Although homozygous deletions of p16 have been reported in several types of malignancies, it has never been detected in esophageal adenocarcinoma, suggesting hypermethylation of the remaining p16 allele as alternative mechanism of inactivation [16].

A study done by Wong *et al.* 1977 has provided evidence that p16 may be inactivated in esophageal adenocarcinoma with 9p21 LOH by at least two different mechanisms: mutation or promoter hypermethylation.19 out of 21 patients with Barrett's esophagus (90%) had LOH and 8 of 21 patients (38%) had p16 promoter hypermethylation suggesting that p16 promoter hypermethylation with 9p21 LOH is a common mechanism for inactivation of the p16 gene in premalignant epithelium and esophageal adenocarcinomas [55].

Multiple authors have studied p16/CDKN2A methylation [16, 26, 28, 31, 41, 42]. The range of methylation in normal tissues varied from 0% to 24.4%, in metaplastic tissues from 7.4% to 27.5%, in dysplastic tissues from 22.2% to 55.0% and EAC from 39.0 % to 59.5%. Wang *et al.* 2009 did a nested case control study and found that APC and P16 are significantly more methylated in progressors than non progressors [42].

SFRP

Secreted frizzled related protei*n* (SFRP) genes are a new class of tumor suppressor genes that

have been found to be methylated in Barrett's esophagus and esophageal adenocarcinoma [27]. SFRPs are secreted glycoproteins that work as inhibitive modulators of a putative tumorigenic Wnt pathway [56]. Five SFRP genes have been identified and SFRPs 1, 4 and 5 appeared to be more consistently methylated in Barrett's esophagus and EAC [27].

In the study performed by Zou *et al.*, the promoter regions of SFRP genes were methylated in 73-93% of esophageal cancers and in 73-89% of Barrett's epithelia. However SFRP methylation was less common (21-32%) in normal appearing epithelia adjacent to Barrett's and least common (0-13%) in normal esophageal or gastric epithelia from individuals without Barrett's. SFRP 2 was the only one that showed high levels of methylation in normal tissues. It was observed that SFRP mRNA and protein expression were absent or markedly decreased in Barrett's esophagus and cancer but generally high in normal epithelia [27]. Based on the study it was concluded that methylation represents a likely mechanism of SFRP gene silencing in Barrett's esophagus, EAC and methylated SFRP1, 4 and 5 genes represent potential tumor markers for Barrett's esophagus and its related neoplasia. Smith *et al.* [41] and Clement *et al.* [28] also showed similar findings (see **Table 1**).

SOCS

SOCS-1 is mapped to chromosome 16p13.13 and SOCS-3 is mapped to 17q25.3 [57]. These genes encode a member of the STAT-induced STAT inhibitors (SSIs), also known as suppressors of cytokine signaling (SOCS). SSI family members are cytokine-inducible negative regulators of cytokine signaling.

Hengge *et al.* studied 19 Barrett's adenocarcinomas, 56 Barrett's intraepithelial neoplasms (29 low grade and 27 high grade), 30 Barrett's mucosa without neoplasia, 20 samples of normal squamous and gastric epithelium and four cell lines [58]. In normal squamous epithelium and normal gastric mucosa, neither SOCS-3 nor SOCS-1 methylation was observed. In Barrett's mucosa without intraepithelial neoplasia, SOCS-3 methylation occurred in 4/30 cases (13%) whereas SOCS-1 was unmethylated. A hypermethylated SOCS-3 promoter was found in 14/19 Barrett's adenocarcinomas (74%) and in 20/29 high and 6/27 low grade intraepithelial neoplasms (69% and 22%, respectively). SOCS-1 promoter hypermethylation occurred in 8/19 adenocarcinomas (42%) and in 6/29 high grade and 1/27 low grade intraepithelial neoplasms (21% and 4%, respectively). They concluded that promoter methylation and subsequent transcript downregulation of SOCS-3 transcripts and, to a much lesser extent, SOCS-1 are involved in the multistep carcinogenesis of Barrett's adenocarcinoma

SST

Somatostatin whose gene is mapped to 3q28 [59] is produced by the thalamus and also by a variety of endocrine and neuroendocrine tissues. In the GI tract, SST regulates endocrine and exocrine secretion, modulates motor activity, and is the primary inhibitor of gastrin-stimulated gastric acid secretion [60]. SST gene promoter methylation was studied by Jin *et al.* in 260 esophageal biopsy specimens of different histologies using MSP. Incremental increases in the frequency of SST hypermethylation were observed during progression from NE (9%) to BE (70%), HGD (71.4%) and EAC (71.6%), whereas LGD (63.2%) demonstrated a slightly lower frequency of SST hypermethylation than did BE or HGD [60] SST promoter hypermethylation was significantly more frequent in Long segment Barrett's esophagus (LSBE), which has a higher malignant potential than does short segment Barrett's esophagus (SSBE). Thus SST methylation represents a molecular correlate of BE segment length, as well as a potential biomarker of the prediction of BE progression. They concluded that hypermethylation of SST promoter was significantly more frequent in the premalignant lesions, metaplastic BE and BE with high-grade dysplasia, as well as in frank EAC and ESCC, than in normal esophageal epithelium.

TAC1

The tachykinin-*1* (TAC) gene has been mapped to chromosome 7q21-22 [61] a locus that frequently undergoes loss of heterozygosity in human cancers including esophageal adenocarcinoma [62]. TAC 1 encodes the neuropeptides substance P, neurokinin A, and neuropeptide K, which act through two types of transmembrane G-protein-coupled receptors, denoted neurokinin-1 and neurokinin-2. The precise involvement of TAC 1 in carcinogenesis is not clear

although the neuropeptides like substance P have been shown to have proliferative and antiapoptotic effects.

Methylation of the TAC 1 gene promoter was studied in 258 human endoscopic esophageal specimens and 126 plasma samples by Jin *et al.* by real-time quantitative methylation-specific PCR from patients or tissues at various stages of neoplastic evolution [63]. The frequency of TAC1 hypermethylation increased dramatically from 7.5% in normal esophagus to 55.6% in BE from patients with Barrett's metaplasia alone, 57.5% in dysplastic Barrett's esophagus and 61.2% in EAC [63]. The segment length of BEs with hypermethylated TAC 1 promoters were significantly longer than the segment lengths of BEs with unmethylated promoters and it was noted that the frequency of TAC1 hypermethylation was significantly higher in LSBE than in SSBE. Overall patient survival correlated significantly with TAC1 hypermethylation in patients with squamous cell esophageal tumors but not in EAC patients. In addition, the data in the study suggested that circulating hypermethylated TAC1 DNA is associated with the presence of EAC. They concluded that TAC1 promoter hypermethylation is a common event in both major histologic types of human esophageal carcinoma, occurs early, correlates with other progression risk factors in esophageal adenocarcinogenesis, and is a tissue biomarker of poor prognosis in SCC of the esophagu [63].

TIMP3

The TIMP-3 gene is located on chromosome 22q12.3, a region in which loss of heterozygosity and silencing of the gene due to CpG island hypermethylation is frequently observed in various cancers [64] TIMP-3 is considered a tumor suppressor gene, because TIMP-3 can inhibit tumor growth, angiogenesis, and invasion and can promote apoptosis [65].

TIMP3 methylation was studied by multiple authors including [16, 26, 28, 31, 41]. They reported methylation of TIMP3 in normal tissues from 0% to 19.3%, metaplastic from 54.1 % to 87.5%, dysplastic tissues from 71.4 to 77.8% and EAC from 19.5 % to 86.3%.

WIF1

Many authors have established the crucial role of the Wnt signaling pathway in the pathogenesis of many human cancer [18, 28, 29]. Overexpression of its signaling components as well as the downregulation of its antagonist (SRFP) have also been confirmed along the EAC carcinogenesis sequence [27-29]

Among other Wnt antagonists, WIF-1 also plays an important role. WIF-1 is mapped to 12q14.3 [66]. Clement *et al.* found that methyaltion of WIF-1 ranged from 0% in normal tissues to 51.6% in metaplastic tissues and 83.3 in EAC. They also showed that WIF-1 hypermethylation was more frequent in BE samples from patients with EAC than in BE samples from patients who had not progressed to EAC. Restoration of WIF-1 in cell lines where WIF-1 was epigenetically silenced resulted in growth suppression. Restoration of WIF-1 could sensitize the EAC cells to the chemotherapy drug cisplatin. They therefore concluded that silencing of WIF-1 through promoter hypermethylation is an early and common event in the carcinogenesis of BE. Restoring functional WIF-1 might be used as a potential new targeted therapy for the treatment of this malignancy [29].

Clinical applicability of DNA methylation in barretts esophagus

Aberrantly methylated DNA can be used as prognostic biomarker

Jin *et al.* conducted a multicenter , retrospective, double blinded trail in 2009 with the 8 genes(p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12, and CDH13) [67]. They had studied the genes earlier and had established that all these genes predicted progression risk. Using MSP they studied 145 nonprogressors (NPs) and 50 progressors at 2 and 4 years, they found that the 8-marker panel is more objective, quantifiable and possesses higher predictive sensitivity and specificity than do clinical features, including age. This is an example of how the genes above can be used in combination to predict progression risk and as an early disease marker. Such studies can also be used in the future to predict endoscopic surveillance interval since only about 0.5% of patients with Barrett's progress to EAC.

Aberrant DNA methylation can be detected in the serum

Ultimately the biomarkers will be more useful if they can be detected in the plasma remote from

the site of the tumor. Kawakami *et al.* published a study showing APC DNA methylation in the plasma could be used as a biomarker [34]. Hypermethylated APC DNA was observed in the plasma of 13 (25%) of 52 adenocarcinoma patients and in two (6.3%) of 32 squamous carcinoma patients. High plasma levels of methylated APC DNA were statistically significantly associated with reduced patient survival [34]. In the year 2007 Jin *et al.* measured TAC1 methylation levels in 126 plasma samples from 35 control subjects, 10 additional patients with Ba, 20 with dysplastic Barrett's esophagus, and 61 with EAC. TAC1 hypermethylation frequencies in plasma samples were significantly higher in EAC patients than in control subjects [63]. Hoffmann *et al.* used APC and DAPK as biomarkers in plasma. Thirty-six of 59 patients (61.0%) with esophageal cancer had detectable levels of methylated DAPK or APC promoter DNA and preoperative detection was significantly associated with an unfavorable prognosis The combination of both markers significantly increased sensitivity and specificity for discriminating between short (<2.5 years) and long survivors [68]. Postoperative APC detection was significantly different if residual tumor was apparent . Therefore some of the genes studied in the paper could be used as a combination to develop a biomarker that can be used remotely from the site of the tumor and be helpful to a clinician in many ways.

Genome wide analysis of methylation reveals hypomethylation occurs during barrett's neoplasia

We conducted the first genome wide analysis of DNA methylation in Barrett's esophagus by using the HELP assay. The *Hpa*II tiny fragment Enrichment by Ligation-mediated PCR (HELP) assay, compares *Hpa*II (methylation-sensitive) and *Msp*I (methylation insensitive) genomic representations to identify hypo- and hypermethylated loci in the genome and has been used extensively to study the methylome of cancer [69]. 10 samples representing Barrett's metaplasia, low and high grade dysplasia and frank adenocarcinoma with normal controls were profiled by gene expression and methylome analysis. Unsupervised hierarchical clustering analyses demonstrated that, at the level of the transcriptome, squamous mucosa clustered discretely from "glandular" epithelium (including gastric cardiac and Barrett's metaplasia, as well as all stages of BE progression). In contrast, at the level of the epigenome, "normal" mucosa (including both normal esophageal squamous and cardiac subtypes) clustered discretely from all "abnormal" (*i.e.,* BE) epithelia. These results suggest some degree of commonality of epigenetic profiles between otherwise normal gastrointestinal tissues, despite obvious morphological differences. Contrary to the hypermethylation reported in previous single-locus studies, we identified significant hypomethylation occurring at a large number of loci genome-wide during the transition of squamous mucosa to Barrett epithelium. Since this epigenetic "shift" is not observed in the comparison of normal esophageal squamous *versus* cardiac mucosal samples, we believe these methylation alterations may be reflective of the actual BE disease process, rather than simply due to acquisition of columnar histology. Validation at the whole-genome level by the Luminometric methylation assay (LUMA) revealed a significantly large increase in unmethylated CpGs in BE samples *versus* matched normal squamous mucosa. These results demonstrate that the previously reported global hypomethylation observed in human cancers can initiate at a very early stage of neoplastic transformation, such as in the non-invasive precursors of EAC. In addition to this panoramic view of epigenetic shifts, we also assessed the nature of the *Hpa*II sites showing altered methylation during BE progression. As the HELP assay microarray design includes both canonical CpG islands and additional CG dinucleotide loci within gene promoters, we sought to test whether CpG islands, long considered the principal target of epigenetic dysregulation in cancer, were disproportionately affected. We compared the proportions of loci at which differential methylation was occurring within CpG islands *versus* other CG dinucleotide loci represented on the microarray. We determined that the majority of *Hpa*II loci exhibiting differential methylation during BE progression lay, paradoxically, outside of canonical CpG islands. In fact, an example of this effect was seen for the gene *Deleted in Malignant Brain Tumor 1 (DMBT1). DMBT1's* promoter lacks a defined CpG island, yet demonstrates progressive hypomethylation accompanied by significant transcript upregulation during BE progression.

In our genome wide analysis, we also integrated differences in transcript expression with both methylation status and DNA copy number at

various loci. This was done to not only identify significant transcriptomic alterations during neoplastic progression, but also to elucidate the relative contributions of genomic and epigenetic factors toward such deregulation. For example, a chromosome 9p21 hemizygous deletion was identified by array CGH analysis of LGD and HGD biopsies obtained from this individual, which was absent in the matched normal esophageal squamous epithelium, consistent with a somatic monoallelic loss, as confirmed by FISH analysis. This region harbors two closely approximated tumor suppressor genes: *CDKN2A/p16* and *CDKN2B/p15.* Prior copy number and other studies have implicated *CDKN2A* as the target of inactivation at this locus in BE. Nevertheless, in this particular example, microarray data demonstrated that the relative fold reduction in gene expression was considerably greater for *CDKN2B* (~100-fold downregulation) than for *CDKN2A* (~4-fold); moreover, this finding was independently validated by qRT-PCR, which confirmed the complete absence of *CDKN2B* transcripts in the dysplastic biopsy samples, while the expression of *CDKN2A*, albeit significantly reduced, was still detectable. Analysis of the methylome by the HELP assay clarified that the *CDKN2B* promoter in the retained allele underwent progressive hypermethylation during BE progression, while the *CDKN2A*promoter maintained its methylation *status quo*. This finding suggests the importance of methylation of the remaining allele in regulating expression, and provides direct experimental evidence for genetic and epigenetic hits acting concurrently and synergistically to downregulate tumor suppressor genes during oncogenesis through bi-allelic inactivation.

We also determined that many genes not previously implicated in esophageal carcinogenesis were significantly upregulated during stepwise progression to cancer. These were upregulated by either loss of methylation, or gene amplification, or both occurring together. For example, transcripts corresponding to a family of chemokine ligands were among the most significantly upregulated, and the corresponding gene cluster is present on the 4q21 chromosomal segment that was amplified in all patient samples during the process of Barrett neoplasia. Integrative analysis revealed that in addition to amplification, the *CXCL1* and *CXCL3* gene promoters were also hypomethylated during transformation, and their relative increase in transcript expression was many fold greater (4–6 fold by array, 10-20 fold by qRT-PCR) than that of *IL-8*, a chemokine gene which was part of the 4q21 amplicon but whose promoter was not affected by loss of methylation. These results demonstrate the combinatorial affect of both genetic and epigenetic alterations on dysregulation of gene expression during carcinogenesis.

Conclusions

These data demonstrate that Barrett's esophagus and esophageal adenocarcinoma are characterized by widespread alterations in DNA methylation. Earlier, single locus studies showed that hypermethylation of promoters of tumor suppressors and other cancer associated genes was seen during neoplastic transformation, Newer genome-wide analysis shows that loss of methylation is the predominant change during this process and only selective hypermethylation is seen during later stages of carcinogenesis. Even though various studies have already revealed prognostic implications of these aberrantly methylated loci, newer higher resolution studies will further refine the clinical applicability of these findings.

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