# Accumulation of Promoter Methylation Suggests Epigenetic Progression in Squamous Cell Carcinoma of the Esophagus

Mingzhou Guo,[1,3] Jingli Ren,[5] Michael G. House,[2] Yu Qi,[4] Malcolm V. Brock,[1,2] and James G. Herman[1]

**Abstract**

**Purpose:** Squamous esophageal cancer is common in non-Western countries and has a well-defined progression of preinvasive dysplasia leading to invasive squamous cell carcinoma. We examined the changes in promoter region methylation occurring during neoplastic progression.

**Experimental Design:** The frequency of epigenetic changes in the promoter region of 14 genes epigenetically silenced in other cancers was determined and examined the most frequent changes in dysplastic lesions using methylation-specific PCR. Invasive squamous carcinomas, low to high grade dysplasia, and normal esophagus were then examined for methylation changes in the promoter region of each of the eight most commonly methylated genes.

**Results:** Methylation was most frequent for CDKN2A/p16INK4a (52%) but was also common for $O^6$-methylguanine-DNA methyltransferase, E-cadherin (CDH1), and retinoic acid receptor β2. Methylation at individual genes increased in frequency from normal to invasive cancer. Methylation of MLH1 was associated with microsatellite instability in most cases. The number of genes methylated in individual lesions increased as cellular atypia increased. In individual patients, cancers adjacent to dysplasia had the same epigenetic alterations as the less advanced lesions but often had additional methylation of other genes.

**Conclusions:** These findings suggest that epigenetic progression parallels the histologic changes observed in the progression of squamous carcinoma of the esophagus.

Esophageal cancer is the seventh most common cancer worldwide, and the incidence of esophageal cancer has increased rapidly over the past 3 decades (1–3). A marked geographic variation exists in the incidence of this cancer, and regional differences exist in the prevalence of adenocarcinoma and squamous cell carcinoma (1, 2). Although most tumors are adenocarcinoma in western societies, squamous cell cancers constitute 80% of esophageal cancer in the world. This tumor type is especially prevalent in East Asia, South Asia, and South Africa. The histologic progression of esophageal squamous cancer includes the transition from normal squamous epithelium to hyperproliferative epithelium, low-grade dysplasia, intermediate-grade dysplasia, high-grade dysplasia/carcinoma *in situ*, and, ultimately, invasive esophageal squamous cell carcinoma (ESCC; ref. 3). Each of these changes represents an increasing atypia compared with normal squamous epithelia and is associated with an increasing risk for the development of invasive squamous cell cancer (4).

Although the histologic progression of ESCC is relatively well established, our knowledge of the molecular progression is more limited. Thus far, studies of loss of heterozygosity have suggested the early involvement of genes on chromosomes 3p, 4p, 9q, and 13q and later involvement of other loci, including 3p24, 8p, 9p, 11p, 13q, and 17p, in this progression (5). Genetic alterations implied from these loci include the tumor suppressor genes *Rb* (13q), *p53* (17p), and *CDKN2A/p16INK4a* (9p). For Rb, loss of heterozygosity is observed in 50% of ESCC (5). For p53, loss of heterozygosity is a frequent albeit relatively late event, occurring in 43% of high-grade dysplasias and ESCC but not in low-grade dysplasias (5). Mutational frequencies of p53 have generally paralleled the rates of loss of heterozygosity and have shown interesting geographic differences (6). Loss of 9p has also been reported to be a later event in ESCC progression and has only rarely been associated with mutational events on the remaining allele (5, 7).

In addition to genetic changes, epigenetic silencing of tumor suppressor genes has become widely recognized as a means of gene inactivation in cancer (8). In esophageal cancer, recent attention to epigenetic changes has focused primarily on studies of adenocarcinoma of the esophagus, where frequent methylation of CDKN2A, adenomatous polyposis coli (APC), and CDH1 has been reported (9–11). Frequent methylation of CDKN2A in preinvasive lesions in esophageal adenocarcinoma (9, 12–14) suggests an important role of CDKN2A hypermethylation in the neoplastic progression of Barrett's esophagus (13, 14). Multigene methylation occurs in esophageal

**Authors' Affiliations:** [1]Cancer Biology Program, Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins; [2]Department of Surgery, The Johns Hopkins University, Baltimore, Maryland and Departments of [3]Gastroenterology and [4]Surgery, The First Teaching Hospital and [5]Department of Pathology, The Second Teaching Hospital, Zhengzhou University, Zhengzhou, People's Republic of China

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**Requests for reprints:** James G. Herman, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, Baltimore, MD 21231. Phone: 410-955-8506; Fax: 410-614-9884; E-mail: hermanji@jhmi.edu.



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**Table 1.** Primers used for methylation analysis

| Gene | External sense (5′-3′) | External antisense (5′-3′) | Internal methylated sense (5′-3′) |
|---|---|---|---|
| *p16* | AGAAAGAGGAGGGGTTGGTTGG | ACRCCCRCACCTCCTCTACC | TTATTAGAGGGTGGGGCGGATCGC |
| *p14* | TTTAGTTTGTAGTTAAGGGGGTAGGAG | CRCTACCCACTCCCCCRTAAACC | GTGTTAAAGGGCGGCGTAGC |
| *p15* | GAYGTYGGTTTTTTGGTTTAGTTGA | AACRCAACCRAACTCAAAACC | GGTTTTTTATTTTGTTAGAGCGAGGC |
| *MGMT* | GYGTTTYGGATATGTTGGGATAGTT | AAACTCCRCACTCTTCCRAAAAC | TTTCGACGTTCGTAGGTTTTCGC |
| *MLH1* | GGAGTGAAGGAGGTTAYGGGTAAGT | AAAAACRATAAAACCCTATACCT-AATCTATC | ACGTAGACGTTTTATTAGGGTCGC |
| *GSTπ* | GGGATTTTAGGGYGTTTTTTTG | ACCTCCRAACCTTATAAAAATAATCCC | TTCGGGGTGTAGCGGTCGTC |
| *RARβ2* | TATGYGAGTTGTTTGAGGATTGGGA | AATAATCATTTACCATTTTCCAAACTTA | TGTCGAGAACGCGAGCGATTC |
| *DAPK* | YGGAGGATAGTYGGATYGAGTTAA | ACRAAAACACAACTAAAAAAT-AAATAAAAAAC | GGATAGTCGGATCGAGTTAACGTC |
| *APC* | TGGGYGGGGTTTTGTGTTTTATT | TACRCCCACACCCAACCAATC | TATTGCGGAGTGCGGGTC |
| *TIMP-3* | GYGGTATTATTTTTTATAAGGATTTGA | ACCRAATAATATAACRCTAAACCCC | CGTTTCGTTATTTTTTGTTTT-CGGTTTC |
| *CDH1* | GTGTTTTYGGGGTTTATTTGGTTGT | TACRACTCCAAAAACCCATAACTAACC | TGTAGTTACGTATTTATTTTT-AGTGGCGTC |
| *BRCA1* | GAGAGGTTGTTGTTTAGYGGTAGTTTT | TCTAAAAAACCCCACAACCTATCC | TCGTGGTAACGGAAAAGCGC |
| *p73* | GGGTYGGGTAGTTYGTTTTGTTTT | CRACCCTAAACCTCCTACCTACAACC | GGACGTAGCGAAATCGGGGTTC |
| *Thrombspondin-1* | GGGTTTTTGTYGTTTTTTA-GGAGTAATT | CTCCAAATAAATATCCCRAACAACTTTA | TCGGACGTATAGGTATTTTTCGC |

Abbreviation: TIMP-3, tissue inhibitor of metalloproteinase-3.

adenocarcinoma (15, 16). In adenocarcinoma of the esophagus, evidence for the epigenetic changes in tumor progression has been presented (16, 17) for some genes occurring early in tumor progression (18).

However, squamous cancers have not been as extensively studied. The *CDKN2A* gene has also been the most widely studied gene for epigenetic changes in ESCC as well (19–21). In invasive tumors, CDKN2A methylation was reported to be present in 40% to 81% of ESCC (19–21). Methylation of CDKN2A (22), p14ARF (22), and retinoic acid receptor β2 (RARβ2; ref. 23) has been described in both squamous esophageal cancer and preinvasive neoplasia. In addition, $O^6$-methylguanine-DNA methyltransferase (MGMT) methylation has been found to be related to mutations in p53 in squamous esophageal cancer (24). Based on these previous studies, we sought to examine multiple genes, which may show aberrant DNA methylation in esophageal cancer, to determine the timing of these events in preinvasive lesions and the potential role they therefore may play in tumor initiation and progression.

## Materials and Methods

***Sample collection and DNA extraction.*** All samples were obtained at The Second Teaching Hospital of Zhengzhou University (Zhengzhou, People's Republic of China). Sixty-nine cases of esophageal squamous cell cancer, 39 cases of low-grade esophageal dysplasia, 12 cases of intermediate-grade esophageal dysplasia, and 9 cases of high-grade esophageal dysplasia were studied as paraffin-embedded samples. These samples were selected for the presence in many cases of adjacent dysplastic lesions. Each surgically removed cancer specimen was examined for evidence of tumor involvement at the surgical margins. When present, dysplastic samples were obtained from the tumor-free margin of the resected specimens. Seventeen cases of normal esophageal mucosa were obtained by endoscopic biopsy of patients with nonulcerative dyspepsia determined to be free of dysplasia or neoplasia. Biopsy specimens were snap frozen before DNA extraction. Paraffin-embedded tissue samples were sectioned at 10 μm, and four to six tissue sections were deparaffinized in xylene and washed twice with 100% ice-cold ethanol. The snap-frozen samples are normal controls from noncancerous tissues, which provide more DNA suited for amplification following bisulfite treatment, to assure that low levels of methylation were not present in normal esophagus. The samples were then extracted DNA by proteinase K method, including overnight digestion followed by DNA phenol-chloroform extraction and ethanol extraction as described (25).

***Bisulfite modification.*** Genomic DNA (1 μg) was diluted in 50 μL water. Sodium bisulfite converts unmethylated cytosine to uracil when DNA is denatured, whereas methylated cytosines are resistant. These differences provide unique sequences for the design of primers to detect methylation differences. The bisulfite treatment was carried out for 16 hours at 50°C according to previously described procedures (25). DNA samples were then purified with the Wizard DNA Clean-Up System (Promega, Madison, WI) and desulfonated with NaOH. Samples were ethanol precipitated and resuspended in 20 μL water.

***Nested PCR assay.*** To facilitate the examination of multiple genes and particularly on the biopsy samples, a nested PCR approach was used. The bisulfite-modified DNA was subjected to a first-stage, multiplex PCR incorporating external primer sets for four to six genes, and positive and negative controls are included throughout nested methylation-specific PCR (MSP). Each multiplex PCR was carried out in a total volume of 25 μL with 0.5 unit of JumpStart Red Taq DNA polymerase (Sigma, St. Louis, MO), 10 pmol of each external primer, and 4 μL of bisulfite-modified DNA. The external primer sequences used for the multiplex PCR are listed in Table 1. PCR conditions included an initial denaturation at 95°C for 5 minutes followed by 35 cycles of amplification (95°C × 30 seconds, 56°C × 30 seconds, 72°C × 30 seconds) and a final elongation at 72°C for 5 minutes. PCR products were analyzed on 6% polyacrylamide gels to confirm adequate template for subsequent second-stage internal MSP.

**Table 1.** Primers used for methylation analysis (Cont'd)

| Internal methylated antisense (5′-3′) | Internal unmethylated sense (5′-3′) | Internal unmethylated antisense (5′-3′) |
|---|---|---|
| GACCCCGAACCGCGACCGTAA | TTATTAGAGGGTGGGGTGGATTGT | CAACCCCAAACCACAACCATAA |
| AAAACCCTCACTCGCGACGA | TTTTTGGTGTTAAAGGGTGGTGTAGT | CACAAAAACCCTCACTCACAACAA |
| TAACCGCAAAATACGAACGCG | GGTTGGTTTTTTATTTTGTTAGAGTGAGGT | AACCACTCTAACCACAAAATACAAACACA |
| GCACTCTTCCGAAAACGAAACG | TTTGTGTTTTGATGTTTGTAGGTTTTTGT | AACTCCACACTCTTCCAAAAACAAAACA |
| CCTCATCGTAACTACCCGCG | TTTTGATGTAGATGTTTTATTAGGGTTGT | ACCACCTCATCATAACTACCCACA |
| GCCCCAATACTAAATCACGACG | GATGTTTGGGGTGTAGTGGTTGTT | CCACCCCAATACTAAATCACAACA |
| CGACCAATCCAACCGAAACGA | TTGGGATGTTGAGAATGTGAGTGATTT | CTTACTCAACCAATCCAACCAAAACAA |
| CCCTCCCAAACGCCGA | GGAGGATAGTTGGATTGAGTTAATGTT | CAAATCCCTCCCAAACACCAA |
| TCGACGAACTCCCGACGA | GTGTTTTATTGTGGAGTGTGGGTT | CCAATCAACAAACTCCCAACAA |
| CCGAAAACCCCGCCTCG | TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT | CCCCCAAAAACCCCACCTCA |
| CGAATACGATCGAATCGAACCG | TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT | ACACCAAATACAATCAAATCAAACCAAA |
| AAATCTCAACGAACTCACGCCG | TTGGTTTTTGTGGTAATGGAAAAGTGT | CAAAAAATCTCAACAAACTCACACCA |
| ACCCCGAACATCGACGTCCG | AGGGGATGTAGTGAAATTGGGGTTT | ATCACAACCCCAAACATCAACATCCA |
| ATCCTCGACGACCGCCG | TTTTTATTTTGGATGTATAGGTATTTTTTGT | CAACTTTAATCCTCAACAACCACCA |

***Methylation-specific PCR.*** MSP was done as described previously (25). Multiplex PCR products were diluted 1:1,000, and 4 μL of this dilution were introduced to second-stage PCR using internal primers to discriminate methylated versus unmethylated templates. Primers were selected and previously shown to determine methylation in regions leading to gene silencing. Conditions were established to provide specific detection of methylation associated with functional silencing as described previously (26). PCR conditions were gene specific as follows: *APC* and *p14ARF* (*p14*) genes ($T_m$ = 60°C × 25 cycles), *MGMT*, *MLH1*, *RARβ2*, *E-cadherin* (*CDH1*), *CDKN2A/p16INK4a* (*p16*), *tissue inhibitor of metalloproteinase-3*, *p73*, *death-associated protein kinase* (*DAPK*), *BRCA1*, *CDKN2B/p15INK4B* (*p15*), and *thrombspondin-1* genes ($T_m$ = 60°C × 30 cycles), and *GSTπ* gene ($T_m$ = 58°C × 30 cycles). Each PCR assay included a positive control (*in vitro* methylated DNA) using DNA treated *in vitro* with *Sss*I methyltransferase (New England Biolabs, Beverly, MA) and a negative control using normal human peripheral lymphocytes. MSP products were analyzed on 6% polyacrylamide gels.

***Microsatellite instability analysis.*** A set of five microsatellite loci (BAT26, BAT25, D2S123, D5S346, and D17S250) with their respective primer pairs has been recommended previously as a reference panel for the determination of microsatellite instability (MSI; ref. 27) and was used as described (28). We used the MSI Test (Roche Molecular Biochemicals, Mannheim, Germany) to facilitate the analysis of MSI in the 18 primary tumors or dysplasias that were compared with normal adjacent esophageal parenchyma. Microsatellite variation was determined when the electropherogram of the informative markers showed extra peaks over a broader size range in the tumor compared with the corresponding normal tissue from each specimen. A colorectal cell line (HCT116) with a replication error phenotype because of biallelic inactivation of *hMLH1* was used as a positive control for MSI, and microsatellite-stable HT29 cells served as the negative control.

***Statistical analysis.*** Differences in methylation frequency according to histologic changes were compared with the Mantel-Haenszel $\chi^2$ test for trends using STATA software.

## Results

***Gene-specific methylation in invasive squamous esophageal carcinoma.*** Using MSP, we examined 42 specimens of ESCC for methylation at the promoter region of 14 genes found to be methylated in other malignancies and previously examined in ESCC (29). The 14 genes were *MGMT*, *APC*, *p73*, *p14*, *p15*, *p16*, *BRCA1*, *MLH1*, *CDH1*, *GSTπ*, *RARβ2*, *DAPK*, and *tissue inhibitor of metalloproteinase-3*. Examples of the methylation analysis are shown in Fig. 1. The frequency of promoter hypermethylation for each of these genes varied greatly among these genes: *p16*, 50%; *MGMT*, 36%; *MLH1*, 33%; *CDH1*, 31%; *BRCA1*, 26%; *RARβ2*, 24%; *DAPK*, 24%; *p73*, 17%; *p15*, 17%; *APC*, 14%; *thrombspondin-1*, 10%; *tissue inhibitor of metalloproteinase-3*, 10%; *p14*, 7%; and *GSTπ*, 5%. Of these 42 carcinomas, methylation of at least one gene was present in 39 (93%) tumors.

Three promoter region CpG islands are located in close proximity at chromosome 9p21, allowing us to examine the relationship of methylation of these genes in this frequently deleted region (5). The methylation frequencies that we observed for each gene were similar to those reported previously for ESCC (20), which were 40% for *p16INK4a*, 15% for *p14ARF*, and 13% for *p15*. The more frequent methylation of *p16INK4a* found in these tumors suggests that the primary target for epigenetic change among these genes is *p16*, clarifying the target for inactivation by deletion.

***Promoter methylation in preinvasive squamous esophageal carcinoma.*** Seven genes among the 14 studied were found to be hypermethylated in >20% of the 42 ESCCs studied: *MGMT*, *p16*, *BRCA1*, *MLH1*, *CDH1*, *RARβ2*, and *DAPK*. In addition, because of the previous studies of frequent hypermethylation of the APC gene in adenocarcinoma of the esophagus, we also included the *APC* gene in this analysis (10, 17). Consequently, we examined these eight genes further to investigate epigenetic alterations in esophageal cancer precursor lesions. A nested MSP approach was applied to determine the methylation status of selected genes in normal esophageal mucosa and esophageal dysplasia. These eight genes were analyzed in 17 cases of normal squamous epithelia, 39 cases of low-grade dysplasia, 12 cases of intermediate-grade

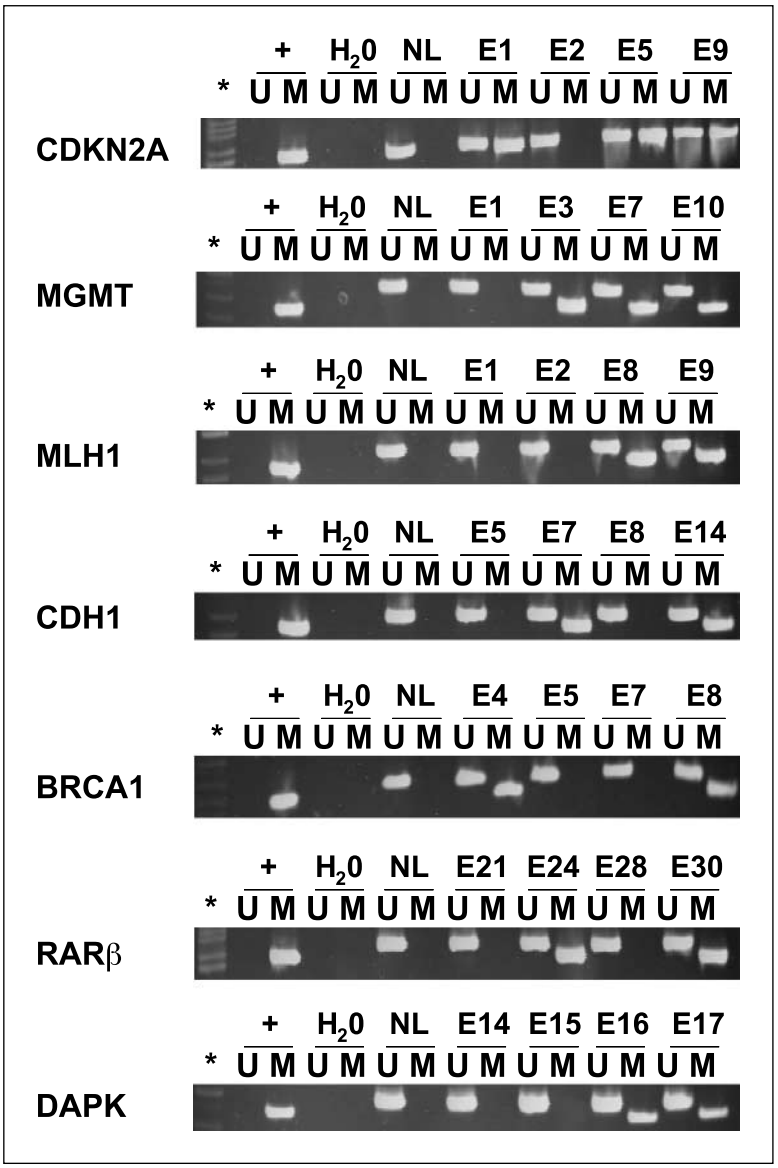


**Fig. 1.** Promoter hypermethylation in squamous carcinoma of the esophagus. Left, gene loci. Lanes U, for each gene, the presence of a visible PCR product indicates the presence of an unmethylated promoter region; lanes M, the presence of product indicates a methylated gene promoter. *In vitro* methylated DNA (+) was used as a positive control for promoter hypermethylation of each gene, and normal lymphocytes (*NL*) were used as a negative control for methylation. Systematic controls for PCRs ($H_20$). Numbers, esophageal tumor samples (e.g., E1) and correspond to data summarized in Fig. 2. Methylation of p16 is seen in samples E1, E5, and E9 but not in E2. MGMT methylation is present in E3, E7, and E10 but not in E1. The presence of U signal in each sample results from normal tissue contamination of the sample and serves as an internal control for the first-round PCR.

dysplasia, 9 cases of high-grade dysplasia/carcinoma *in situ*, and 27 ESCCs from which these earlier lesions were derived. This, including the 42 ESCCs described above, results in the analysis of 69 invasive cancers.

Methylation at these eight loci increased in frequency from normal mucosa through invasive cancer (Table 2). The specific methylation pattern for each lesion is shown in Fig. 2. Normal esophagus was not methylated at any locus in the 17 samples

**Table 2.** Methylation frequency (%) of eight gene loci according to histology

| | *p16* | *MGMT* | *MLH1* | *APC* | *BRCA1* | *RARβ2* | *CDH1* | *DAPK* | Meth ≥1 gene | Meth ≥2 genes |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal ($n = 17$) | 0 | 6 | 6 | 0 | 0 | 0 | 18 | 0 | 29 | 0 |
| ED1 ($n = 39$) | 31 | 23 | 8 | 3 | 3 | 13 | 10 | 28 | 51 | 33 |
| ED2 ($n = 12$) | 42 | 17 | 17 | 0 | 0 | 33 | 17 | 25 | 58 | 42 |
| ED3 ($n = 9$) | 33 | 11 | 33 | 0 | 0 | 44 | 33 | 11 | 56 | 56 |
| ESC ($n = 69$) | 52 | 33 | 23 | 13 | 28 | 36 | 34 | 26 | 91 | 70 |
| $P^*$ | <0.001 | 0.013 | 0.1 | 0.19 | <0.001 | 0.001 | 0.06 | 0.09 | <0.001 | <0.001 |

Abbreviations: Normal, normal esophagus; ED1, low-grade esophageal dysplasia; ED2, intermediate-grade dysplasia; ED3, high-grade esophageal dysplasia/carcinoma *in situ*.

*Using Mantel-Haenszel $\chi^2$ test for trends.

examined, with few exceptions. In five cases of normal esophagus, one gene in each case had promoter region methylation, including CDH1 methylation in three of the normal tissues. No normal esophagus had methylation of more than one gene. Although methylation of BRCA1 and APC had been observed in the invasive cancers (28% and 13%, respectively), methylation was found for each gene in only one dysplastic lesion in all of the samples of dysplasia examined, suggesting that these events develop later in the progression of ESCC. DAPK methylation, on the other hand, appeared with similar frequencies in dysplasia and invasive cancer. p16/CDKN2A methylation, commonly present in dysplasia (33%), was found even more frequently among the invasive lesions (52%), suggesting early but progressive changes in this gene as has been seen in other tumor types (30, 31). Other genes, including *MGMT*, *MLH1*, *RARβ2*, and *CDH1*, show increases in methylation frequency as cellular atypia advanced (Table 2; Fig. 2).

***Multiple gene methylation increases during progression.*** In addition to the observed differences in methylation frequency at specific loci, we observed an increase in the number of methylated genes in each sample according to histologic atypia. This can be seen by examination of the individual lesions displayed in Fig. 2 but is more apparent by examining cumulative methylation frequencies for each level of dysplasia as shown in Fig. 3. For simplification and because of the smaller numbers of esophageal lesions with intermediate and high-grade dysplasia, these groups were combined. The percentage of samples without a methylated locus decreased from normal esophagus (70%) through each stage of dysplasia and finally to invasive cancer (10%).

***Intraindividual epigenetic progression.*** A comparison of the methylation patterns in dysplastic and neoplastic lesions from 31 patients with multiple atypical lesions is presented in Table 3. Epigenetic changes (i.e., promoter methylation) seem to be acquired during the histologic progression associated with neoplastic transformation. The majority of individuals experienced an overall gain of methylation; however, there was occasional loss of individual gene methylation in six individuals. Five of the six methylation losses were associated with DAPK, which in other patients was associated with progression. With this exception of DAPK methylation, however, there was clear evidence of the accumulation of methylation events at additional loci in the progression of histologic atypia from low-grade dysplasia to invasive ESCC. In particular, many patients retained methylation of genes present in dysplastic lesions while adding methylation at other loci, most of the patients at the bottom of Table 3.

***MLH1 methylation and MSI.*** Few studies have examined either the presence of methylation of MLH1 or MSI in squamous esophageal cancer, and these studies have reported widely different frequencies of MSI (32–36). In the current study, we found methylation of MLH1 rarely in the low-grade dysplasia but more frequently in moderate/high-grade and invasive cancers. To address this issue, we examined DNA

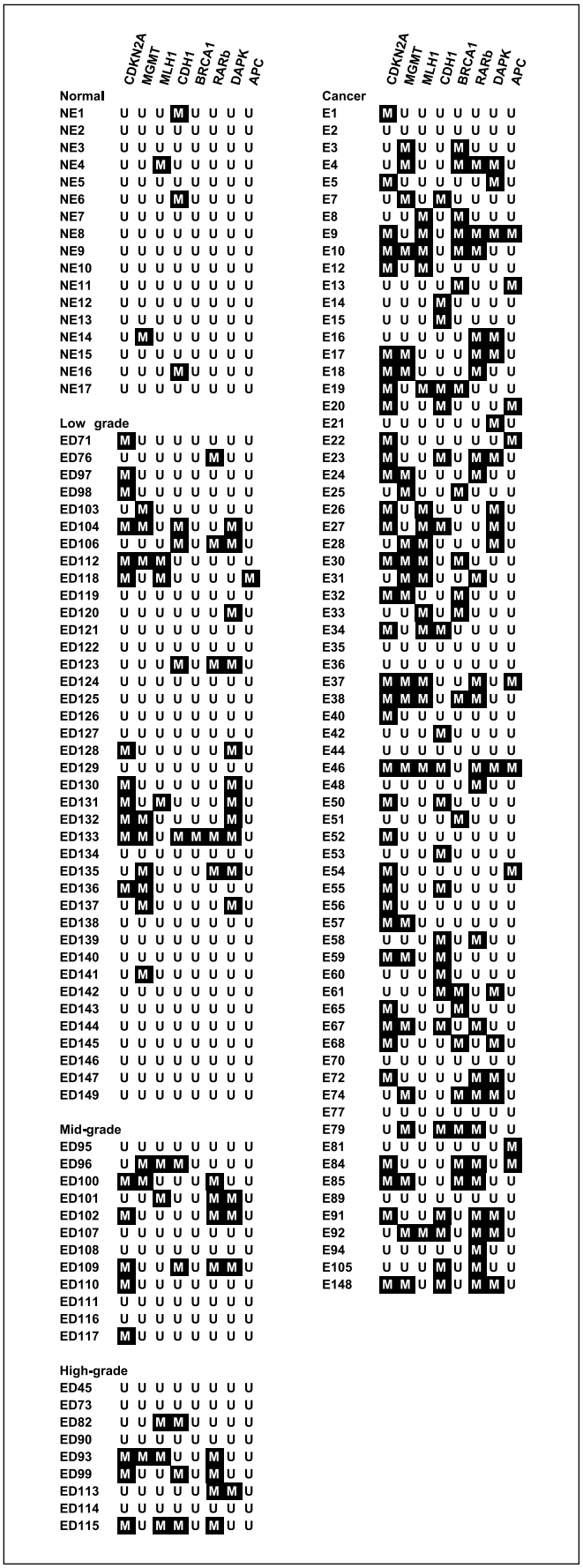


**Fig. 2.** Gene methylation patterns for individual lesions during progression of ESCC. Top, gene loci with lesions grouped according to histologic diagnosis. Left, individual lesions corresponding to the methylation data. M, methylation present at this loci; U, lack of methylation at this loci.

from 18 dysplasias and cancers with MLH1 methylation for MSI. Eleven of the 18 neoplasia with MLH1 methylation showed MSI, suggesting that MLH1 methylation was associated with the development of MSI in esophageal cancer as has been observed previously in other tumors (37). In two patients, we were able to examine lesions with methylation of MLH1 of different histologies for MSI. In one patient, both the intermediate-grade dysplasia and the invasive component had methylation of the MLH1 promoter region, and both the invasive cancer (E92) and the dysplasia had MSI (E82). In the second case, MSI was present in the mid-grade dysplasia but somewhat equivocal but was clearly present in the invasive esophageal cancer (E38). This accumulation of mutational changes during progression of preneoplasia with MLH1 methylation appears similar to that seen in endometrial cancer (38).

## Discussion

The molecular changes associated with progression continue to be defined for adenocarcinoma of the esophagus (3, 39). Studies of both the colon and esophagus support a defined molecular progression underlying the histologic changes apparent to the pathologist in these forms of adenomatous gastrointestinal malignancies. Although squamous cancer of the esophagus shares a similar metaplasia-dysplasia-carcinoma sequence, much less is known about the molecular progression in ESCC. Our results suggest that the increasing atypia observed at the histologic level is associated with distinct molecular events, including an increasing number of methylated CpG islands associated with gene promoter regions.

Our results for the genes studied on the 9p21 locus confirm previous studies, suggesting that CDKN2A/p16INK4a methylation is a frequent and early event in ESCC and that the primary locus for epigenetic change on 9p21 is CDKN2A itself and not the adjacent promoters for the CDKN2B/p15 or p14ARF loci (19–21). The *APC* gene was rarely methylated in this study but has been reported to be frequently hypermethylated in esophageal adenocarcinoma and in other types of gastrointestinal carcinoma (17, 40). Likewise, we (41) and others (15) have previously found higher rates of APC methylation in adenocarcinoma of the esophagus than the frequency found for squamous carcinomas reported in this study, suggesting that the alterations of APC are perhaps more important in adenocarcinomas. We found that methylation of the RARβ2 locus was not observed in normal esophagus but in 13% of the low-grade dysplasia. Higher-grade dysplasia and cancers showed even higher frequencies of methylation (33-44%), numbers that are consistent with the reported loss of expression of this gene determined by *in situ* hybridization (42) and confirm results of loss of expression and promoter methylation in a previous study of ESCC (23).

For many of the genes examined in the current report, previous studies have not characterized methylation in ESCC. Methylation of MLH1 has not been studied in squamous esophageal cancer, but MSI has indeed been reported in these tumors (43, 44). The finding of MSI following the methylation of MLH1 observed here shows the functional consequences of loss of MLH1 in the development of MSI as described previously (37, 38). Methylation of *BRCA1* was not expected, given the previous suggestion that this gene was most commonly affected in breast and ovarian, not esophageal, cancers (45). However, the observation that BRCA1 methylation was nearly exclusive for invasive tumors and not in normal or dysplastic esophagus, this occurs as a late event in the progression of ESCC.

Our results show that acquisition of aberrant tumor suppressor gene hypermethylation is similar to the classic gene

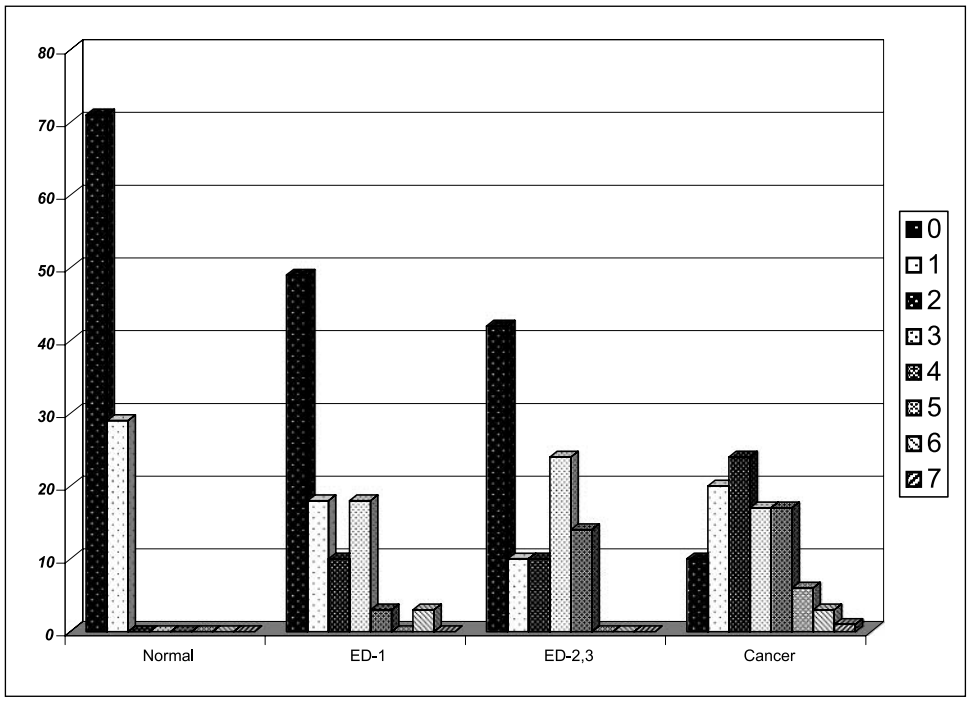


**Fig. 3.** Epigenetic progression of ESCC. The frequency of the number of methylated genes is plotted according to histologic grade. Shaded columns, number of methylated genes for tumors categorized according to histologic grade. All normal samples had zero to one methylated genes. Dysplastic lesions varied, with most low-grade lesions (*ED-1*) showing zero to three changes and higher-grade lesions (*ED-2,3*) with zero to four changes. Most cancers were found to have one to four changes. Intermediate-grade (ED2) and high-grade (ED3) dysplasias were combined into one group.

**Table 3.** Methylation changes of eight loci in multiple lesions from individual patients

| ED1 | Gene(s) methylated | ED2 | Gene(s) methylated | ED3 | Gene(s) methylated | ESC | Gene(s) methylated | Change |
|---|---|---|---|---|---|---|---|---|
| | | **108** | None | | | **36** | None | 0 |
| **119** | None | | | | | **77** | None | 0 |
| **127** | None | | | | | **89** | None | 0 |
| **120** | D | | | | | **44** | None | −1 |
| **129** | None | | | | | **51** | B | 1 |
| **125** | None | | | | | **94** | R | 1 |
| **121** | None | | | | | **40** | C | 1 |
| **144** | None | **95** | None | | | **48** | R | 1 |
| | | | | **90** | None | **81** | A | 1 |
| **142** | None | | | | | **58** | R, E | 2 |
| **124** | None | | | | | **105** | E, R | 2 |
| **126** | None | | | | | **25** | M, B | 2 |
| **122** | None | | | **73** | None | **65** | C, B | 2 |
| **146** | None | | | | | **61** | E, B, D | 3 |
| **140** | None | | | | | **91** | C, E, R, D | 4 |
| **147** | None | | | | | **84** | A, R, B, C | 4 |
| **98** | C | | | | | **52** | C | 0 |
| **71** | C | | | | | **56** | C | 0 |
| **130** | C, D | **117** | C | | | **23** | C, A | 1, −1 |
| **131** | C, L, D | | | | | **50** | C, E | 1, −2 |
| **128** | C, D | **102** | C, D, R | **113** | D, R | **72** | C, D, R | 1 |
| | | **110** | C | | | **68** | C, B, D | 2 |
| **136** | C, M | | | | | **67** | C, M, E, R | 2 |
| **97** | C | | | **93** | C, M, L, R | | | 3 |
| **103** | M | | | | | **79** | M, B, R, E | 3 |
| | | **109** | C, R, D, E | | | **148** | C, R, D, E, M | 1 |
| **123** | R, E, D | | | | | **22** | R, E, D, C | 1 |
| **106** | R, E, D | **82** | E, L | | | **92** | R, E, L, M, D | 2 |
| | | **101** | R, D, L | | | **38** | R, L, M, E, B, C | 4, −1 |
| **132** | C, M, D | | | | | **85** | C, M, B, E | 2, −1 |
| **133** | M, R, B, D, C, E | | | | | **74** | M, R, B, D | −2 |

NOTE: Each line represents an individual with sample number (in bold) to the left of the alterations observed at each loci. The number of changes during progression is summarized to the right with negative numbers indicating losses of methylation.
Abbreviations: C, CDKN2A/p16; M, MGMT; L, MLH1; D, DAPK; E, E-cadherin/CDH1; R, RARβ2; B, BRCA1; A, APC.

mutation accumulation that occurs during tumor progression (46). During this multistep process, some events often appear early whereas others are typically later. Previous studies have suggested that methylation of CDKN2A/p16 occurs early in the development of lung (30) and esophageal cancers (12, 21), underscoring the importance of loss of cell cycle checkpoint control early in tumor development. Methylation of MGMT also seems to be early as in other tumors (47, 48) perhaps due to relationship between loss of MGMT and formation of mutations in p53 in ESCC (24) as was also seen in colon cancer (47).

The progression in methylation of CDH1, most frequent in high-grade dysplasia and invasive cancer, may be attributed to the role of this gene in invasion and metastasis. For other genes with later changes (e.g., *BRCA1*, *MLH1*, and *APC*), the reasons behind this later timing are not clear. There seems to be no absolute, specific order of molecular events occurring in for individual patients as seen in Table 3. However, the progression in the number of genes methylated support for the clonal evolution of these lesions during tumor progression (49), further supported by the acquisition of additional methylated genes in matched samples from patients with dysplasia and cancer. This report shows the use of esophageal squamous cell cancer as a model to study the genetic and epigenetic alterations involved in the development and later progression of human neoplasia. In addition, an understanding of these changes is necessary to develop early detection markers that may detect not only cancer but also high-risk preinvasive lesions because increases in methylation of these genes parallel the histologic progression observed. Such information has been useful in the detection of methylation changes for the early detection of cancer (50).

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