Association of Global Levels of Histone Modifications with Recurrence-Free Survival in Stage IIB and III Esophageal Squamous Cell Carcinomas

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Abstract

This study was aimed at understanding the effects of histone modifications on recurrence-free survival (RFS) after esophagectomy in esophageal squamous cell carcinoma (ESCC). The acetylation of histone H3 lysine (H3K9Ac), histone H3 lysine 18 (H3K18Ac), and histone H4 lysine 12 (H4K12Ac), and the dimethylation of histone H3 lysine 9 (H3K9diMe) and histone H4 arginine 3 (H4R3diMe) were analyzed by immunohistochemistry in 237 ESCCs. The K-means clustering algorithm was used to identify unique patterns of histone modifications. At a median follow-up of 5.1 years, 109 (46%) of 237 patients had developed recurrence of disease. Mean global levels of H3K9Ac, H3K18Ac, H3K9diMe, H4K12Ac, and H4R3diMe were 81.5%, 65.1%, 80.3%, 45.9%, and 27.4%, respectively. In the analysis of individual histones, a 1% increase in the global level of H3K18Ac in pathologic stage III worsened RFS at 1.009 times [95% confidence interval (CI), 1.001-1.016; P = 0.03], after adjusting for age, sex, and operative method. Cluster analysis also showed significant effects of histone modifications on RFS. For stage IIB cancers, Cox proportional hazards analysis showed that RFS of cluster 1, with high global levels of H3K18Ac and H4R3diMe, was 2.79 times poorer (95% CI, 1.14-6.27; P = 0.008) than that of cluster 2, with low levels. RFS for stage III cancers was also poorer in cluster 1 than cluster 2 (adjusted hazard ratio, 2.42; 95% CI, 1.10-5.34; P = 0.02). In conclusion, the present study suggests that global levels of histone modifications in ESCC may be an independent prognostic factor of RFS. Cancer Epidemiol Biomarkers Prev; 19(2); 566–73. ©2010 AACR.

Introduction

Esophageal cancer is characterized by one of the highest fatality rates of all cancers (1). Worldwide, 300,000 new cases are diagnosed annually; moreover, its incidence has risen in recent decades in Western countries. Although the risk of esophageal resection has been reduced to an acceptable level over the past 10 years with advances in surgical and anesthetic techniques and improvements in perioperative management, the current postoperative prognosis remains grave, and the 5-year survival rate is under 25%, even in patients undergoing surgery with curative intent (1, 2). The poor prognosis is partially due to the high rates of recurrence after surgical resection of all gross tumors, with residual cancer cells frequently present at the resection margins. Thus, it is crucial to develop biomarkers for predicting recurrence and to develop efficient treatments for preventing recurrence after surgery in these patients.

Histones are subject to posttranslational modifications, including acetylation and methylation of lysines (K) and arginines (R). Histone modification is involved not only in tumorigenesis, but also in tumor invasion and metastasis. The global levels of acetylated histone H4 are reduced in most gastric and colorectal cancers (reviewed in ref. 3), and global loss of monoacetylation and trimethylation of histone H4 also occurs in human cancer cells (4). Recently, several groups have reported that global levels of histone modifications predict clinical outcomes in human cancers such as prostate (5, 6), kidney (6), lung (6-8), ovary (9), pancreas (9), breast (9, 10), and stomach (11). In esophageal cancer, histone H4 is significantly hyperacetylated in the early stage, and changed into a hypoacetylated state with cancer progression (12). Reduced expression of acetylated histone H4 is correlated with nodal involvement and metastasis in patients with esophageal squamous cell carcinoma (ESCC; ref. 13). In addition, low levels of histone H3 lysine 18 acetylation (H3K18Ac) and histone H3 lysine 27 trimethylation (H3K27triMe) are significantly correlated with better survival in early stages of ESCC (14).
H3K9 modification is important in the epigenetic silencing of tumor suppressor genes but its effect on cancer outcomes is not clear in ESCC (15, 16). To define potential prognostic indicators for recurrence-free survival (RFS) in ESCC, we investigated the modification patterns of five histones in 237 ESCCs, including H3K9, using immunohistochemistry. Among the five histones studied, the acetylation of H3K9, H3K18, H4K12, and the dimethylation of H4R3 are associated with active transcription, whereas the dimethylation of H3K9 was associated with inactive transcription.

Materials and Methods

Study Population

From September 1994 to December 2001, 340 patients underwent esophagectomy for esophageal cancer at the Department of Thoracic and Cardiovascular Surgery at Samsung Medical Center in Seoul, Korea. Esophagectomy specimens were grossly and histologically examined to determine the pathologic tumor-node-metastasis classification as established by the American Joint Committee on Cancer (17). All patients were followed up at 1 mo after operation, at 3-mo intervals for the first 2 y, at 6-mo intervals for the next 2 y, and annually thereafter. Follow-up evaluation included routine blood biochemistry, chest radiography, and chest computed tomography (CT) from the lower neck to the upper abdomen. Endoscopy, abdominal ultrasonography, brain magnetic resonant imaging, bone scans, or positron emission tomography-CT were done if the patient was symptomatic.

Recurrence was defined as the reappearance of a tumor beyond 1 mo after surgery. The evidence of a tumor within 1 mo was considered to be persistent neoplastic disease. Histologic, cytologic, or unequivocal radiologic proof was required before a diagnosis of recurrence was made. Lymph node metastasis was defined by the following criteria: (a) a short-axis diameter over 1 cm, or (b) marginal enhancement with central necrosis on computed tomography, or (c) persistent enlargement during follow-up. Recurrences supported by clinical impressions alone were not included. Recurrence or death was evaluated from the information obtained from our and other hospital records, as of September 30, 2008. Patients with histology other than squamous cell carcinoma and patients who died in the hospital after operation were excluded for the analysis of RFS. Two hundred and thirty-seven of 340 patients were included in the final data analysis.

Surgical Approach

The surgical approach included transhiatal esophagectomy; the Ivor Lewis operation for middle or lower thoracic esophageal cancer; the three-hole (modified McKeown) operation for upper thoracic esophageal cancer; and three-field lymph node dissection for upper thoracic or cervical esophageal cancer with an abdominal lymphadenectomy, a total mediastinal lymphadenectomy, or bilateral cervical lymphadenectomy. The three-hole operation was completed with a cervical incision for esophagogastric anastomosis. Lymphadenectomy of subcarinal, periesophageal, subaortic, and both recurrent laryngeal lymph nodes was done. Abdominal lymphadenectomy comprised en bloc resection of all lymphatic tissue in the lower posterior mediastinum, in the left and right paracardial regions, along the lesser curvature, and along the celiac and left gastric arteries. Cervical lymphadenectomy was composed of en bloc resection of all lymphatic tissue along with deep internal and external cervical nodes, and supraclavicular nodes.

Tissue Microarray and Immunohistochemistry

Tissue microarrays (TMA) of ESCC were prepared as previously described (18). Briefly, three tumor areas were carefully selected from a donor block using H&E staining, and 2.0-mm-size cores were reembedded into a recipient paraffin block using a Tissue Microarrayer (Beecher Instruments). Four-micrometer-thick tissue sections were taken from the TMA blocks for immunohistochemistry, deparaffinized in xylene, and rehydrated through an alcohol series. The rehydrated sections were treated with Hydrogen Peroxide Block (Lab Vision) and Ultra V Block (Lab Vision) for 5 min each to block endogenous peroxidase. To unmask the antigens, sections were microwaved in citrate buffer [0.01 mol/L citric acid (pH 6)] for a total of 5 min and were incubated for blocking nonspecific proteins with 5% goat serum in PBS for 1 h. The sections were then incubated overnight at 4°C with primary antibodies to H3K9Ac (Abcam) at 1:200, H3K18Ac (Cell Signaling Technology, Inc.) at 1:200, H4K12Ac (Abcam) at 1:100, H3K9diMe (Upstate) at 1:100, and H4R3diMe (Upstate) at 1:25. Detection of immunoreactivity by each antibody was done by the Vectastain Elite ABC reagent (Vector Laboratories), and 3,3′-diaminobenzidine tetrahydrochloride was used as a chromogen. All sections were counterstained with Mayer’s hematoxylin, and negative controls were included in each staining sequence. The intensity and global level of staining were scored semiquantitatively for each TAM by a pathologist (EY Cho) who was blinded to all clinicopathologic variables. The global level of staining refers to the percentage of tumor cells that stained positively for an antibody within each TAM at ×200 magnification using a light microscope.

Statistical Analysis

Pearson’s correlation coefficient or Spearman’s rank correlation coefficient was used to test the relationships between the global expression levels of five histones. Comparisons of two groups with respect to global expression levels of histones were done using the Student’s t test or the Mann-Whitney test or the Kruskal-Wallis test. The K-means clustering algorithm was applied to identify unique patterns of histone modifications. K-means clustering is a method of cluster analysis that aims to partition n observations into k clusters by iterative reallocation of candidate members such that the sum of
squares from points to the assigned cluster centers is minimized (19). The RFS function was estimated by the Kaplan-Meier method, and the log-rank test was used to compare RFS curves. Cox proportional hazards regression analysis was used to estimate the hazard ratios (HR) of independent factors for RFS, after controlling for potential confounding factors such as age, sex, operative methods, pT, pN, and pM. All statistical analyses were

Figure 1. Immunohistochemical analysis of histone modification in ESCC samples. Left, representative examples of diffuse and strong nuclear staining (original magnification, ×400) H3K9Ac (A), H3K18Ac (B), H3K9diMe (C), H4K12Ac (D), and H4R3diMe (E). Right, distribution of positive staining of each histone. X-axis, percentage of cells stained positively for each histone. Y-axis, the corresponding frequencies of particular data values within each interval of the X-axis.
two sided, with a 5% type I error rate. The Statistical Package for the Social Sciences for Windows (release 13.0, SPSS, Inc.) was used to analyze data.

Results

Characteristics of Histone Modifications

Representative immunostaining for five histones, and the frequencies of patients with the indicated global levels of each histone, are shown in Fig. 1. Only nuclear staining for the five histones was regarded as positive. Positive staining of H3K9Ac and H3K9diMe was found in 232 (97.9%) and 236 (99.6%) of 237 patients. The expression of H3K18Ac, H4K12Ac, and H4R3diMe occurred in 60% to 80% of patients. Staining intensities of H4K12Ac and H4R3diMe were weak in >50% of samples, in contrast to that of H3K9Ac, H3K18Ac, and H3K9diMe. The mean global levels of H3K9Ac, H3K18Ac, H3K9diMe, H4K12Ac, and H4R3diMe were 81%, 65%, 80%, 45%, and 27%, respectively.

Global levels of H3K9Ac and H3K18Ac and H3K9diMe had very similar distributions, with >60% of the samples showing 90% to 100% staining of the tumor cells (Fig. 1A-C). H3K18Ac and H4R3diMe showed broad distributions at global levels (Fig. 1D-E). The global levels of each histone modification were an independent prognostic factor for RFS after adjusting confounding factors (Table 3). Among the five histones studied, high global levels of H3K18Ac were significantly associated with patient age and sex. Fifty-eight percent (122 of 210) of the carcinomas were moderately differentiated and 18% (37 of 210) were poorly differentiated. The global levels of H3K9Ac, H3K9diMe, H4K12Ac, and H4R3diMe in well-differentiated cases showed a tendency to be greater than those in moderately or poorly differentiated cases. However, these levels were not found to be statistically significant. Of 237 patients, 2% (4 of 237) of the patients were in pathologic stage 0, 13% (30 of 237) of the patients were in pathologic stage I, 27% (65 of 237) were in pathologic stage IIA, 10% (23 of 237) were in pathologic stage IIB, and 32% were (76 of 237) in pathologic stage III. No associations were found between the global levels of the five histones and pathologic stage.

RFS Analysis

The median duration of follow-up was 5.1 years, and recurrence was observed in 109 (46%) of 237 patients. RFS was analyzed with respect to global levels of histone modifications individually or in combination. Global levels of individual histones in univariate analysis were not associated with RFS (data not shown), but clustering analysis showed a significant relationship between clusters with distinct patterns of histone modifications and RFS. The median RFS time of cluster 1 was 2.3 years (SEM, 0.427) and 6.2 years (SEM = 2.0) for cluster 2. Recurrence-free 5-year survival rates for cluster 1 and cluster 2 were 38% and 78%, respectively, and this difference was statistically significant (P = 0.01). Because tumor recurrence was found to be significantly associated with pathologic stage at the time of curative resection, we reanalyzed RFS after stratifying the data according to pathologic stage. RFS was not significantly different between clusters in stage I (Fig. 3A) and stage IIA (Fig. 3B), and IV, but cluster 1 showed significantly poorer RFS in stage IIB and stage III cancers. The recurrence-free 5-year survival rates for the 23 stage IIB cancers were 34% and 78% in cluster 1 and cluster 2, respectively, and this difference was statistically significant (P = 0.002; Fig. 3C). For the 76 stage III cancers, the recurrence-free 5-year survival rates also showed a significant difference between cluster 1 and cluster 2 (18% versus 52%, respectively; P = 0.005; Fig. 3D).

Cox Proportional Hazards Analysis

Stratified Cox proportional hazards regression analysis was conducted to determine whether any of the patterns of histone modifications were an independent prognostic factor for RFS after adjusting confounding factors (Table 3). Among the five histones studied, high global levels of H3K18Ac were significantly associated with

| Table 1. Correlation between global levels of each histone modification |
|------------------------|-----------------|-----------------|-----------------|-----------------|
|                        | H3K18Ac | H3K9diMe | H4K12Ac | H4R3diMe |
| H3K9Ac                 | 0.092   | 0.184*   | 0.301*   | 0.172*   |
| H3K18Ac                | 0.091   | 0.111    | 0.184*   | 0.167*   |
| H3K9diMe               | 0.206*  | 0.117    | 0.244*   | 0.185*   |
| H4K12Ac                | 0.185*  | 0.117    | 0.244*   | 0.185*   |

NOTE: Numbers indicate r coefficient.

*Correlation is significant at the 0.01 level.

†Correlation is significant at the 0.05 level.
poor RFS [adjusted HR, 1.009; 95% confidence interval (CI), 1.001-1.016; \( P = 0.03 \)] in stage III cases, after controlling for age, sex, and operative method. For stage IIB cancers, RFS of cluster 1 was 2.79 times poorer (95 CI%, 1.14-6.27; \( P = 0.008 \)) than that of cluster 2, after controlling for the same confounding factors. In stage III cancers, cluster 1 was also significantly associated with short RFS (adjusted HR, 2.42; 95 CI%, 1.10-5.34; \( P = 0.02 \)).

**Discussion**

Although recent works suggest that global patterns of histone modifications can be used to predict patient prognoses in a variety of cancers, histone modifications relative to DNA methylation are less well characterized as prognostic indicators in ESCC. We retrospectively analyzed the relationship between RFS and histone modifications in 237 ESCCs to identify a recurrence-associated epigenetic prognostic indicator after esophagectomy in patients with ESCC. We found that the relationships between histone modifications and RFS were significantly different according to pathologic stage. Of the five histones studied, the global level of H3K18Ac was significantly associated with RFS in stage III cases. Clustering analysis showed that cluster 1, with high global levels of H3K18Ac and H4R3diMe, had a poor RFS in stage IIB and stage III cases. These observations suggest that histone modifications might be an independent risk factor for RFS in ESCC, according to pathologic stage, and that precise information can be obtained by a combinatory analysis.

The histone code hypothesis suggests that distinct modifications of individual histone residues on the same or different histone tails can act sequentially or cooperatively in a combinatorial fashion to form a “histone code” for distinct chromatin structure, and to specify a biological event (20). Distinct patterns of lysine acetylation that define groups of biologically related genes were generated using the K-means clustering algorithm (19). In addition, histone modifications in cells tend to colocalize in different regions of genes for transcriptional regulation, and to correlate...
with each other at an individual nucleosome level (21). Several groups have reported the importance of the combinatorial effect of histone modifications in cancer prognosis since Seligson and colleagues (5) identified specific patterns of histone modifications that were related to patient outcome by applying the random forest clustering algorithm in prostate cancer. Classification of stage I non–small cell lung carcinoma patients by a recursive partitioning analysis also found that patient prognosis differed according to global levels of H3K9Ac and H3K4Me2 (7). Recently, Elsheikh and coworkers (10) applied for the K-means and the partitioning around medoids algorithms for clustering analysis, and identified distinct patterns of histone modifications showing significant difference in cancer outcome in 880 breast cancers.

Although modifications of all five histones in this study contributed to patient grouping by the K-means clustering algorithm, the most significant difference between the two clusters was found in H3K18Ac and H4R3diMe: mean global levels of H3K18Ac in cluster 1 and cluster 2 were 91.5% and 2.7%, respectively ($P < 0.0001$), and global levels of H4R3diMe were 30.0% in cluster 1 and 20.5% in cluster 2 ($P = 0.03$). This suggests that global levels of H3K18Ac and H4R3diMe might be sufficient to distinguish two groups of patients with distinct clinical outcomes and, moreover, that they may play a major role in determining RFS in ESCC. However, the mechanism underlying poor RFS in ESCC patients with high global levels of H3K18Ac and H4R3diMe is not clear. Adenovirus E1A is known to induce global hypoacetylation of H3K18 by redirecting the recruitment of histone acetyltransferase p300/CBP and repressors such as Rb to specific gene promoters/enhancers (22, 23). In addition, the modification of H3K18Ac is detected only at genes that promote entry into the cell cycle. These findings are in contrast with our results but instead support previous studies reporting poor prognostic effects of H3K18 hypoacetylation in prostate cancer (5), adenocarcinomas of lung and kidney (6), and breast cancer including basal carcinomas and human epidermal growth factor receptor-2–positive tumors (10).

In this study, the hyperacetylation of H3K18 was significantly associated with poor RFS in ESCC, supporting the results of a report by Tzao and colleagues (14) that low expression of H3K18Ac and H3K27triMe correlated with better prognosis of patients with ESCC. The cause of the tissue-specific differences in the effects of H3K18Ac on cancer outcomes is not clear. One possibility is that the effect of H3K18Ac on cancer outcome is modified by a third factor in different types of cancers. In this study, global levels of H3K18Ac were significantly correlated with those of H4R3diMe ($P < 0.01$).

H3K18Ac in human CD+ T cells is mainly located in the region surrounding a transcriptional start site (21), and symmetrical methylation of H4R3diMe by protein methyltransferase serves as a direct binding target for the DNA methyltransferase DNMT3A and contributes to gene silencing by subsequent DNA methylation.

Table 2. Relationships between clinicopathologic characteristics and the global expression levels of five histones ($n = 237$)

| Variables      | H3K9Ac  |  | H3K18Ac  |  | H3K9diMe |  | H4K12Ac  |  | H4R3diMe |  |
|----------------|---------|  |----------|  |----------|  |----------|  |----------|  |
| Age            |         |  |          |  |          |  |          |  |          |  |
| <62 (116)      | 81 ± 22 | 0.54 | 62 ± 41  | 0.08 | 79 ± 24  | 0.08 | 44 ± 35  | 0.08 | 26 ± 35  | 0.08 |
| ≥62 (121)      | 81 ± 21 | 67 ± 41 | 1.00 | 81 ± 24  | 0.00 | 46 ± 34  | 0.00 | 26 ± 34  | 0.00 |
| Sex            |         |  |          |  |          |  |          |  |          |  |
| Men (221)      | 80 ± 22 | 0.08 | 65 ± 41  | 0.08 | 80 ± 25  | 0.08 | 46 ± 34  | 0.08 | 26 ± 34  | 0.08 |
| Women (16)     | 91 ± 8  | 58 ± 46  | 0.00 | 83 ± 19  | 0.00 | 37 ± 35  | 0.00 | 27 ± 30  | 0.00 |
| Differentiation | |  |          |  |          |  |          |  |          |  |
| Well (51)      | 89 ± 9  | 60 ± 44  | 0.00 | 87 ± 20  | 0.00 | 53 ± 32  | 0.00 | 37 ± 39  | 0.00 |
| Moderate (122)| 79 ± 24 | 64 ± 42  | 0.00 | 78 ± 24  | 0.00 | 41 ± 34  | 0.00 | 23 ± 33  | 0.00 |
| Poor (37)      | 78 ± 22 | 64 ± 39  | 0.00 | 77 ± 26  | 0.00 | 46 ± 36  | 0.00 | 22 ± 32  | 0.00 |
| Pathologic stage | |  |          |  |          |  |          |  |          |  |
| 0, I (34)      | 79 ± 23 | 74 ± 38  | 0.00 | 81 ± 25  | 0.00 | 45 ± 34  | 0.00 | 25 ± 36  | 0.00 |
| II (65)        | 83 ± 19 | 69 ± 40  | 0.00 | 85 ± 17  | 0.00 | 48 ± 36  | 0.00 | 29 ± 35  | 0.00 |
| IIIB (23)      | 75 ± 24 | 54 ± 44  | 0.00 | 78 ± 25  | 0.00 | 46 ± 36  | 0.00 | 32 ± 38  | 0.00 |
| III (76)       | 81 ± 24 | 61 ± 43  | 0.00 | 82 ± 23  | 0.00 | 43 ± 35  | 0.00 | 23 ± 31  | 0.00 |
| IV (39)        | 82 ± 19 | 62 ± 42  | 0.00 | 68 ± 32  | 0.00 | 42 ± 31  | 0.00 | 23 ± 34  | 0.00 |

*Numbers in parentheses indicate the number of cases.

1 All values indicate mean global levels of each histone ± SD (%).

2 Differentiation data are missing for 27 patients.

3P values are based on the Mann-Whitney test or the Kruskal-Wallis test.
Accordingly, the CpG island methylation of genes involved in the prevention of cell proliferation and tumor invasion may affect the association between global levels of H3K18Ac and H4R3diMe and RFS in ESCC. Considering the enormous number of histone modification sites, information on the global modification patterns of other histones and on their interaction with specific oncogenes or tumor suppressor genes might be helpful in understanding the tissue-specific effects of H3K18Ac modification on clinical outcome.

The effects of histone modification on patient prognosis depend on cell type as well as pathologic stage. Seligson and coworkers (5) reported that patterns of histone modifications were predictors of tumor recurrence independently of tumor stage in patients with low-grade prostate cancer. However, Barlesi and colleagues (7) reported that H3K4diMe in large-cell or squamous cell type and H3K9Ac in adenocarcinoma influenced patient survival for stage I non–small cell lung cancer patients. They also found that patient outcomes for pathologic stage II patients were associated with expression levels of H2AK5Ac. Tzao and colleagues (14) also reported that low expression of H3K18Ac and H3K27triMe correlated with better prognosis of patients with ESCC, especially for early stages. In this study, a significant relationship between histone modifications and RFS was found only in stage IIB and III. These different effects of histone modifications on cancer outcome according to cell type and pathologic stage may be attributable to (a) the difference in biological behavior of the tumors, (b) cellular epigenetic heterogeneity, or (c) differences in genetic background.

Histone modification is involved in tumorigenesis, but the timing of histone modifications during a process of tumorigenesis is not clear yet. In this study, the global expression levels of each histone were not significantly

Table 3. Stratified Cox proportional hazards model for RFS

<table>
<thead>
<tr>
<th></th>
<th>HR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>(1) H3K18Ac</td>
<td></td>
<td></td>
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<tr>
<td>Stage IIB</td>
<td>1.008 (0.990-1.026)</td>
<td>0.39</td>
</tr>
<tr>
<td>Stage III</td>
<td>1.009 (1.001-1.016)</td>
<td>0.03</td>
</tr>
<tr>
<td>(2) Cluster analysis*</td>
<td></td>
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<tr>
<td>Stage IIB</td>
<td>2.79 (1.14-6.27)</td>
<td>0.008</td>
</tr>
<tr>
<td>Stage III</td>
<td>2.42 (1.10-5.34)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE: Cox proportional hazards model was adjusted for age, sex, operative method, and separately analyzed according to pathologic stage.

*Reference in cluster analysis; cluster 2.
associated with pathologic stage, which suggest that the modifications of five histones analyzed in this study may be an early event in ESCC. However, further study is needed in premalignant lesions to clearly understand the timing that histone modifications occur in ESCC. This study was limited by the small number of samples included, and additional work including other modification sites in a larger sample is required to precisely determine the relationship between global levels of histone modifications and RFS in ESCC. In addition, possible interactions between histone modifications and major risk factors for ESCC, including heavy alcohol consumption, tobacco use, and previous caustic injury, require further study. In conclusion, the results of the present study suggest that global levels of histone modifications may be an independent risk factor of RFS after esophagectomy in resected ESCC. In addition, ESCC patients with high global levels of H3K18Ac and/or H4R3diMe might benefit from the development of a more aggressive regimen of epigenetic therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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