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MAL hypermethylation is a tissue-specific event that correlates with MAL mRNA expression in esophageal carcinoma

Zhe Jin^{1,3,4*}, Liang Wang^{1*}, Yuan Zhang^{1*}, Yulan Cheng², Yan Gao⁵, Xianling Feng¹, Ming Dong¹, Ziyi Cao¹, Si Chen¹, Huimin Yu¹, Zhenfu Zhao¹, Xiaojing Zhang¹, Jie Liu¹, Yuriko Mori², Xinmin Fan¹ & Stephen J. Meltzer²

¹Department of Pathology, The Shenzhen University School of Medicine, Shenzhen, Guangdong, People's Republic of China, ²Division of Gastroenterology, Department of Medicine, The Johns Hopkins University School of Medicine and Sidney Kimmel Comprehensive Cancer Center, Baltimore, Maryland, USA, ³Shenzhen Key Laboratory of Micromolecule Innovative Drugs, Shenzhen, Guangdong, People's Republic of China, ⁴Laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, Guangdong, People's Republic of China, ⁵Nanshan Hospital, Guangdong Medical College, Shenzhen.

MAL promoter hypermethylation was examined in 260 human esophageal specimens using real-time quantitative methylation-specific PCR (qMSP). MAL hypermethylation showed highly discriminative ROC curve profiles which clearly distinguished esophageal adenocarcinomas (EAC) from both esophageal squamous cell carcinomas (ESCC) and normal esophagus (NE). Both MAL methylation frequency and normalized methylation value (NMV) were significantly higher in Barrett's esophagus (BE), dysplastic BE, and EAC than in ESCC or in NE. Among matched NE and EAC samples, MAL NMVs in EAC were significantly higher than in corresponding NE. There was a significant correlation between MAL hypermethylation and BE segment length. Treatment with 5-aza-2'-deoxycytidine reversed MAL methylation and reactivated MAL mRNA expression in OE33 EAC cells. MAL mRNA levels in EACs with unmethylated MAL were significantly higher than in EACs with methylated MAL. MAL hypermethylation is a common, tissue-specific event in human EAC and correlates with clinical neoplastic progression risk factors.

Esophageal cancer ranks as the 8th most-frequent and 6th most-fatal cancer type worldwide, with an estimated 480,000 new cases diagnosed and 400,000 deaths globally in 2008¹. This cancer consists of two major histologic subtypes: esophageal adenocarcinoma (EAC), which is more prevalent in Western countries, with a rapidly increasing incidence; and esophageal squamous cell carcinoma (ESCC), which is frequent in developing countries, especially in Asia, and including China². Since both types of esophageal cancer exhibit highly aggressive behavior, with rapid progression to death³, a better understanding of the molecular events underlying their pathogenesis is essential to achieving improved survival. Therefore, we sought to discover molecular events with potential early detection biomarkers or targets of chemoprevention and therapy.

Myelin and Lymphocyte protein (MAL, also known as mal or T-cell differentiation protein), a 17 kDa hydrophobic membrane protein, is widely expressed in a variety of cell types, including T-lymphocytes⁴, myelin-forming cells^{5,6}, and epithelial cells of the kidney, stomach, and large intestine^{7,8}. It has recently been clarified that MAL constitutes an essential component of glycolipid-enriched membrane micro-domains or lipid rafts involved in the apical transport of membrane and secretory proteins in polarized epithelial cells^{9,10}. Apical transport is essential for the proper functioning of epithelial cells, and the neoplastic transformation process is frequently associated with loss of this polarized phenotype¹¹. Several investigations have indicated that down-regulation of MAL may constitute as a common molecular event contributing to the initiation and/or progression of several cancers, including those arising in the digestive tract. For example, using tissue microarrays, Guro *et al.* found that epithelial cells of colorectal carcinomas were MAL-negative, whereas in normal colonic tissues cytoplasmic expression of MAL occurred in both epithelial and connective tissues¹². Koshi *et al.* showed that MAL expression was reduced or absent in esophageal cancers vs. normal cells, using differential display experiments¹³. Dysregulation of MAL has also been implicated in several other malignancies, including breast¹⁴, cervical¹⁵ and HNSCC cancers¹⁶. These results suggest that MAL possesses tumour-suppressive capabilities.

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Correspondence and requests for materials should be addressed to Z.J. (zhejin1995@yahoo.com) or S.J.M. (smeltzer@jhmi.edu)

* These authors contributed equally to this work.

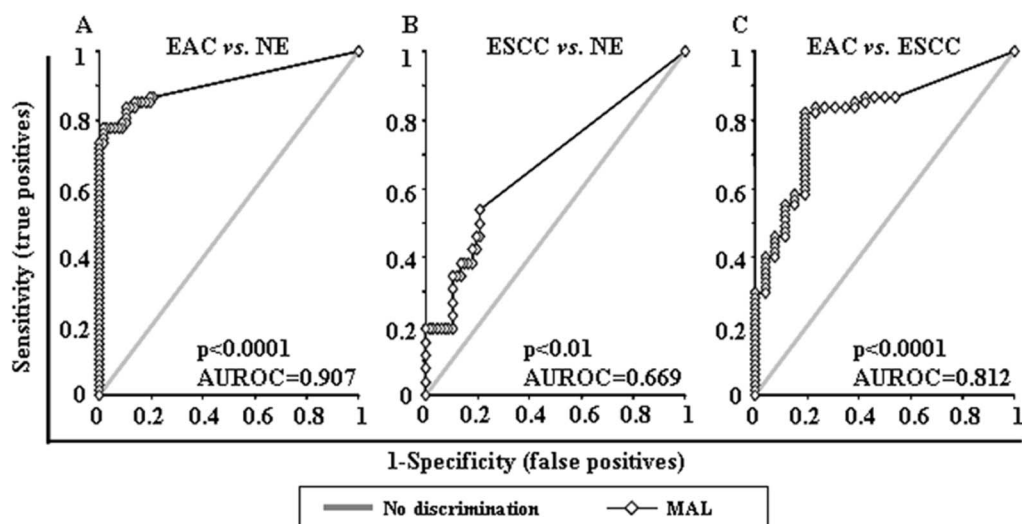


Figure 1 | Receiver-operator characteristic (ROC) curve analysis of normalized methylation value (NMV). ROC curve analysis of *MAL* NMVs of normal esophagus (NE) vs. esophageal adenocarcinoma (EAC) (A), NE vs. esophageal squamous cell carcinoma (ESCC) (B), and EAC vs. ESCC (C). The area under the ROC curve (AUROC) conveys this biomarker's accuracy in distinguishing EAC from NE and from ESCC in terms of its sensitivity and specificity.

Aberrant methylation of promoter CpG islands upstream of tumor suppressor genes is now well-established as a major mechanism of gene inactivation in human tumorigenesis¹⁷, including ESCC and EAC¹⁸, where it plays an important role in pathogenesis^{19–22}. Some of these methylation events appear to represent useful prognostic markers, as they precede and thus predict the progression of Barrett's esophagus (BE) to EAC²³. Aberrant promoter methylation of *MAL* is indeed associated with inactivation of its expression in breast and colorectal cancers^{12,14}. Therefore, we hypothesized that *MAL* might be inactivated via promoter hypermethylation in human esophageal cancers, and that hypermethylation of *MAL* could constitute an early event in the genesis of EAC.

Results

MAL promoter hypermethylation in different esophageal tissues.

Promoter hypermethylation of the *MAL* gene was analyzed in 67 normal esophagus (NE), 60 BE, 40 dysplasias occurring in BE [D, 19 low-grade (LGD) and 21 high-grade (HGD)], 67 EAC and 26 ESCC samples. *MAL* promoter hypermethylation showed highly discriminative receiver-operator characteristic (ROC) curve profiles, which clearly distinguished EAC from both NE and ESCC. ROC curves with corresponding the area under the ROC curve

(AUROC) for *MAL* of EAC vs. NE, ESCC vs. NE and EAC vs. ESCC are shown in **Figure 1**.

The cutoff normalized methylation value (NMV) for *MAL* (0.02) was chosen from ROC curves to maximize sensitivity and specificity. Mean NMV and frequency of *MAL* hypermethylation for each tissue type are shown in **Table 1**. The mean NMV of *MAL* was significantly higher in BE (0.0681, $p = 0.000001$), LGD (0.0945, $p = 0.000004$), HGD (0.0549, $p = 0.000001$), D (0.0737, $p = 0.0000001$), EAC (0.0459, $p = 0.000002$), and ESCC (0.0042, $p = 0.009$) than in NE (0.0001; Student's t-test). The frequency of *MAL* hypermethylation was increased in BE (53.3%), LGD (63.2%), HGD (57.1%), D (60%), and EAC (40.3%) vs. NE (0.0%; all $p < 0.01$; Fisher's exact probability test). Among 28 cases with matched NE and EAC, the *MAL* NMVs in EAC (mean = 0.0610) were significantly higher than in corresponding NE (mean = 0.0001; $p = 0.0015$, Student's paired t-test; **Figure 2A**). However, Among 13 cases with matched NE and ESCC, the difference of *MAL* NMVs in ESCC (mean = 0.0023) and in corresponding NE (mean = 0.0003) were not significant ($p = 0.29$, Student's paired t-test; **Figure 2B**). Finally, both NMVs and hypermethylation frequency of *MAL* were significantly higher in BE, D and EAC than in ESCC (**Table 1**).

According to generally accepted criteria²⁴, BE was defined as long-segment (LSBE) if it was equal to or greater than 3 cm in length, or

Table 1 | Clinicopathologic characteristics and methylation status of *MAL* in human esophageal tissues

Histological type	No. of samples	Age (year) mean	Gender M/F	Race W/B/A/unknown	NMV	Methylation Frequency
					mean \pm SD*	cut off 0.02**
Normal esophagus	67	64.4	60/7	40/11/4/12	0.00014 \pm 0.00039	0
Barrett's metaplasia	60	63.7	56/4	43/3/1/13	0.0681 \pm 0.09951 ^{#/\$}	53.3% (32/60) ^{#/\$}
Dysplasia in Barrett's esophagus	40	65.3	35/5	29/3/2/6	0.0737 \pm 0.11781 ^{#/\$}	60% (24/40) ^{#/\$}
Low-grade dysplasia	19	65.3	17/2	13/2/1/3	0.0945 \pm 0.15812 ^{#/\$}	63.2% (12/19) ^{#/\$}
High-grade dysplasia	21	65.2	18/3	16/1/1/3	0.0549 \pm 0.06149 ^{#/\$}	57.1% (12/21) ^{#/\$}
Esophageal adenocarcinoma	67	65.1	58/9	57/1/2/7	0.0459 \pm 0.07453 ^{#/\$}	40.3% (27/67) ^{#/\$}
Esophageal squamous cell carcinoma	26	62.5	19/7	8/13/2/3	0.0042 \pm 0.01248 [#]	7.7% (2/26)

M, male; F, female; W, white; B, black; A, asian; NMV: normalized methylation value.

* $p < 0.01$, Student's t test.

** $p < 0.01$, Fisher's exact probability test.

[#]comparisons made to normal esophagus.

^{\$}comparisons made to esophageal squamous cell carcinoma.

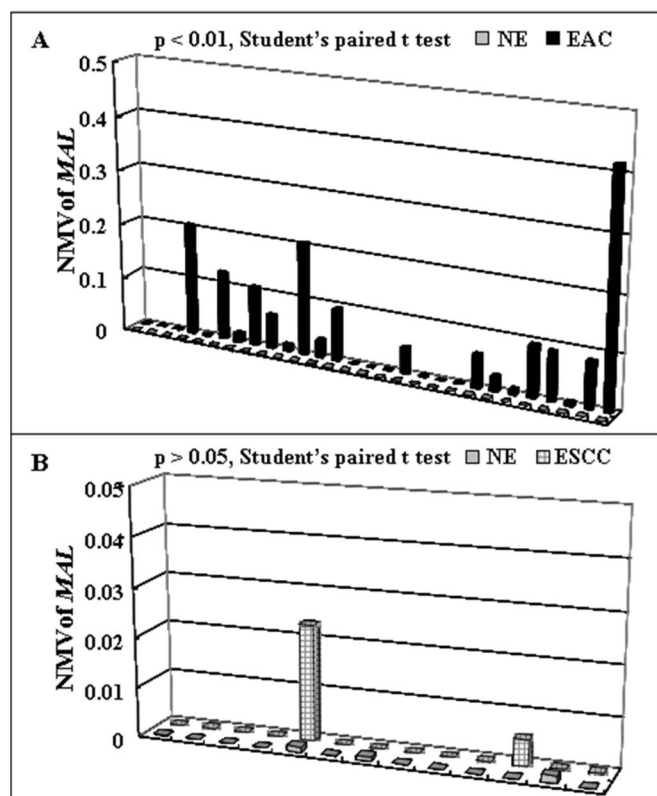


Figure 2 | Methylation status of *MAL* in corresponding esophageal samples. (A) In 28 patients with corresponding NE and EAC, *MAL* normalized methylation values for EAC (mean = 0.0610) were significantly higher than those for matching NE (mean = 0.0001; $p < 0.01$, Student t test for paired data). (B) In 13 patients with corresponding NE and ESCC, the differences between *MAL* NMVs for ESCC (mean = 0.0023) and those for matching NE (mean = 0.0003) were not significantly ($p > 0.05$, Student t test for paired data).

short-segment (SSBE) if less than 3 cm. The mean NMV of *MAL* was significantly higher in LSBE (mean = 0.1235) than in SSBE (mean = 0.0269; $p = 0.03$, Student's t-test, **Figure 3A**). Similarly, segment lengths of BEs with hypermethylated *MAL* promoters (mean =

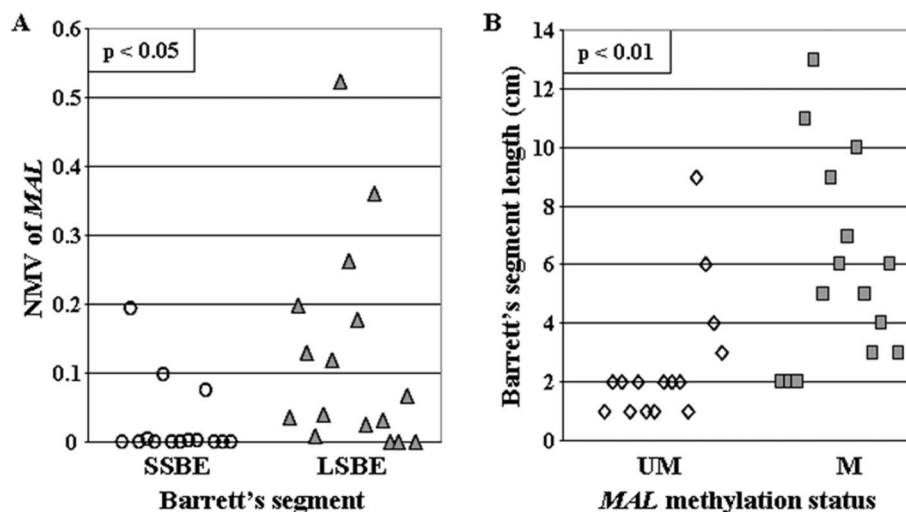


Figure 3 | Correlation between Barrett's segment length and *MAL* hypermethylation. (A) Mean NMV of *MAL* was significantly higher in LSBE (mean = 0.1235, black triangle) than in SSBE (mean = 0.0269, white circle; $p < 0.05$, Student's t test). (B) segment lengths of BEs with hypermethylated *MAL* promoters (mean = 5.87 cm, black square) were significantly greater than segment lengths of BEs with unmethylated *MAL* promoters (mean = 2.60 cm, white rhombus; $p < 0.01$, Student's t-test).

5.87 cm) were significantly greater than segment lengths of BEs with unmethylated *MAL* promoters (mean = 2.60 cm; $p = 0.005$, Student's t-test; **Figure 3B**).

No significant associations were observed between *MAL* promoter hypermethylation and patient age, survival, smoking or alcohol consumption status, tumor stage or lymph node metastasis, histologic tumor differentiation, or histologic type of esophageal carcinoma (data not shown).

***MAL* methylation and mRNA levels in OE33 cells after 5-Aza-dC treatment.** OE33 cells were subjected to demethylation by 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *MAL* was diminished (0.4264, 0.1647 and 0.1414 on day 0, 2 and 4, respectively), while the mRNA level of *MAL* (0.0184 on day 0) was increased on day 2 (0.0643) and further increased on day 4 (0.0773, **Figure 4**).

Inverse correlation between hypermethylation and mRNA expression of *MAL* in human esophageal tissues. To further elucidate the relationship between DNA hypermethylation and mRNA expression of *MAL*, we determined the normalized mRNA value (NRV) of *MAL* in 10 NE and 28 EAC samples using qRT-PCR. The mean NRV of *MAL* in EAC (mean = 0.0819) was significantly lower than in NE (mean = 0.4128; $p = 0.00015$, Mann-Whitney U test; **Figure 5A**). The mean NRV of *MAL* in specimens with unmethylated *MAL* (mean = 0.2418, $n = 24$) was significantly higher than in specimens with hypermethylated *MAL* (mean = 0.0441, $n = 14$; $p = 0.0084$, Mann-Whitney U test; **Figure 5B**). A significant negative correlation (Pearson correlation coefficient $r = -0.363$, $p = 0.025$) between NMVs and NRVs of *MAL* was also found among these samples (**Figure 5C**).

Discussion

MAL down-regulation has been reported in many types of cancer, including breast, cervical, colorectal and esophagus^{14,15,25,26}. These results suggest that low expression of *MAL* may represent a general characteristic or even a requirement of transformed cells in many kinds of carcinogenesis. Potential mechanisms underlying this suppression of expression include posttranscriptional and epigenetic changes, such as aberrant DNA methylation^{20,27}. Koshi *et al.* reported that the expression of *MAL* was extinguished in ESCC, and speculated that DNA methylation and histone deacetylation in the *MAL*

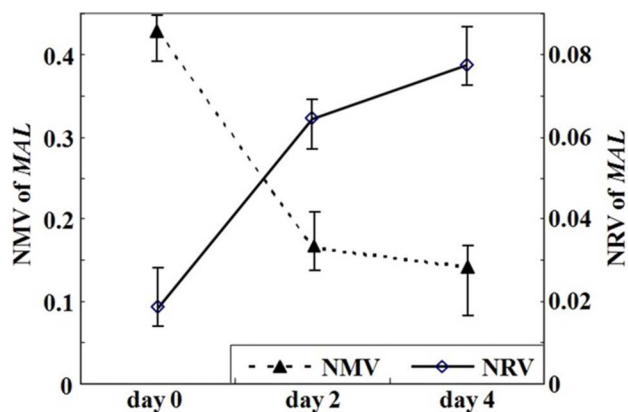


Figure 4 | *MAL* methylation level and mRNA expression in esophageal cancer cell line (OE33) after treatment with the demethylating agent 5-aza-20-deoxycytidine (5-Aza-dC). After 5-Aza-dC treatment, the NMV of *MAL* was diminished, while the NRV of *MAL* was increased.

promoter may act as synergistic controls to silence the *MAL* gene in esophageal cancer¹³. However, evidence for this conclusion was based on 5-aza-20-deoxycytidine and trichostatin A-treated esophageal carcinoma cell lines. Whether hypermethylation of the *MAL*

gene was a common event in esophageal carcinoma was thus still unknown. Further evidence based on sufficient clinical samples was therefore needed.

In the current study, by studying 260 primary human tissue specimens, we systematically investigated hypermethylation of the *MAL* gene promoter in primary human esophageal lesions of differing histological types and grades using real-time quantitative methylation-specific PCR (qMSP). DNA methylation levels were not validated by other methods, such as pyrosequencing. Our results demonstrate that *MAL* promoter hypermethylation occurs frequently in human EAC, but not in ESCC (Table 1). *MAL* NMVs in EAC were significantly higher than in corresponding NE in 28 cases with matched NE and EAC (Figure 2A). However, no significant difference of *MAL* NMVs was found between NE and ESCC in 13 cases with matched NE and ESCC (Figure 2B). In addition, both NMV and hypermethylation frequency of *MAL* were significantly higher in BE, D and EAC than in ESCC (Table 1). These results suggest that hypermethylation of *MAL* may represent an early epigenetic event in these subjects, and that this event is highly prevalent in human esophageal adenocarcinomas.

BE is the major risk factor for esophageal adenocarcinoma²⁸. Although it is somewhat arbitrary to separate SSBE from LSBE, this distinction persists in clinical practice^{29,30}. To date, length of BE as a predictive factor for BE progression remains controversial. While

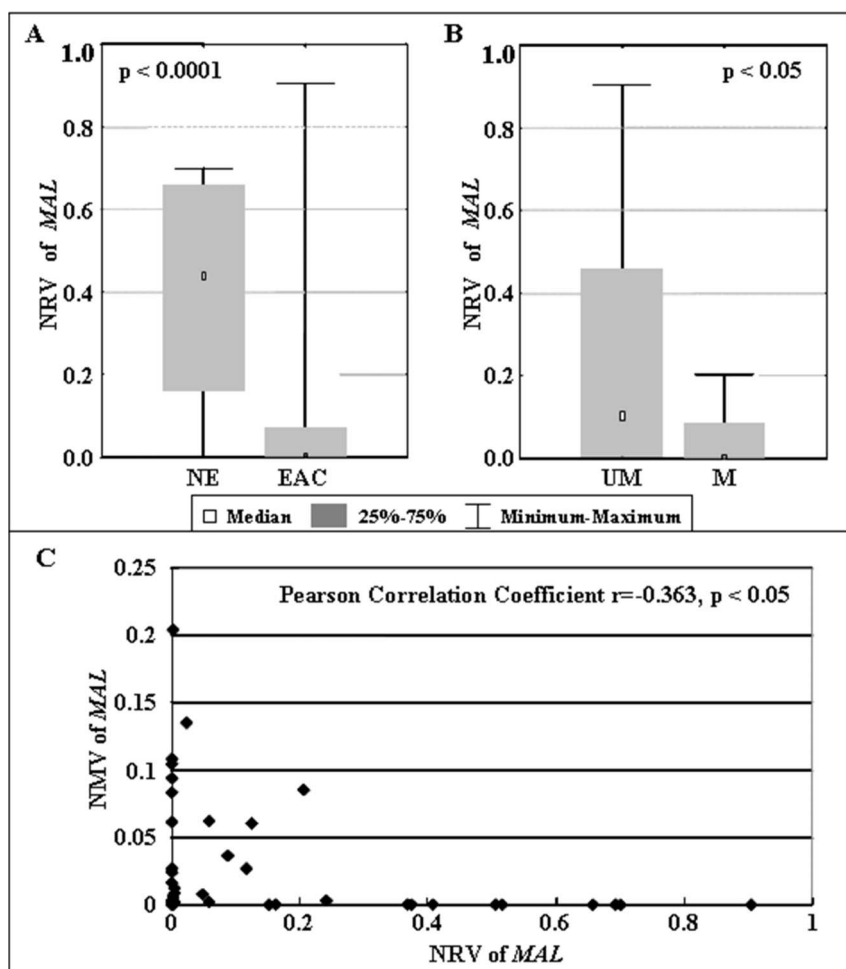


Figure 5 | Correlation between *MAL* methylation status and mRNA expression levels in human esophageal tissues. (A) The mean NRV of *MAL* in EAC (mean = 0.0819, $n = 28$) was significant lower than in NE (mean = 0.4128, $n = 10$; $p < 0.0001$, Mann-Whitney U test). (B) The mean NRV of *MAL* in specimens with unmethylated *MAL* (mean = 0.2418, $n = 24$) was significantly higher than in specimens with hypermethylated *MAL* (mean = 0.0441, $n = 14$; $p < 0.05$, Mann-Whitney U test). (C) Significant negative correlation (Pearson correlation coefficient $r = -0.363$, $p < 0.05$) between NMV and NRV of *MAL* in human esophageal tissues.



several previous studies found that patients with SSBE can develop dysplasia and cancer^{31,32}, the statement that an increased risk of EAC development exists only in patients with LSBE^{33,34} is not accepted universally. Giovanni *et al.* reported that complete-type intestinal metaplasia (IM), which is considered the least prone to cancerization, was significantly prevalent in SSBE patients at baseline, whereas the incomplete type of IM, which is more closely associated with cancer progression, significantly prevailed in LSBE³⁵. In a prospective cohort study of 309 Barrett's patients followed in the Seattle Barrett's Esophagus Project, a strong trend was observed, with a 5 cm difference in length associated with a 1.7-fold increase in cancer risk (95% CI, 0.8–3.8-fold), when patients with HGD at entrance were excluded³⁶. Several other researchers also demonstrated significant differences between SSBE and LSBE in the frequency of both dysplasia and EAC^{33,34}. Thus, it is likely that length of Barrett's epithelium is an important risk factor for both the prevalence of concurrent dysplasia or cancer and the incidence of future malignant progression. Notably, *MAL* hypermethylation exhibited a strong correlation with BE segment length in the current study (Figure 3). Therefore, *MAL* methylation may constitute both a molecular correlate of BE segment length, and a potential biomarker for the prediction of future BE progression.

We further analyzed the methylation status and mRNA expression level of *MAL*, both in 5-Aza-dC treated EAC cells and in primary human EAC specimens. These experiments revealed that reversal of methylation and restoration of *MAL* expression were induced in OE33 cells by 5-Aza-dC treatment (Figure 4). Restoration of *MAL* mRNA expression due to 5-Aza-dC treatment is consistent with the interpretation that DNA promoter hypermethylation silences the *MAL* gene. This experiment was performed in only one EAC cell line, and not in any BE or ESCC cell lines. Moreover, in 38 esophageal specimens, the mean NRV of *MAL* was significantly higher in specimens with unmethylated *MAL* promoters than in specimens with hypermethylated *MAL* (Figure 5B), and a significant negative correlation between NMV and NRV of *MAL* was also observed (Figure 5C). It is worthwhile to note that many previous studies have proposed epigenetic regulation of *MAL* expression^{12,37,38}. These previous observations, together with our own new data, suggest that epigenetic regulation (such as promoter hypermethylation) is essential in silencing of *MAL* expression in human esophageal adenocarcinoma, and that the *MAL* gene is a candidate tumor suppressor gene in this disease.

In conclusion, the current study shows that hypermethylation of the *MAL* gene promoter, leading to gene silencing, is a common event in human Barrett's-associated EAC and is associated with clinical risk factors of neoplastic progression. In addition, *MAL* hypermethylation is uncommon in human ESCC, thus making this event a potential cell type-specific biomarker for EAC.

Methods

Tissue samples. In the current study, 67 NE, 60 BE samples without dysplasia, 19 low-grade and 21 high-grade dysplasias occurring in BE (D), 67 EACs, and 26 ESCCs were examined. All patients provided written informed consent under a protocol approved by the Institutional Review Boards at the University of Maryland and Baltimore Veterans Affairs Medical Centers, where all esophagogastroduodenoscopies were performed. Biopsies were taken using a standardized biopsy protocol, as previously described¹⁹. Research tissues were obtained from grossly apparent Barrett's epithelium or from mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel aliquots obtained at endoscopy. All biopsy specimens were stored in liquid nitrogen before DNA/RNA extraction. Clinicopathologic characteristics are summarized in Table 1.

Cell lines. The EAC (OE33) cell line was obtained from the ATCC (Manassas, VA). These cells were cultured in 47.5% RPMI 1640, 47.5% F-12 supplemented with 5% fetal bovine serum.

DNA and RNA extraction. Genomic DNA was extracted from biopsies and cultured cells using a DNeasy Tissue Kit (Qiagen, Valencia, CA). Total RNA was isolated cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). DNAs and RNAs were stored at -80°C before analysis.

Bisulfite treatment and real-time quantitative methylation-specific PCR. DNA was treated with bisulfite to convert unmethylated cytosines to uracils prior to qMSP, as described previously¹⁹. Promoter methylation levels of *MAL* were determined with the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA), using primers and probes as described previously²⁰. A standard curve was generated using serial dilutions of CpGenome Universal Methylated DNA (CHEMICON, Temecula, CA). The NMV was defined as follows: $\text{NMV} = (\text{MAL-S}/\text{MAL-FM}) / (\text{ACTB-S}/\text{ACTB-FM})$, where *MAL-S* and *MAL-FM* represent the methylation levels of *MAL* in sample and universal methylated DNAs, respectively, while *ACTB-S* and *ACTB-FM* correspond to β -Actin in sample and universal methylated DNAs, respectively²¹.

Real-time quantitative reverse-transcriptase polymerase chain reaction. To determine *MAL* mRNA levels, one-step real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed using a Qiagen QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes were the same as previously reported²⁰. β -Actin was used for normalization of data. A standard curve was generated using serial dilutions of qPCR Reference Total RNA (Clontech, Mountainview, CA). The NRV was calculated according to the following formula for relative expression of target mRNA: $\text{NRV} = (\text{TarS}/\text{TarC}) / (\text{ACTB-S}/\text{ACTB-C})$, where *TarS* and *TarC* represent levels of mRNA expression for the target gene in sample and control mRNAs, respectively, whereas *ACTB-S* and *ACTB-C* correspond to amplified β -Actin levels in sample and control mRNAs, respectively²¹.

5-Aza-dC treatment of esophageal cancer cell lines. To determine whether *MAL* inactivation was due to promoter hypermethylation in esophageal cancer, OE33 EAC cells were subjected to 5-Aza-dC (Sigma, St. Louis, MO) treatment as previously described²¹. Briefly, 1×10^5 cells/ml were seeded onto a 100 mm dish and grown for 24 h. Then, 1 μl of 5 mM 5-Aza-dC per ml of cells was added every 24 hours for 6 days. DNA and RNA were harvested on day 4.

Data analysis and statistics. ROC curve analysis³⁹ was performed using NMVs for the 67 EAC, 26 ESCC and 67 NE by Analyse-it software (Version 1.71, Analyse-it Software, Leeds, UK). Using this approach, the AUROC yielded optimal sensitivity and specificity to distinguish normal from malignant esophageal tissues, and corresponding NMV thresholds were calculated for *MAL*. The cutoff value determined from this ROC curve was applied to determine the frequency of *MAL* methylation in each tissue type included in the present study. For all other tests, Statistica (version 6.1; StatSoft, Inc., Tulsa, OK) was used. Differences with $p < 0.05$ were deemed significant.

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Author contributions

Dr. Liang Wang as L.W., Dr. Yuan Zhang as Y. Z., Dr. Yulan Cheng as Y.C., Dr. Yan Gao as Y.G., Dr. Xianling Feng as X.Fe., Dr. Ming Dong as M.D., Dr. Ziyi Cao as Z.C., Dr. Si Chen as S.C., Dr. Huimin Yu as H.Y., Dr. Zhenfu Zhao as Z.Z., Dr. Xiaojing Zhang as X.Z., Dr. Jie Liu as J.L., Dr. Xinmin Fan as X.Fa., Dr. Yuriko Mori as Y.M., Dr. Stephen J. Meltzer as S.J.M., Prof. Zhe Jin as Z.J., Z.J. and S.J.M. designed the study. Z.J. and L.W. wrote the main manuscript text. Y. Z., Y.C., Y.G., X.Fe., M.D., Z.C., S.C., H.Y., Z.Z., X.Z., J.L., X.Fa. and Y.M. analyzed and interpreted the data. All authors reviewed the manuscript.

Additional information

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