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Indoleamine 2, 3-dioxygenase 1 promoter hypomethylation is associated with poor prognosis in patients with esophageal cancer

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Abstract

Indoleamine 2, 3-dioxygenase 1 (IDO1) is a primary enzyme that generates immunosuppressive metabolites. It plays a major role in tumor immunology and is a potential immune-based therapeutic target. We have reported that IDO1 protein expression was associated with an unfavorable clinical outcome in esophageal cancer. Recently, it has been reported that IDO1 expression is regulated by methylation of the IDO1 promoter. Thus, the aim of this study was to examine the relationship between IDO1 expression, IDO1 promoter methylation, and clinicopathological features in esophageal cancer. We first confirmed changes in IDO1 expression levels in vitro by treating cells with 5-azacytidine. We then evaluated the relationship between IDO1 expression levels, IDO1 promoter methylation (bisulfite pyrosequencing), and clinicopathological features using 40 frozen samples and 242 formalin-fixed, paraffin-embedded samples resected from esophageal cancer patients. We treated cell lines with 5-azacytidine, and the resulting hypomethylation induced significantly higher IDO1 expression ($P < .001$). In frozen samples, IDO1 expression levels correlated inversely with IDO1 promoter methylation levels ($R = -0.47$, $P = .0019$). Furthermore, patients in the IDO1 promoter hypomethylation group (n = 67) had a poor prognosis compared with those in the IDO1 promoter hypermethylation group (n = 175) (overall survival, $P = .011$). Our results showed that IDO1 promoter hypomethylation regulated IDO1 expression and was associated with a poor prognosis in esophageal cancer patients.

KEYWORDS

esophageal cancer, IDO1, immunotherapy, methylation, PD-L1

1 | INTRODUCTION

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer mortality worldwide, resulting in approximately 400 000 deaths per year.[1] Despite recent remarkable advances in multidisciplinary therapies including surgery, chemotherapy, radiotherapy, and chemoradiotherapy, the prognosis of esophageal cancer patients remains poor.[2]

Immunotherapy has important clinical applications with potential favorable outcomes. Specifically, immunotherapy targeting the programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) checkpoints has already been approved for many types of cancer.[3,4] In fact, several innovative clinical trials evaluating PD-1/PD-L1 signal blocking agents have reported efficacy in patients with numerous types of malignancies, including gastrointestinal cancer, in recent years.[5-8] However, the majority of patients with certain types of cancer do not respond, strongly suggesting that additional immunoregulatory pathways control the effectiveness of immunosurveillance in human cancers.

Indoleamine 2, 3-dioxygenase 1 (IDO1) is a primary enzyme that generates immunosuppressive metabolites. It oxidizes tryptophan into kynurenine and modulates the immune response by limiting T cell function and engaging mechanisms of immune tolerance after immune activation by pro-inflammatory stimuli such as γ-interferon (IFNγ). Indoleamine 2, 3-dioxygenase 1 plays a major role in tumor immunology and is a potential immune-based therapeutic target[9]; it has recently been the focus of drug discovery efforts as a potential therapeutic target.

In a previous study, we reported that IDO1 expression was associated with an unfavorable clinical outcome in esophageal cancer.[10] Other studies have also reported on the relationship between IDO1 expression and clinical outcomes in several types of cancer[11-16] (Table 1). However, the molecular mechanisms that regulate IDO1 expression are not completely understood, including in esophageal cancer. Recently, it has been reported that IDO1 expression was regulated by promoter methylation in breast cancer.[17,18] Promoter methylation is associated with gene expression levels and changes in transcriptional function or malignant behavior of cancer cells.

Thus, we investigated the mechanism of regulation of IDO1 by examining the relationship between IDO1 expression, IDO1 promoter methylation levels, and clinicopathological features and identified the mechanisms involved in regulating its expression pattern in esophageal cancer.

2 | MATERIALS AND METHODS

2.1 | Specimens

We analyzed 242 formalin-fixed, paraffin-embedded (FFPE) esophageal cancer specimens from consecutive patients who underwent esophagectomy at Kumamoto University Hospital (Kumamoto, Japan) between January 2005 and June 2013. Tumor staging was undertaken

TABLE 1 Studies on indoleamine 2, 3-dioxygenase 1 (IDO1) and prognosis

Article: journal, year	Cancer type	Sample no.	Method	Role of IDO1 in prognosis	Reference
Clin Cancer Res, 2006	Colorectal	143	IHC	No correlation	32
Oncol Rep, 2007	Ovarian	122	IHC	No correlation	33
Clin Cancer Res, 2007	Renal	52	PCR	Better	34
Br J Cancer, 2012	Colorectal	265	IHC	Worse	11
Cancer Immunol Immunother, 2013	Breast	203	IHC	Worse	12
Oncotarget, 2014	AML	37	WB	Worse	13
J Immunother Cancer, 2017	Breast	362	IF	Worse	14
Ann Surg, 2018	Esophageal	305	IHC	Worse	10
Oncotarget, 2018	Esophageal	87	PCR	Worse	16
World J Gatroenterol, 2018	Colorectal	95	IHC	Worse	15
Current study	Esophageal	305	Pyro	Worse	

IF, immunofluorescence; IHC, immunohistochemistry; Pyro, pyrosequencing; WB, western blot analysis.

as prescribed in the American Joint Committee on Cancer Staging Manual (7th edition).[19] The commonest histologic type was squamous cell carcinoma (219 cases, 90.5%), followed by adenocarcinoma (13 cases, 5.4%), and others (10 cases, 4.1%). Eighty-three patients (34.3%) had undergone preoperative treatment (53 chemotherapy [cisplatin and 5-fluorouracil either with or without docetaxel] and 30 chemoradiotherapy [chemotherapy + 39.6-70 Gy radiation therapy, which was delivered with megavoltage equipment (6-10 MV), using opposing portal or multiple field irradiation techniques]). Patients were followed up at the outpatient clinic every 1-3 months after discharge until death or January 1, 2018, whichever came first. Overall survival and cancer-specific survival were defined as the period from the date of surgery to the date of death. Disease-free survival was defined as the period from the date of surgery to the date of recurrence. Written informed consent was obtained from each subject. All procedures were approved by the Institutional Review Board of Kumamoto University. Throughout this article, the term "prognostic marker" is defined according to the REMARK guidelines.[20]

2.2 | Quantitative RT-PCR

Total RNA extraction, cDNA synthesis, and quantitative RT-PCR (qRT-PCR) were carried out as previously described.[21] Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), and cDNA was synthesized using the SuperScript III Transcriptor First-Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The qRT-PCR was carried out using a LightCycler 480 II instrument (Roche, Basel, Switzerland). To determine differences in gene expression levels among specimens, fold changes in samples were measured using the $2^{-\Delta\Delta CT}$ method. The qRT-PCR primers were designed using the Universal Probe Library (Genenet, Fukuoka, Japan), following the manufacturer's recommendations. The primer sequences and probes used in real-time PCR were: IDO1 (IDO1_#22), 5′-TTCAGTGCTTTGACGTCCTG-3′ and 5′-ATGTCCTGGAGGAACTGAGC-3′, and β-actin (ACTB_#11), 5′-ATTGGCAATGAGCGGTTC-3′ and 5′-CGTGGATGCCACAGGACT-3′.

2.3 | Measurement of IDO1 promoter methylation and long interspersed nucleotide element-1 using pyrosequencing

Genomic DNA was collected from frozen esophageal cancer specimens using a QIAamp DNA Mini Kit (Qiagen). Genomic DNA unmethylated cytosines were converted to uracil with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen). To measure IDO1 promoter methylation, we undertook PCR and pyrosequencing using a PyroMark Q24 System (Qiagen). Pyrosequencing reactions were carried out with the reverse primer biotinylated at the 5′-end (forward primer 5′-GTAAGTTTGTGGTTTATTTTAGAGGTATTG-3′, reverse primer [biotin] 5′-ACTATTTCTCTTTTCTCCTTTTAATCA-3′, sequencing primer 5′-GGAAGTTAAAGAAGAAATTAAG-3′). Polymerase chain reaction was carried out using the PyroMark PCR Kit (Qiagen), following the manufacturer's recommendations with an annealing temperature of 50°C (Figure 1). We also performed PCR and pyrosequencing of long interspersed nucleotide element-1 (LINE-1) as previously described.[22]

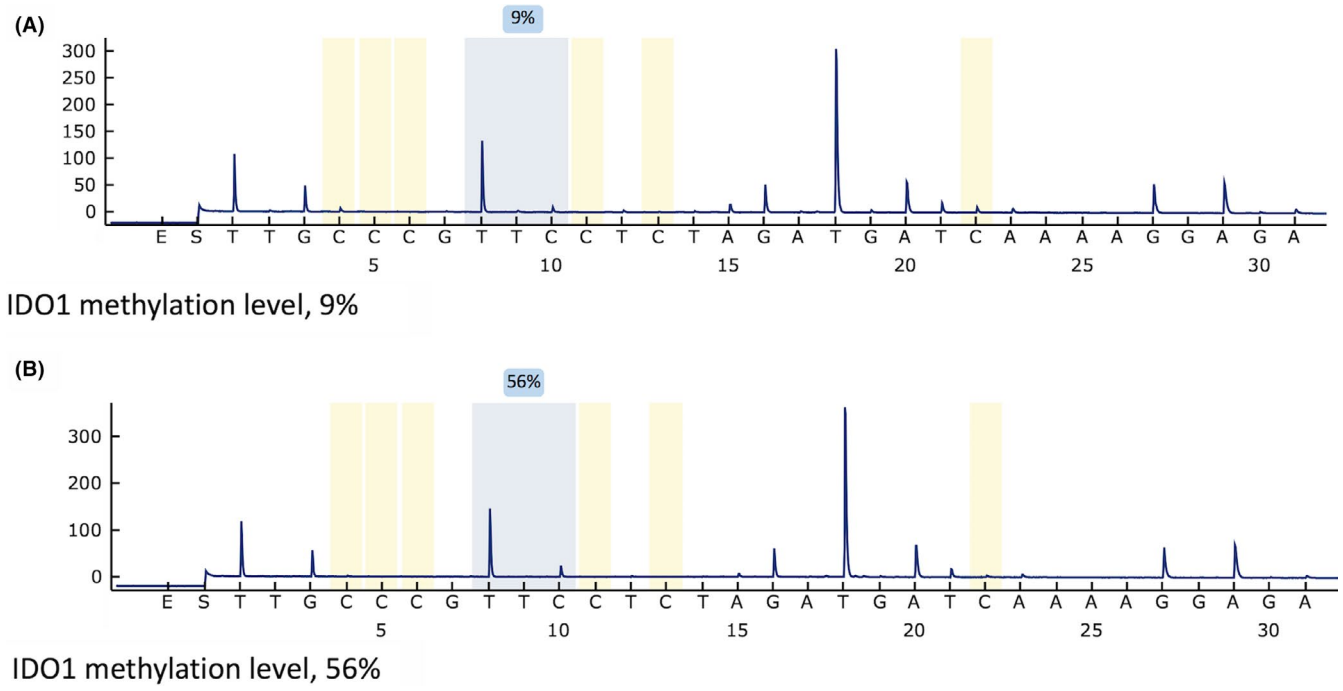


FIGURE 1 Pyrosequencing assay used to measure the indoleamine 2, 3-dioxygenase 1 (IDO1) promoter methylation level. A, IDO1 promoter hypomethylated tumor (methylation level, 9%). B, IDO1 promoter hypermethylated tumor (methylation level, 56%). The percentages (in blue) are the proportion of C at the CpG site after bisulfite conversion, and the methylation level of the CpG site was estimated by the proportion of C (%)

2.4 | Cell lines

Human esophageal squamous cell carcinoma (ESCC) cell lines (KYSE-30 and TE series) were acquired from the Japanese Collection of Research Bioresources Cell Bank, the Cell Resource Center for Biomedical Research, and the Riken BioResource Center Cell Bank (Osaka, Japan). These cell lines were cultured in RPMI-1640 or DMEM, supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C.

2.5 | Treatment with 5-azacytidine

Cells were seeded in a 100-mm dish and cultured for 24 hours. To demethylate methylated CpG sites, cells were continuously treated with 5-azacytidine (5-AZA; 100 nmol/L-concentration) (Wako, Osaka, Japan) for an additional 72 hours. The medium was replaced every 24 hours.

2.6 | Statistical methods

All statistical calculations were carried out using JMP version 10 (SAS Institute, Cary, NC, USA) and Excel for Windows 2013 (Microsoft). All *P* values were 2-sided. Mean values were compared using Student's *t* test for age and body mass index (BMI), and the χ^2 or Fisher's exact test was used for all other variables. In the survival analysis, the survival time distribution was evaluated using the Kaplan-Meier method and the log-rank test was used for comparisons. To obtain the hazard ratio (HR), we constructed a multivariate Cox proportional hazards model of IDO1 expression status, containing age at surgery (continuous variable), gender (male vs female), BMI (continuous variable), tobacco use (yes vs no), alcohol use (yes vs no), comorbidity (present vs absent), performance status (PS) (0 vs 1+), preoperative treatment (present vs absent), and tumor stage (I vs II vs III). Interactions were assessed by including the cross-product of the IDO1 status and another variable of interest in a Cox model. We considered $P < .05$ as statistically significant.

3 | RESULTS

3.1 | Changes in IDO1 expression levels in esophageal cancer cell lines treated with 5-AZA

To confirm that the decrease in the DNA methylation level affected IDO1 expression levels as in other types of cancer, we treated 5 types of ESCC lines with 5-AZA. Although IDO1 promoter methylation levels decreased in all ESCC cell lines after 5-AZA treatment, there were significant and more substantial changes in IDO1 mRNA expression (Figure 2). We confirmed the effect of 5-AZA treatment by measurement of LINE-1. Because LINE-1 represents a considerable part of the human genome (approximately 17%), LINE-1 methylation levels have been considered as a surrogate marker of global DNA methylation.[23] The IDO1 promoter methylation levels were also decreased by 5-AZA treatment. In addition, we determined whether IFNγ influences the IDO1 promoter methylation levels and IDO1 expression levels (Figure S1). From those experiments, we found that IFNγ did not influence IDO1 promoter methylation levels, but did influence IDO1 expression levels. These findings suggested that the changes in DNA methylation levels influenced IDO1 expression levels apart from the influence of IFNγ.

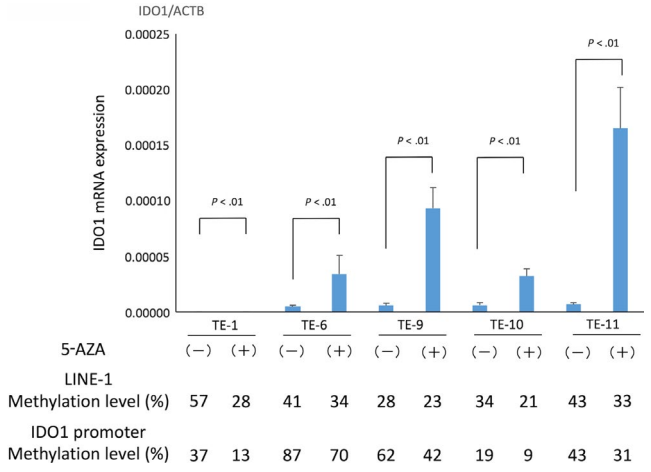


FIGURE 2 Indoleamine 2, 3-dioxygenase 1 (IDO1) mRNA expression levels and IDO1 promoter methylation levels in 5 esophageal cell lines were measured before and after 5-azacytidine (5-AZA) treatment. Global DNA methylation levels were evaluated by measuring long interspersed nucleotide element-1 (LINE-1) methylation levels

3.2 | Correlation between IDO1 expression and IDO1 promoter methylation levels

To confirm the association between IDO1 expression and IDO1 methylation levels, we measured methylation levels of the CpG site in the IDO1 promoter in 40 frozen samples from esophageal cancer patients. We found that the methylation level of the CpG site in the IDO1 promoter inversely correlated with the IDO1 mRNA expression level (correlation rate: −0.47, $P = .0019$) (Figure 3). These data suggest that DNA hypermethylation in the IDO1 promoter might indeed be involved in the reduction of IDO1 transcription observed in esophageal cancer.

3.3 | Evaluation of the association of IDO1 methylation levels and clinicopathological variables

Next, we quantified IDO1 methylation in 242 FFPE cancer specimens. The distribution of IDO1 methylation levels in the 242 samples (Figure 1) was as follows: mean, 36.0; median, 46.0; SD, 16.5; range, 6-98; interquartile range, 24-46 (all in 0-100 scale). The IDO1 methylation level was then divided into the hypermethylation group (>24, n = 175) and the hypomethylation group (≤24, n = 67)] for further analysis (dot/whisker plot, Figure S2). There were no significant differences in age, gender, BMI, PS, tobacco use, alcohol use, comorbidity, tumor location, histological type, pathological stage, and postoperative treatment between the IDO1 promoter hypermethylation and IDO1 promoter hypomethylation groups. Subsequently, we found that the IDO1 methylation level was associated with the

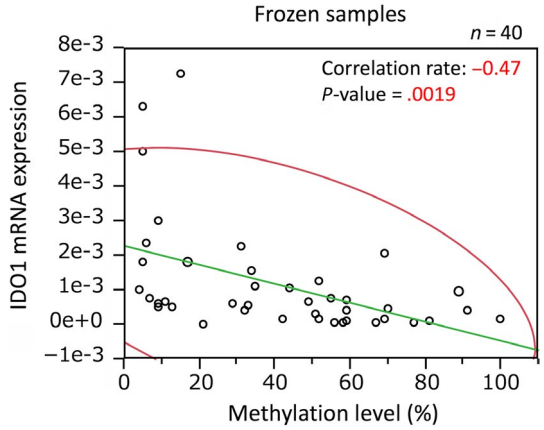


FIGURE 3 Methylation of CpGs in the indoleamine 2, 3-dioxygenase 1 (IDO1) promoter inversely correlated with IDO1 mRNA expression derived from 40 frozen samples from curatively resected esophageal cancer patients

presence of preoperative treatment (chemotherapy, n = 53; chemoradiotherapy, n = 30) (Table 2).

3.4 | Hypomethylation of IDO1 promoter and patient survival

During follow-up of the 242 patients, there were a total of 116 esophageal cancer recurrences and 91 deaths that were confirmed to be attributable to esophageal cancer. The median follow-up time for censored patients was 3.9 years. In the Kaplan-Meier analysis, the IDO1 hypomethylation group showed a significantly shorter overall survival (OS) (log-rank $P = .011$) (Figure 4). In univariate Cox regression analysis, patients with IDO1 promoter hypomethylation showed significantly higher overall mortality than those with IDO1-negative tumors (HR 1.75; 95% confidence interval, 1.120-2.677; $P = .015$].

3.5 | Survival analysis of interaction between IDO1 and other variables

We next determined whether the influence of IDO1 promoter methylation on OS was affected by any of the clinical, pathological, or epidemiological variables (Figure 5). The relationship between IDO1 promoter methylation and OS rate was apparently unmodified by PS, tumor location, preoperative chemotherapy, tumor stage, or the presence of $CD8^+$ tumor infiltrating lymphocytes (TIL) ($P > .10$ for all interactions). Conversely, age ($P = .021$) and the absence of comorbidity ($P = .001$) influenced the relationship between IDO1 promoter methylation and OS rates.

4 | DISCUSSION

We undertook this study to examine the mechanism involved in regulating IDO1 expression in curatively resected esophageal cancer patients. Indoleamine 2, 3-dioxygenase 1 is one of the most important immunological metabolic enzymes that induce immune tolerance.[24] Therefore, many clinical trials have been carried out to investigate the effects of the IDO1 inhibitors epacadostat and indoximod in several cancer types, including gastrointestinal cancer.[25-27] Although these studies are ongoing with some encouraging results, expectations are high that IDO1 will be an important therapeutic target. Indoleamine 2, 3-dioxygenase 1 is also recognized as a resistance mechanism to immune checkpoint blockade in cancer[28]; therefore, elucidation of the mechanism of regulation of IDO1 expression in esophageal cancer could help improve immunotherapeutic strategies for this disease.

Some previous studies have reported on the mechanism of IDO1 expression. In dendritic cells, IFNβ, IFNγ, and tumor necrosis factor-α activate the JAK/STAT pathway that results in the activation of IDO1.[29] These inflammatory cytokines have also been shown to stimulate the activity of IDO1 in cancer cells. Furthermore, in breast cancer, it has been reported that IDO1 expression was regulated by IDO1 promoter methylation in estrogen receptor-positive cases. Specifically, hypomethylation of CpGs in the IDO1 promoter was associated with JAK/STAT pathway signaling and increased IDO1 activity.[17] In lung cancer, sustained IDO1 activity was reported to occur, resulting from sustained activity of the aryl hydrocarbon receptor, interleukin-6, and STAT3 signaling loop.[30] In cervical cancer, it has been suggested that the expression of IDO1 was induced by inflammatory cytokines that were produced in the tumor stroma; however, this has not yet been proved.[31]

To our knowledge, there has been no report of a particular mechanism involved in regulating IDO1 activity in esophageal cancer. Based on past studies in other types of cancer, we considered that the most important mechanism might involve methylation of CpGs in the IDO1 promoter and therefore investigated the relationship between methylation level and IDO1 expression level. We found that demethylation significantly induced higher expression of IDO1 in all esophageal cancer cell lines. In addition, we discoveres IDO1 mRNA expression levels and IDO1 promoter mthylation level have inverse correretion in frozen samples. Furthermore, experiments using 242 FFPE samples showed that there was a strong association between methylation level and poor prognosis in patients with esophageal cancer. Therefore, our results are evidence that epigenetic hypomethylation induces high expression of IDO1 and contributes to malignant behavior in esophageal cancer. As we summarized in Table 1, the relationship between IDO1 expression and prognosis has been examined by various methods, including PCR and immunohistochemistry. Most reports have concluded that mRNA, protein, and hypomethylation were associated with poor prognosis, regardless of the type of cancer. However, even in the same cancer, the reported significance of IDO1 expression differs, depending on tissue type or gene type.[32,33] Conversely, it has been reported that IDO1 expression prolongs OS in renal cancer.[34] Thus, it would be necessary to validate the significance if IDO1 expression using the same samples with multiple measurement methods.

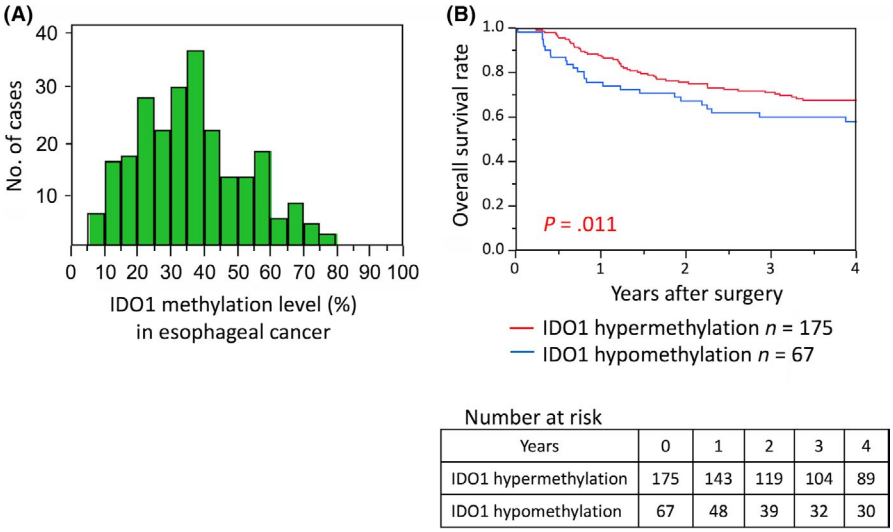
In the clinicopathological analysis, only preoperative treatment was associated with IDO1 promoter methylation levels.

TABLE 2 Indoleamine 2, 3-dioxygenase 1 (IDO1) promoter methylation and association with expression and clinicopathological features

Clinicopathological feature	Total N	IDO1 promotor methylation		*P* value
		Hyper	Hypo	
All cases	242	175	67	
Age (y), mean ± SD	66 ± 9.21	65 ± 9.76	68 ± 7.46	0.100
Gender				
Male	214 (88)	156 (89)	58 (87)	0.570
Female	28 (12)	19 (11)	9 (13)	
Body mass index, median ± SD	21.7 ± 2.9	21.7 ± 2.7	21.7 ± 3.1	0.830
Performance status				
0	182 (75)	134 (77)	48 (72)	0.420
1+	60 (25)	41 (23)	19 (18)	
Tobacco use				
Yes	197 (81)	147 (84)	50 (75)	0.100
No	45 (19)	28 (16)	17 (25)	
Alcohol use				
Yes	205 (85)	151 (86)	54 (81)	0.280
No	37 (15)	24 (14)	13 (19)	
Comorbidity				
Present	171 (71)	120 (69)	51 (76)	0.240
Absent	71 (29)	55 (31)	16 (24)	
Tumor location				
Upper	40 (16)	28 (16)	12 (18)	0.730
Middle	111 (46)	83 (47)	28 (42)	
Lower	91 (38)	64 (37)	27 (40)	
Histological type				
Squamous cell carcinoma	219 (91)	158 (90)	61 (91)	0.270
Adenocarcinoma	13 (5)	8 (4)	5 (7)	
Others	10 (4)	9 (6)	1 (2)	
Preoperative treatment				
Present	83 (34)	53 (30)	30 (45)	**0.036**
Absent	159 (56)	122 (70)	37 (55)	
Pathological stage				
I	93 (38)	71 (41)	22 (33)	0.440
II	62 (26)	45 (26)	17 (25)	
III	87 (36)	59 (33)	28 (42)	
Postoperative treatment				
Present	61(25)	47 (27)	14 (21)	0.330
Absent	181 (75)	128 (73)	53 (79)	

Values in parentheses are percentages.
Bold value is $P < 0.05$.

Although there have been no reports that confirm the relationship between chemotherapy or radiotherapy and IDO1 expression, some studies have investigated IDO1 expression and immunological factors, including PD-1 and PD-L1.[35,36] Thus, it is conceivable that preoperative treatment might affect IDO1 expression. In this respect, our findings could have clinical implications. The relationship between IDO1 promoter hypomethylation, preoperative therapy, and patient outcomes needs to be confirmed in independent cohorts in the future. As another point of view, we analyzed the relationship between IDO1 promoter methylation levels, presence of CD8+ TIL, and prognosis because we reported the importance of the role of $CD8^+$ TIL for esophageal cancer in



Number at risk

Years	0	1	2	3	4
IDO1 hypermethylation	175	143	119	104	89
IDO1 hypomethylation	67	48	39	32	30

FIGURE 4 A, Distribution of indoleamine 2, 3-dioxygenase 1 (IDO1) promoter methylation levels in 242 esophageal cancers. B, Kaplan-Meier curves for overall survival among patients with esophageal cancer according to IDO1 promoter methylation levels

previous studies.[10,37] However, we found no direct association between $CD8^+$ TIL status (Figure S3), IDO1 promoter methylation, and OS (Figure 5). Therefore, it is suggested that there are complex mechanisms that determine the influence of methylation on IDO1 protein expression, malignant behavior from IDO1, and absence of $CD8^+$ TIL.

Our analysis has revealed that age and the absence of comorbidity influenced the relationship between IDO1 promoter methylation and OS rates. Interestingly, a past report has suggested that IDO1 expression was higher in young, compared to old, prostate cancer patients.[38] Furthermore, IDO1 depletion has been reported to be associated with development of pulmonary hypertension[39] or diabetes.[40] From these results, *IDO1* was identified as an oncogene in esophageal cancer, but it could involve very complicated mechanisms in relation to various diseases. In addition, in the multivariate analysis, IDO1 promoter hypomethylation was not a statistically independent prognostic factor (Table S1). In our previous study, we showed that IDO1 protein expression was an independent prognostic factor.[10] Therefore, further studies are necessary to examine whether histological type, type of preoperative treatment, or other factors influence the characterization of IDO1 promoter methylation.

Our present study has several limitations. A larger cohort of patients with other histological types or various immunological factors and further analysis are required to verify the impact of IDO1 promoter methylation in esophageal cancer. Additionally, it is necessary to analyze factors that change with IDO1 expression, including kynurenine or tryptophan, to confirm the mechanism in more detail.

In summary, this study suggests that methylation of CpG sites in the IDO1 promoter regulated IDO1 expression levels and was associated with poor prognosis in esophageal cancer patients. Thus, additional studies are needed to test this mechanism as a potentially new therapeutic target or prognostic biomarker for esophageal cancer. In future, development of a multidisciplinary treatment strategy, including immunotherapy, is expected to contribute to developing individualized therapeutic regimens in esophageal cancer.

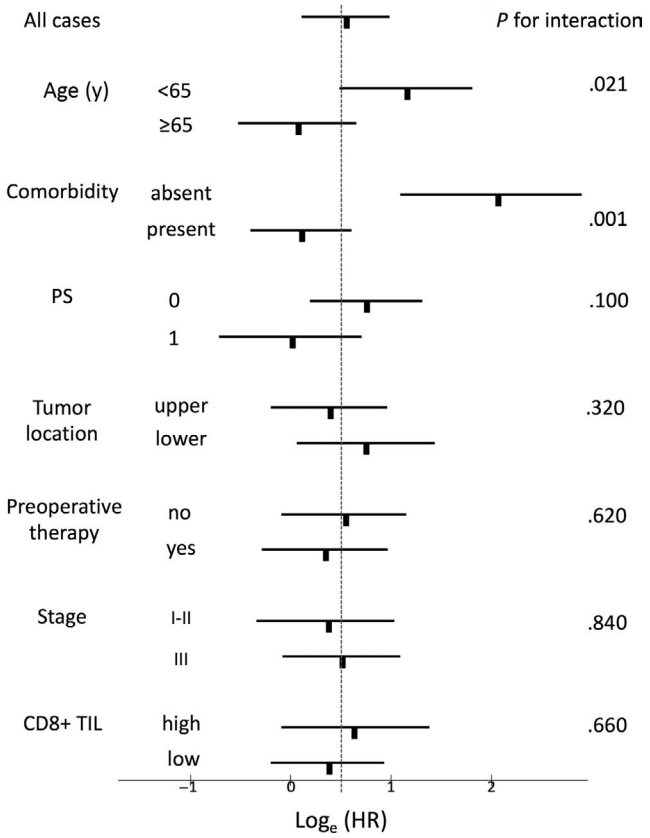


FIGURE 5 Relationship between indoleamine 2, 3-dioxygenase 1 (IDO1) promoter methylation in esophageal cancer and overall survival. Shown are the $\log_e$ (hazard ratio [HR]) plots of the overall survival rates in the IDO1 promoter hypomethylation and IDO1 promoter hypermethylation groups. PS, performance status; TIL, tumor infiltrating lymphocyte

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DISCLOSURE

The authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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