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Type
Research paper

Keywords
prognosis, esophageal cancer, methylation, IDO1

Abstract

Introduction
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Material and methods
Data from The Cancer Genome Atlas (TCGA)-ESCA and The Genotype-Tissue Expression (GTEx) project were obtained for analysis. Subgroup analysis was performed in esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (ESAD) respectively.

Results
IDO1 expression was significantly upregulated in ESAD and ESCC tissues than in normal esophagus. Although gene-level copy number alterations were common in both ESAD and ESCC, they were not associated with IDO1 dysregulation. Among 3 CpG sites (cg10262052 and cg08465774 in promoter and cg24188163 in gene body) in IDO1 gene locus examined, only cg10262052 was hypomethylated in cancerous tissues than in normal tissues in ESAD. All 3 sites showed significantly different methylation in ESCC than in normal tissues, among which cg10262052 and cg08465774 were hypomethylated, while cg24188163 was hypomethylated. Correlation analysis confirmed negative correlations between cg10262052/cg08465774 methylation and IDO1 expression while cg24188163 methylation was positively correlated with IDO1 expression (Pearson’s r=0.45) in ESCC patients. Genomic study confirmed that cg24188163 is in the flanking region of an intragenic promoter of IDO1. IDO1 expression had an independent prognostic value in terms of OS in ESCC patients (HR: 1.183, 95%CI: 1.025-1.367, p=0.022), but was not a risk factor of unfavorable OS in ESAD patients.

Conclusions
IDO1 mRNA upregulation was associated with both promoter hypomethylation and gene body hypermethylation in ESCC. Its expression has a specific prognostic value in terms of OS in ESCC, but not in ESAD patients.
IDO1 mRNA upregulation was associated with gene body hypermethylation and poor overall survival in esophageal squamous cell carcinoma

Chang-Qing Zhang¹, Xue-Bin Wang¹, Shu-Ping Li¹, Guo-Ying Miao¹, Rong Wang¹, Wen Ma¹,²*

1. Department of Tumor Center, Gansu Provincial Hospital, Lanzhou, China, 730000.
2. Medical School, Xi'an Jiaotong University, Xi'an, China, 710000.

Corresponding author: We Ma
Email: mawen2018@sina.com

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Key words: IDO1; esophageal cancer; methylation; prognosis
**Introduction**

Indoleamine 2, 3-dioxygenase 1 (IDO1) is an enzyme encoded by the IDO1 gene. Basically, it functions as a rate-limiting enzyme that catalyzes the catabolism of tryptophan to N-formyl-kynurenine (Kyn). It is well illustrated that IDO1 upregulation induces an immune suppressive effect in the tumor microenvironment \(^1\), which was related to several possible mechanisms. For example, tryptophan depletion results in the activation of the amino-acid-sensitive GCN2 and/or mTOR signaling pathways of T cells, leading to subsequent T cell cycle arrest and autophagy \(^1\). Secondly, the metabolic products of tryptophan, such as L-Kyn and 3-hydroxy-L-Kyn can activate the aryl hydrocarbon receptor (AhR), which leads to induced IDO1 expression in some dendritic cells (DCs) and also the conversion of naive CD4\(^+\) T cells into regulatory T cells (Treg) \(^2,3\).

In the past decades, immune checkpoint blockade therapy has shown promising therapeutic effects in some solid tumors, including esophageal cancer (ESCA) \(^4\). Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) are the three targets with drugs approved for clinical application. Although the antibody targeting PD-1 significantly prolonged overall survival (OS) and recurrence-free survival (RFS) compared with traditional chemotherapy in patients with advanced ESCA, the overall response rate (ORR) is only around 30% \(^5\). Some recent studies found that IDO1 activity is also involved in the resistance to anti-PD-1 in non-small cell lung cancer \(^6\) and glioblastoma \(^7\).

ESCA has well demonstrated IDO1 upregulation \(^8\)\(^-\)\(^10\). The involvement of IDO1 in immune tolerance and poor prognosis in patients with ESCA is also confirmed \(^8\)\(^-\)\(^11\). These findings suggest that IDO1 might act as an important immune modulator and a prognostic marker in ESCA. Several studies indicated that promoter hypomethylation plays a critical role in enhanced IDO1 transcription in breast cancer...
and ESCA. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (ESAD) are the two dominant histological subgroups of ESCA, which have distinct differences in terms of origins, molecular background (such as copy number abnormalities, mutational patterns and driver genes) and prognosis. Therefore, it is meaningful to figure out the differences of IDO1 in expression profiles, genetic/epigenetic alterations, and prognostic value of in ESCC and ESAD. In this study, we tried to explore these differences using data from The Cancer Genome Atlas (TCGA)-ESCA and the Genotype-Tissue Expression (GTEx) project.

Materials and methods

Data retrieving from TCGA-ESCA

The level-3 data in TCGA-ESCA were acquired as introduced in our previous publication, by using the UCSC Xena browser (https://xenabrowser.net/). Briefly, 96 esophageal squamous cell carcinoma (ESCC) cases, 89 esophageal adenocarcinoma (ESAD) cases and 18 normal esophageal tissues were included. No patient received neoadjuvant treatment. All tumor cases and 16 normal tissues cases had gene methylation data measured by Infinium Human Methylation 450k BeadChip. All ESAD cases, 95 ESCC, and 11 normal tissues cases had RNA-seq data of gene expression (calculated as Log2RSEM+1). 88 ESAD and 95 ESCC cases had gene-level copy number alteration (CNA) data. CNA was defined as homozygous deletion (-2), heterozygous loss (-1), copy-neutral (0), low-level copy gain (+1), high-level amplification (+2), by the GISTIC2 method. The clinicopathological and overall survival (OS) data of the patients were also extracted for re-analysis.

IDO1 transcript analysis in TCGA-ESCA and The Genotype-Tissue Expression (GTEx) project

The transcript profile of IDO1 in ESCA tissues and in normal esophageal tissues was analyzed using the UCSC Xena browser (https://xenabrowser.net/). IDO1 transcript information in normal esophageal tissues were provided by the Genotype-Tissue
Expression (GTEx) project, which is a large database of tissue-specific gene expression\textsuperscript{20,21}. Over 600 cases of normal esophageal tissues were included in this database for gene transcript analysis. \text{Log}_2\text{Transcript per Million (TPM)} was calculated and compared.

The genomic structure of \textit{IDO1} gene was examined by using the human chromosomal map provided by Ensembl\textsuperscript{22}.

**Immunohistochemistry (IHC) staining of \textit{IDO1}**

Human paraffin-embedded ESAD and ESCC tissue array was purchased from Alenabio (Xian, China), which includes 11 ESAD cases and 17 ESCC cases. IHC staining was conducted as described in one previous study\textsuperscript{23}. In brief, the tissue array was treated with 3\% \text{H}_2\text{O}_2 for inactivating tissue peroxidases. The primary antibody used was anti-\textit{IDO1} (1:500, Cat#HPA027772, Sigma-Aldrich, St. Louis, MO, USA). Labeling was performed by biotinylated secondary antibodies (SP-9001, ZSGB-BIO, Beijing, China) and DAB kit (ZSGB-BIO), with hematoxylin used for counterstaining. Staining score was performed by two experienced pathologists without authorship in this study. The expression score is a combination of staining intensity and cell fractions, according to the standards proposed by the Human Protein Atlas\textsuperscript{24}. The final staining scores were defined as not detected, low, medium and high.

**Statistical analysis**

Statistical analysis was performed by using GraphPad Prism 8.04 (GraphPad Inc., La Jolla, CA, USA) or SPSS 25.0 software package (SPSS Inc., Chicago, IL, USA). One-way ANOVA with following Tukey's multiple comparison test was performed. Pearson’s correlation coefficient was calculated for correlation analysis. Kaplan-Meier (K-M) curves were generated for OS comparison between patients with the highest and the lowest tertile \textit{IDO1} expression, with the log-rank test to check the statistical differences. Univariate and multivariate Cox regression models were used to evaluate
the prognostic significance of IDO1 expression in terms of OS in ESCC and ESAD patients respectively, by setting its expression as a continuous variable. $p<0.05$ was considered statistically significant.

**Results**

**Analysis of IDO1 expression in ESCC, ESAD and normal esophageal tissues**

Using RNA-seq data of tumor tissues in TCGA-ESCA and normal tissues in GTEx-Esophagus, we found that IDO1 expression was significantly upregulated in ESCC (N=95) and ESAD (N=89) tissues compared to the respective normal esophageal tissues (mucosa and gastroesophageal junction, N=273 and 137 respectively, $p<0.001$, Figure 1A). Then, we performed IHC staining to check IDO1 expression at the protein level. 10/17 ESCC cases (3 low, 5 medium and 2 high) and 6/11 EASD cases (1 low, 3 medium and 2 high) had IDO1 protein expression (Figure 1B). The representative images of medium IDO1 staining in EASD and ESCC were shown in figure 1C.

**IDO1 expression was irrelevant to the gene-level copy numbers**

By checking gene-level CNA of IDO1 in ESAD and ESCC respectively, we found that among 88 ESAD cases with CNA data, there were 3 high-level amplification (+2), 29 low-level amplification (+1), 32 copy-neutral (0), 22 heterozygous deletions (-1), and 2 homozygous deletions (-2) (Figure 2A). Among 95 ESCC cases, there were 8 high-level amplification, 30 low-level amplification, 30 copy-neutral and 27 heterozygous deletions (Figure 2A). However, neither the amplification group nor the deletion group was associated with dysregulated IDO1 expression, compared to the copy-neutral group in ESAD (Figure 2B) and in ESCC (Figure 2C) cases.

**Analysis of the methylation profile of CpG sites in IDO1 locus in ESAD and ESCC patients**

Some recent studies reported that the expression of IDO1 in ESCC was regulated by promoter methylation in multiple cancer, including ESCC\textsuperscript{10,12}. Using methylation 450k
BeadChip data, we checked the methylation profile of 3 CpG sites in the *IDO1* gene locus (Figure 3A). In ESAD cases, only cg10262052 was hypomethylated in cancerous tissues than in normal tissues (Figure 3B), no significant difference was observed in the other two CpG sites, between cancerous and normal tissues (Figure 3C-D). In ESCC cases, cg10262052 and cg08465774 were hypomethylated, while cg24188163 was hypermethylated in cancerous tissues than in normal tissues (Figure 3B-D).

By performed Pearson’s correlation analysis, we found a moderately negative correlation (Pearson’s r=−0.42) between cg10262052 methylation and *IDO1* expression in ESAD patients (Figure 3E). Correlation analysis also confirmed negative correlations between cg10262052/cg08465774 methylation and *IDO1* expression in ESCC patients (Figure 3F-G). Interestingly, cg24188163 methylation was positively correlated with *IDO1* expression (Pearson’s r=0.45) in these patients (Figure 3H).

**Transcript analysis of *IDO1* in ESCA and normal esophageal tissues**

The transcriptional profile of *IDO1* between cancerous and normal tissues was compared, using data from both TCGA and GTEx. Results indicated that *IDO1* gene has 9 splice variants. Three transcripts were significantly upregulated, including ENST00000522495, which is the dominant and the canonical protein-coding transcript of *IDO1*, and two non-protein-coding transcripts, ENST00000253513 and ENST00000523779 (Figure 4A). By checking the genomic structure of *IDO1* gene using Ensembl ([https://grch37.ensembl.org/Homo_sapiens/Location/View?r=8:39759794-39786309](https://grch37.ensembl.org/Homo_sapiens/Location/View?r=8:39759794-39786309)), we checked the transcript profiles of *IDO1* and genomic locations of the 3 CpG sites. Interestingly, we observed that *IDO1* has multiple promoter regions in its gene locus (Figure 4B). Among the 3 CpG sites, cg10262052 and cg08465774 are promoter-associated, while cg24188163 locates within the gene body of *IDO1*, in the intron between the protein-coding exon 7 and 8, which is also a flanking region of an intragenic alternative promoter (Figure 4B).
**IDO1 expression independently predicts shorter OS in ESCC, but not in ESAD patients**

K-M survival curves were developed to compare the OS of patients with the highest tertile and lowest tertile *IDO1* expression. Log-rank test indicated high *IDO1* expression was associated with significantly shorter OS in ESCC patients (Figure 5A), but not in ESAD patients (Figure 5B).

Univariate and multivariate analysis revealed the independent prognostic value of *IDO1* expression in terms of OS in ESCC patients (HR: 1.183, 95%CI: 1.025-1.367, *p*=0.022), after adjustment for gender and pathological stages (Table 1). In comparison, *IDO1* expression was not a risk factor of unfavorable OS in ESAD patients (Table 1).

**Discussion**

IDO1 positive ESCA tissues have a significantly higher proportion of FOXP3-positive cells, suggesting that its expression is closely associated with immune-suppressive tumor microenvironment. Therefore, a clear understanding of the mechanisms leading to its dysregulation would provide new rationale to make better immunotherapeutic strategies.

Type I (IFNα and IFNβ) and type II (IFNγ) interferons are potent IDO1 inducers in human cancer tissues. Activation of AhR in DCs induced by the metabolic products of tryptophan also stimulates the generation of IDO1 by DCs, thereby forming a positive feedback regulatory network. Recent studies showed that *IDO1* expression is modulated by promoter methylation in some cancers. Specifically, in ER-positive breast cancer cells, cg10262052 methylation is inversely correlated with *IDO1* expression. In ESCA, *IDO1* expression is also regulated by promoter methylation. However, it is not clear whether there are different methylation patterns between ESAD and ESCC.
By analyzing the correlation between CpG sites methylation and *IDO1* expression in ESAD and ESCC patients respectively, we observed different trends of the correlations. In ESAD cases, only cg10262052 was significantly hypomethylated in cancer tissues and its methylation level was negatively correlated with *IDO1* expression. In comparison, all 3 CpG sites in ESCC were related to *IDO1* dysregulation. cg10262052 and cg08465774 were hypomethylated, while cg24188163 were hypermethylated in cancerous tissues. Correlation analysis showed negative correlations between cg10262052/cg08465774 methylation and *IDO1* expression and a positive correlation between cg24188163 methylation and *IDO1* expression. Therefore, we infer that the methylation mediated *IDO1* dysregulation might be tissue-specific.

Although promoter hypermethylation is a well-characterized mechanism leading to gene suppression, some recent studies reported that gene body methylation has distinct regulatory mechanisms on gene expression, which may lead to enhanced gene transcription\(^\text{28}\). However, the mechanisms underlying this phenomenon are quite complex and far from been fully understood. Some mechanisms have been proposed to illustrate the association. Gene body methylation might suppress the initiation of intragenic promoters\(^\text{29}\), contributing to the formation of an ordered structure within the transcribed unit that facilitates transcription by supporting elongation or splicing\(^\text{30}\). One recent study demonstrated that intragenic DNA methylation is directly associated with pre-mRNA slicing\(^\text{31,32}\). Binding of the methyl-sensitive zinc-finger protein CCCTC-binding factor (CTCF) to intragenic DNA leads to local pol II accumulation and favors the inclusion of weak upstream exons in spliced mRNA through kinetic regulation\(^\text{31}\). In comparison, 5-methylcytosine (5mC) evicts CTCF and results in exon exclusion and splicing\(^\text{31}\). In addition, it might contribute to the formation of borders at enhancers or promoters, thereby enhancing transcription of specific transcripts\(^\text{33,34}\). In *IDO1* gene locus, cg10262052 and cg08465774 are promoter-associated, while cg24188163 locates within the gene body of *IDO1* between exon 7 and 8, where is also
a flanking region of an alternative intragenic promoter. Among the three transcripts upregulated in ESCA, ENST00000523779 only contains exon 8, 9 and 10, suggesting that there is a splicing event before exon 8 after transcription. Based on the mechanisms discussed above, we hypothesized that cg24188163 methylation might contribute to the suppressed initiation of the intragenic promoter and contribute to the splicing events associated with ENST00000523779 in ESCC cases. However, future molecular studies should be conducted to validate the hypothesis and to explore the different regulatory effects of the CpG sites on IDO1 expression in ESAD and ESCC respectively.

IDO1 expression has been identified as a potential prognostic biomarker in ESCA \(^{8-11}\). Kiyozumi et al. reported that in patients with ESAD, IDO1 expression and its promoter hypomethylation were associated with unfavorable OS \(^{10,11}\). However, no subgroup analysis was performed to separate ESAD and ESCC in these studies. Rosenberg et al. reported a link between high IDO1 expression and worse OS in both ESAD and ESCC subgroups \(^8\). However, the prognostic value was not adjusted for other well-established risk factors, such as gender and pathologic stages. One very recent study showed that in ESCC cases after neoadjuvant chemoradiotherapy, IDO1 expression was an independent prognostic factor for RFS \(^9\). But OS was not assessed in this study. In the current study, we generated K-M survival curves and performed univariate and multivariate analyses to assess the prognostic value of IDO1 mRNA expression in terms of OS in ESAD and ESCC patients, respectively. Our data showed that IDO1 expression might only have independent prognostic value in ESCC patients, but not in ESAD patients.

The current study made a good supplementation to understand the methylation profiles and prognostic significance of IDO1 in ESCC and ESAD separately. In combination with previous studies, we might infer that IDO1 expression might serve as an independent prognostic biomarker in OS and RFS in ESCC. However, its prognostic value in ESAD is not certain. This study also has some limitations. Firstly, no validation
cohort was used to verify the prognostic significance of IDO1 mRNA expression. Secondly, the prognostic significance was only assessed at the mRNA level. Therefore, validation at both mRNA and protein expression levels in another large cohort is required in future studies.

Conclusions
IDO1 mRNA upregulation was associated with both promoter hypomethylation and gene body hypermethylation in ESCC. Its expression has specific prognostic value in terms of OS in ESCC, but not in ESAD patients.

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Figure 1. IDO1 expression was significantly upregulated in both ESAD and ESCC compared with normal esophageal tissue
A. Plot chart comparing the expression of IDO1 in ESAD/ESCC tissues in TCGA and normal esophageal tissues in GTEx. B. Summary of IHC staining score of IDO1 expression in 11 ESAD and 17 ESCC cases. C. Representative images of medium IDO1 staining in EASD (up) and ESCC (down) tissues.

Figure 2. IDO1 expression was irrelevant to the gene-level copy numbers
A. A heat map showing the correlation between IDO1 expression and its gene-level CNAs in ESAD and ESCC cases respectively. B-C. Violin plot chart showing the expression of IDO1 in different CNA groups in ESAD (B) and ESCC (C) cases. CNAs were defined as homozygous deletion (-2), heterozygous loss (-1), copy-neutral (0) and low-level copy gain (+1), high-level amplification (+2).

Figure 3. Analysis of the methylation profile of CpG sites in IDO1 locus in ESAD
and ESCC patients

A. A heat map showing IDO1 expression and the methylation profile of 3 CpG sites in IDO1 gene locus in ESAD and ESCC cases respectively. B-D. Plot charts showing the methylation level of cg10262052 (B) cg08465774 (C) and cg24188163 (D) in normal esophageal, ESAD and ESCC tissues.

Figure 4. IDO1 transcript profile in ESCA and genomic structure

A. Comparison of the expression profile of IDO1 transcripts in cancerous tissues in TCGA-ESCA and in normal esophageal tissues in GTEx-Esophagus. TPM: Transcripts Per Kilobase Million. B. genomic structure of IDO1 gene, which was accessed via: https://grch37.ensembl.org/Homo_sapiens/Location/View?r=8:39759794-39786309. The annotations were as marked at the bottom of the image.

Figure 5. K-M survival curves of OS in ESCC and ESAD patients

K-M survival curves of OS in ESCC (A) and ESAD (B) patients. Comparisons were performed between patients with the highest tertile and lowest tertile IDO1 expression.

Table 1. Univariate and multivariate analysis of OS in ESCC/ESAD patients

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Table I. Univariate and multivariate analysis of OS in ESCC/ESAD patients

| Parameters                     | Univariate analysis | Multivariate analysis |
|--------------------------------|---------------------|-----------------------|
|                                | $p$     | HR     | 95% CI (lower/upper) | $p$     | HR     | 95% CI (lower/upper) |
| ESCC                           |         |        |                     |         |        |                     |
| Age                            | 0.052   | 1.036  | 1.000               | 1.074   |
| Gender                         |         |        |                     |         |        |                     |
| Female vs Male                 | 0.026   | 0.190  | 0.044               | 0.819   |
| Histologic grade               |         |        |                     |         |        |                     |
| G1/G2 vs. G3                   | 0.454   | 1.452  | 0.547               | 3.851   |
| History of esophageal cancer   |         |        |                     |         |        |                     |
| No vs. Yes                     | 0.098   | 0.359  | 0.107               | 1.209   |
| Pathologic stages              |         |        |                     |         |        |                     |
| I/II vs. III/IV                | 0.017   | 0.418  | 0.204               | 0.857   |
| Reflux History                 |         |        |                     |         |        |                     |
| No vs. Yes                     | 0.386   | 0.680  | 0.285               | 1.625   |
| Smoking History                |         |        |                     |         |        |                     |
| Yes vs. No                     | 0.320   | 1.538  | 0.659               | 3.588   |
| Radiation therapy              |         |        |                     |         |        |                     |
| No vs. Yes                     | 0.671   | 0.831  | 0.355               | 1.947   |
| Postoperative drug therapy     |         |        |                     |         |        |                     |
| No vs. Yes                     | 0.561   | 1.309  | 0.528               | 3.245   |
| IDO1 expression                | 0.036   | 1.148  | 1.009               | 1.307   | 0.022 | 1.183  | 1.025  | 1.367   |
| ESAD                           |         |        |                     |         |        |                     |
| IDO1 expression                | 0.667   | 1.024  | 0.92                | 1.14    |
