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Screening of potential key genes in esophageal cancer based on RBP and expression verification of HENMT1

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Abstract

To screen key biomarkers of esophageal cancer (ESCA) by bioinformatics and analyze the correlation between key genes and immune infiltration. Expression profile data of ESCA was downloaded from TCGA database, and DEGs in ESCA were screened with R software. After the RNA binding proteins (RBPs) in DEGs were screened, the protein interaction network was constructed using tools such as STRING and Cytoscape and the key genes (HENMT1) were screened. Survival analysis of HENMT1 was performed by Kaplan–Meier method. Functional enrichment analysis of HENMT1 interacting proteins was performed using the DAVID website, and GSEA predicted the signal pathways involved by HENMT1. CIBERSORT algorithm was used to analyze the infiltration of immune cells in ESCA. The expression of HENMT1 in ESCA was detected by immunohistochemistry. A total of 105 RNA binding proteins (RBPs) were differentially expressed in ESCA, and a PPI network was constructed to screen the key gene HENMT1. The expression level of hemmt1 gene was closely related to the infiltration of B cells naive, T cells regulatory (Tregs), neutrophils, T cells CD4 memory activated, master cells resting and dendritic cells resting in ESCA tissues ($P < .05$). Immunohistochemical results showed that HENMT1 was highly expressed in ESCA tissues and was positively correlated with the expression of MKI67. HENMT1 is related to the occurrence and prognosis of ESCA, and is also related to the infiltration of immune cells in ESCA tissue, which may provide a new idea for the targeted treatment of ESCA.

Abbreviations: DEGs = differentially expressed genes, ESCA = esophageal carcinoma, FC = fold change, GO = Gene Ontology, IHC = immunohistochemistry, KEGG = Kyoto Encyclopedia of Genes and Genomes, KM = Kaplan–Meier, PPI = protein protein interaction, RBP = RNA binding protein, TCGA = the Cancer Genome Atlas.

Keywords: esophageal carcinoma, HENMT1, immune infiltration, immunohistochemistry

1. Introduction

Esophageal cancer (ESCA) is listed as the seventh largest cancer with the sixth highest mortality rate.[1] The common types of ESCA are esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC).[2] The malignant histology of ESCA makes it a lethal malignant tumor with low survival rate.[3] Although there are advanced diagnosis and treatment methods, most cases are diagnosed at an advanced stage, and the prognosis is poor due to rapid metastasis.[4] Therefore, the research in the field of esophageal cancer has great social value and profound practical significance.

There is a class of proteins a with high affinity with mRNA in cells, which can directly or indirectly bind to cis regulatory

AR and FX contribution equal to this work.

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The datasets generated during and/or analyzed during the current study are publicly available.

This experiment was approved by the ethics committee of the affiliated Huaian No.1 People Hospital of Nanjing Medical University (KY-2022-014-01).

The datasets supporting the conclusion of this article are included within the article.

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elements to promote deadenylation and degradation of mRNA. Such proteins are called RNA binding proteins (RBPs).[5] RNA binding proteins (RBPs) play an important role in the regulation of gene expression after transcription and participate in RNA metabolism processes.[6] Most RNA binding proteins (RBPs) bind to mRNA through RNA binding regulatory domains and regulate cell function. The role of most RNA binding proteins (RBPs) in malignant tumors is to maintain the ability of proliferation, escape cell death and invasion.[7] Therefore, research of RNA binding protein (RBP) in ESCA is particularly significant.

In this study, we downloaded the mRNA expression data of ESCA from the Cancer Genome Atlas (TCGA) database, screened the differentially expressed genes (DEGs) of ESCA by

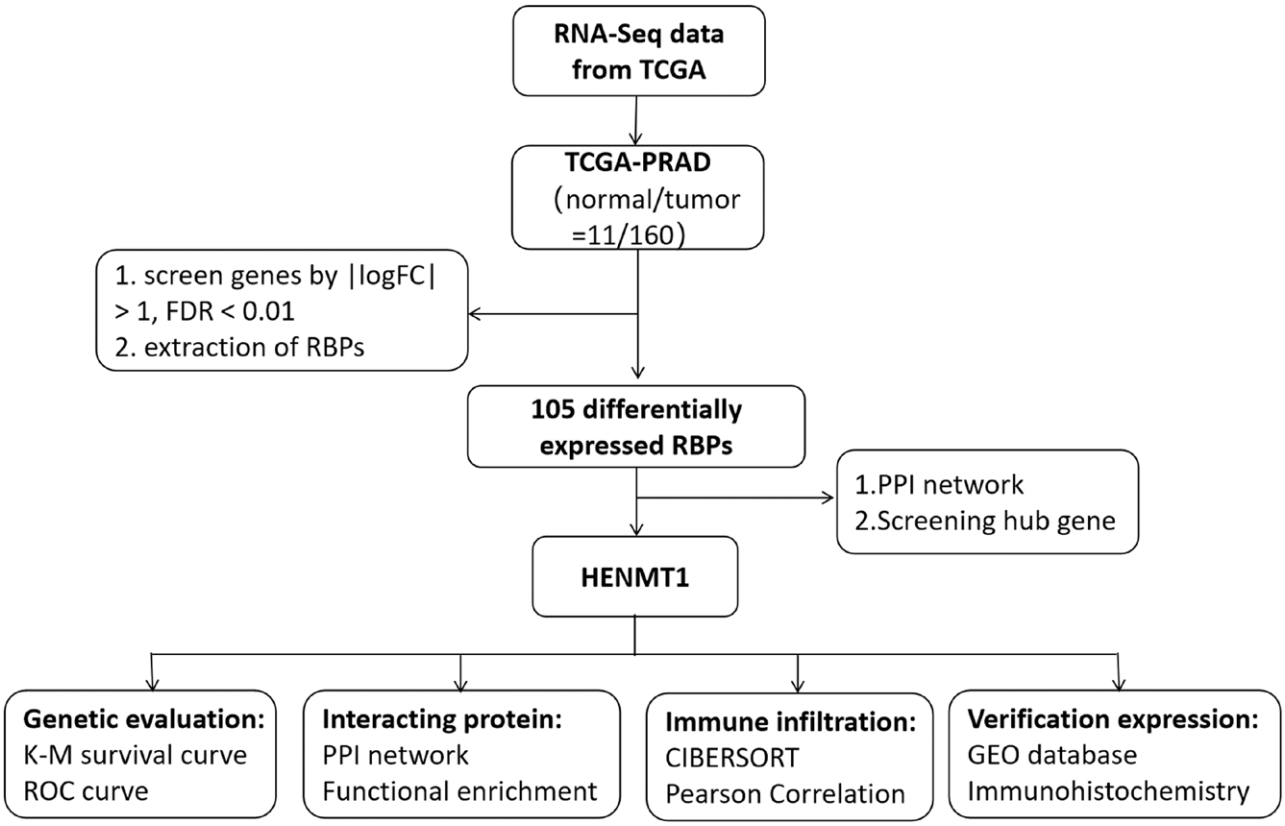


Figure 1. Research design and process of this study.

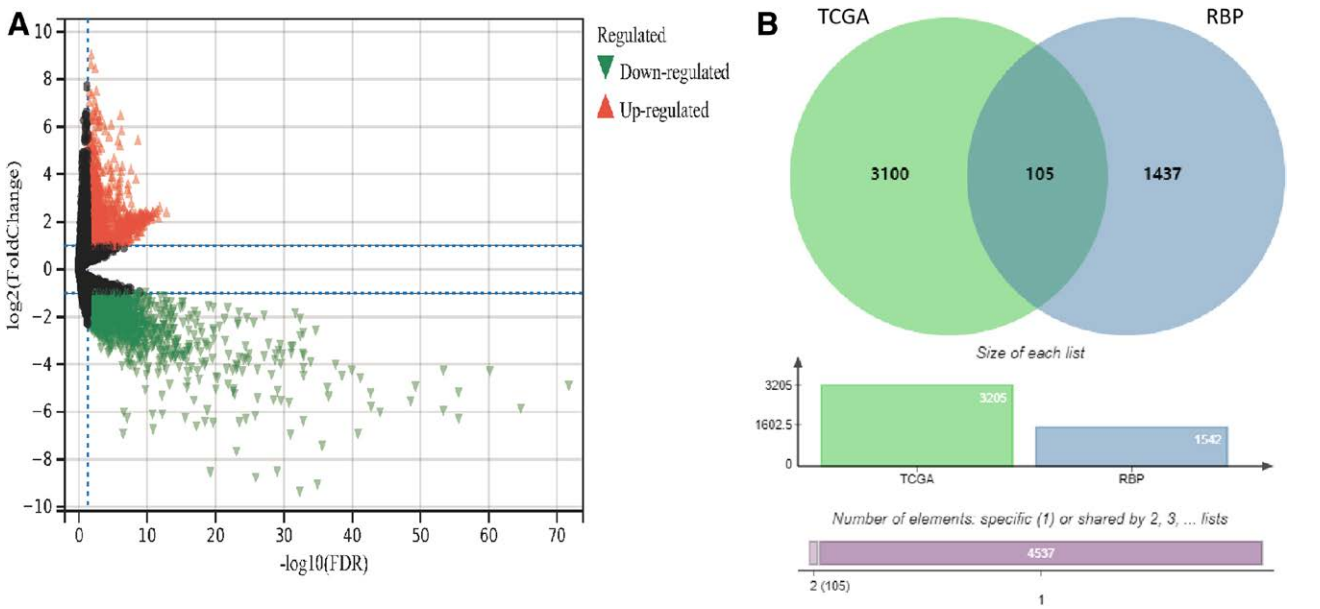


Figure 2. Screening of differentially expressed RBPs in ESCA. (A) volcano map of DEGs screening between ESCA and normal tissues. The selection criteria were |logFC| > 1 and FDR value < 0.05 (red and green represent up-regulated and down-regulated genes, respectively). Wayne diagram of differentially expressed RBPs in ESCA. DEGs = differentially expressed genes. ESCA = esophageal carcinoma, RBP = RNA binding protein.

bioinformatics methods, systematically analyzed the differentially expressed RBPs, and screened the key genes (HENMT1). To explore the interaction protein of HENMT1 and analyze the pathway mechanism. CIBERSORT algorithm was used to analyze the infiltration degree of different kinds of immune cells in ESCA, and the correlation analysis between HENMT1 and immune microenvironment was analyzed. Finally, the expression of HENMT1 in ESCA was verified by immunohistochemistry, and the correlation between HENMT1 and clinical markers of ESCA was analyzed. Exploring biomarkers for early diagnosis of ESCA provides a new idea for targeted therapy of ESCA.

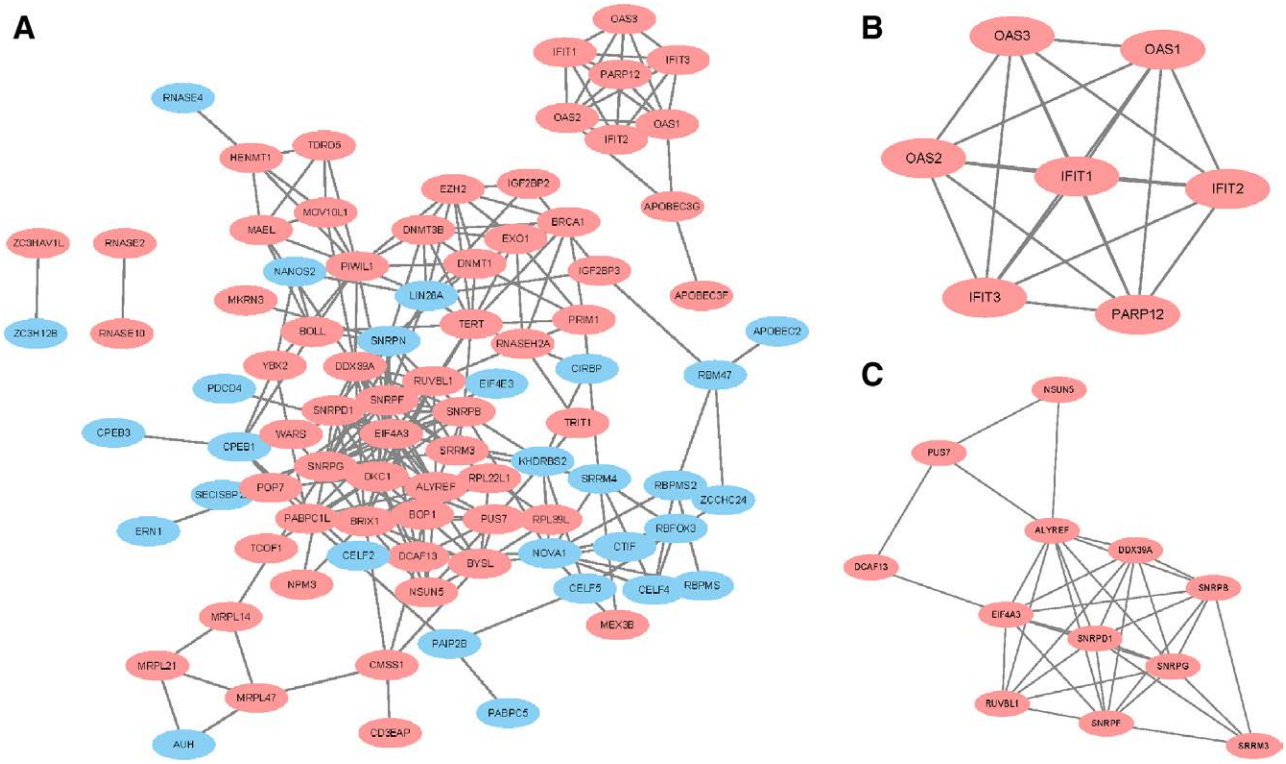


Figure 3. PPI network and significant module analysis among differentially expressed RBPs. (A) Protein interaction network of differentially expressed RBPs. (B) The first important module in PPI network. (C) The second important module in PPI network. Red nodes represent up-regulated genes, and blue nodes represent down-regulated genes. PPI = protein protein interaction, RBP = RNA binding protein.

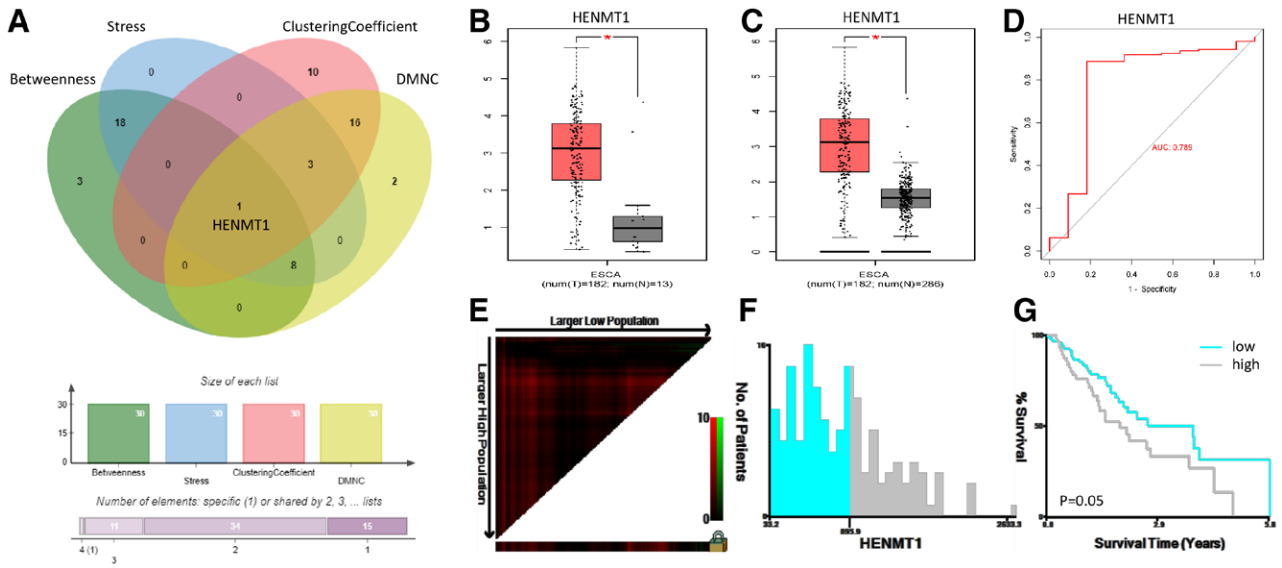


Figure 4. Screening and analysis of key genes. (A) Wayne map screening of key genes common to 4 algorithms (Betweenness, DMNC, Stress, ClusteringCoefficient). (B) Expression difference of HENMT1 in ESCA and normal tissues (TCGA). (C) Expression difference of HENMT1 in ESCA and normal tissues (TCGA + GTEx). *Represents $P < .05$. (D) ROC curve was used to analyze the diagnostic efficacy of HENMT1 for ESCA. (E) The survival data of ESCA patients were analyzed by X-tile software to determine the optimal threshold of HENMT1 expression value. (F) The data histogram showed that the optimal cutoff point for the expression value of HENMT1 was 895.9. (G) Kaplan–Meier survival curve of high and low expression group. ESCA = esophageal carcinoma, TCGA = the Cancer Genome Atlas.

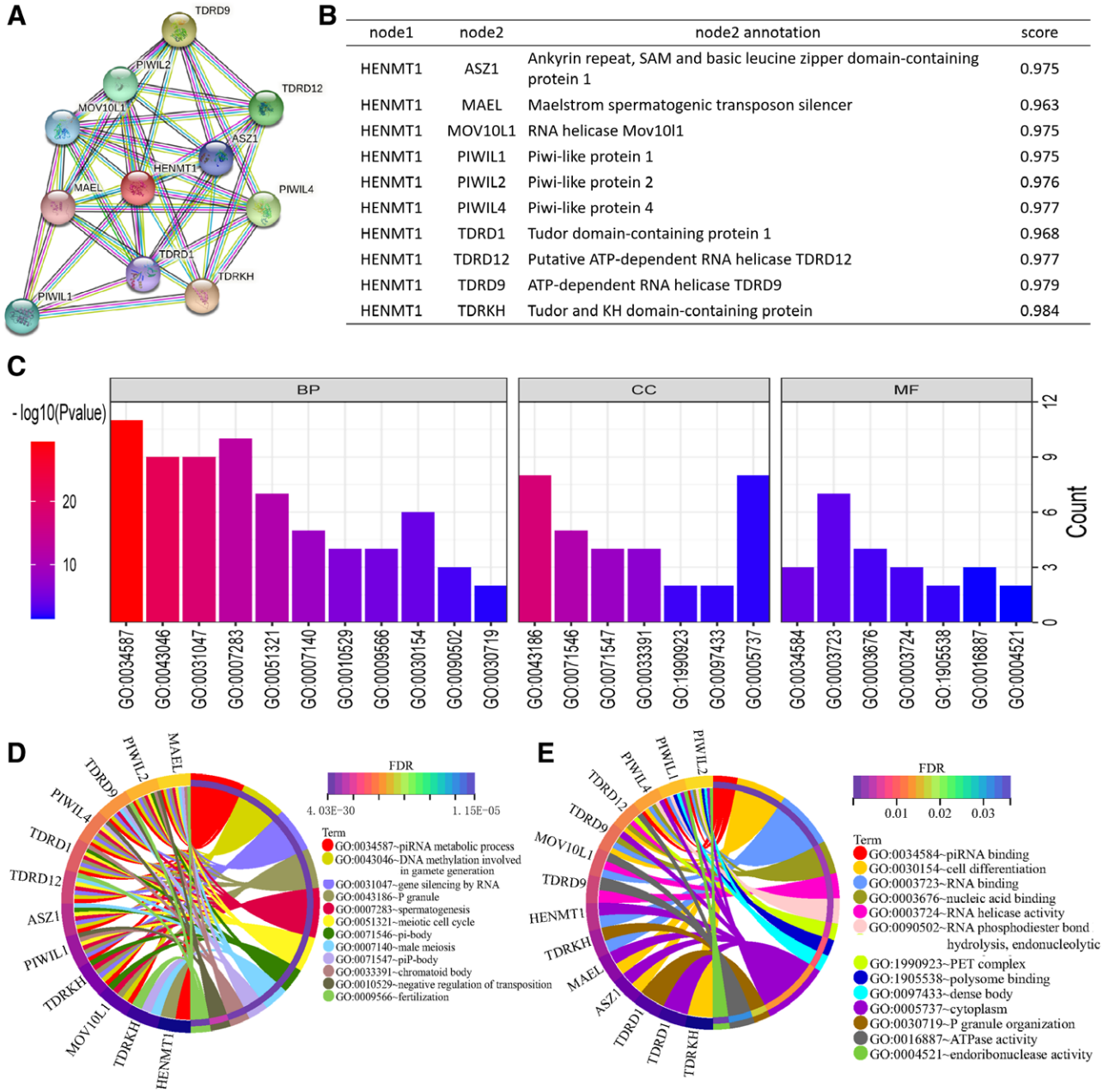
2. Materials and methods

2.1. Data acquisition and preprocessing

ESCA gene transcripts and data were obtained from TCGA database,[8] including 160 esophageal cancer tissue samples and 11 normal esophageal tissue samples. DEGs between ESCA and normal samples were screened by edgeR program.[9] False discovery rate (FDR) < 0.05 and |log2FC (fold change)| > 1.0 were considered as significant differential expression of genes. The analysis was performed on the basis of combining 1542 RBPs found in previous studies[10] (supplementary data 1, 2, 3, http://links.lww.com/MD/L12, http://links.lww.com/MD/L13, http://links.lww.com/MD/L14). Differentially expressed RBPs were extracted from DEGs for further analysis.

2.2. Construction of PPI network and screening of key genes

Differentially expressed RBPs were input into STRING database to construct PPI network diagram, and then imported into Cytoscape 3.8.2 software.[11,12] The module analysis was carried out using the software own molecular complex detection plug-in.[13] Then, the cytohubba plug-in was used to screen the differential genes with 4 conventional algorithms (Betweenness, DMNC, Stress, ClusteringCoefficient). The top 30 genes obtained by each algorithm were key genes (supplementary data 4, http://links.lww.com/MD/L15). The gene HENMT1 shared by the 4 algorithms was screened according to the Wayne map.



node1	node2	node2 annotation	score
HENMT1	ASZ1	Ankyrin repeat, SAM and basic leucine zipper domain-containing protein 1	0.975
HENMT1	MAEL	Maelstrom spermatogenic transposon silencer	0.963
HENMT1	MOV10L1	RNA helicase Mov10l1	0.975
HENMT1	PIWIL1	Piwi-like protein 1	0.975
HENMT1	PIWIL2	Piwi-like protein 2	0.976
HENMT1	PIWIL4	Piwi-like protein 4	0.977
HENMT1	TDRD1	Tudor domain-containing protein 1	0.968
HENMT1	TDRD12	Putative ATP-dependent RNA helicase TDRD12	0.977
HENMT1	TDRD9	ATP-dependent RNA helicase TDRD9	0.979
HENMT1	TDRKH	Tudor and KH domain-containing protein	0.984

Figure 5. HENMT1 interaction protein and functional enrichment analysis. (A) PPI network of proteins interacting with HENMT1. (B) Gene annotation of interacting proteins with HENMT1. (C) The function of HENMT1 and interacting proteins was divided into 3 functional groups in GO enrichment analysis: biological process (BP), cell composition (CC), and molecular function (MF). The color increases from red to blue according to the P value from small to large. (D) Distribution of HENMT1 and interacting proteins in GO enrichment function. (E) Distribution of HENMT1 and interacting proteome in GO enrichment function. GO = Gene Ontology, PPI = protein protein interaction.

2.3. Expression and prognosis analysis of key gene

The GEPIA database was used for online analysis, including TCGA data and GTEx data. The expression difference of HENMT1 in ESCA and normal tissues was analyzed. The ability of HENMT1 to distinguish ESCA from normal samples was tested with the ROC curve. The optimal truncated expression value of HENMT1 in ESCA patients analyzed by X-tile software was 895.9.[14] Patients with ESCA were divided into 2 groups according to the cutoff value.

2.4. HENMT1 protein interaction network and functional enrichment

Ten proteins that closely interact with HENMT1 were screened using the string database. In order to explore their functional mechanism, DAVID database was used for GO enrichment analysis.[15] The protein corresponding to each function is displayed with the chord diagram. Gene pathway analysis was performed using GSEA software.[16] ESCA transcription data were included in GSEA, and samples were divided into high expression group (> = 50%) and low expression group (<50%) according to the expression level of HENMT1. The gene set (c2.cp.kegg.v7.4.symbols.gmt) from the MSigDB database was used as the background gene set for KEGG enrichment analysis.[17]Satisfying both $P < .05$ and FDR < 0.25 was regarded as a significantly enriched pathway.

2.5. Evaluation of immune cell infiltration

In order to evaluate the infiltration of immune cells in ESCA, 22 immune infiltrating cell score of ESCA samples was calculated with CIBERSORT algorithm.[18] The relationship between the expression of HENMT1 and the infiltration of immune cells in ESCA was analyzed by using the Pearson correlation test.

2.6. Immunohistochemistry (IHC) staining

Clinical data of 150 ESCA pathological specimens and patients archived in our hospital from 2016 to 2017 were collected. None of the patients had received adjuvant chemotherapy before surgery. Tissue micro arrays were prepared by extracting the cancer and adjacent tissues of the patients to prepare for subsequent immunohistochemistry. The specimens were fixed in 10% neutral formalin, embedded in paraffin, and 4 μm thick serial sections, sections dewaxing and hydration, citric acid high-pressure antigen repair, PBS buffer washing, dropping primary antibody, overnight incubation at 4 °C, secondary antibody incubation at room temperature for 20min, DAB color development, hematoxylin staining, dehydration and transparency, and microscopic observation. Result interpretation: according to the percentage of the positive area and the staining intensity of positive area, pathological experts will score and evaluate. The staining results were negative (0–1), weak positive (1–2), moderate (2–3) and strong positive (≥ 3).

3. Results

3.1. Screening and identification of differentially expressed RBPs in ESCA

The research route of this paper is shown in Figure 1. Gene expression profile data of ESCA and normal tissues in TCGA database were analyzed by edgeR package. A total of 3205 DEGs were found in ESCA (Fig. 2A), including 105 RBPs (Fig. 2B). The screened 105 differentially expressed RBPs were imported into the STRING database to construct the PPI network (Fig. 3A), and then the molecular complex detection plug-in of Cytoscape 3.8.2 software was used to obtain 2 important templates (Fig. 3B and 3C).

3.2. Acquisition and analysis of key gene

The first 30 hub genes were output based on the 4 algorithms of Betweenness, DMNC, Stress and ClusteringCoefficient in

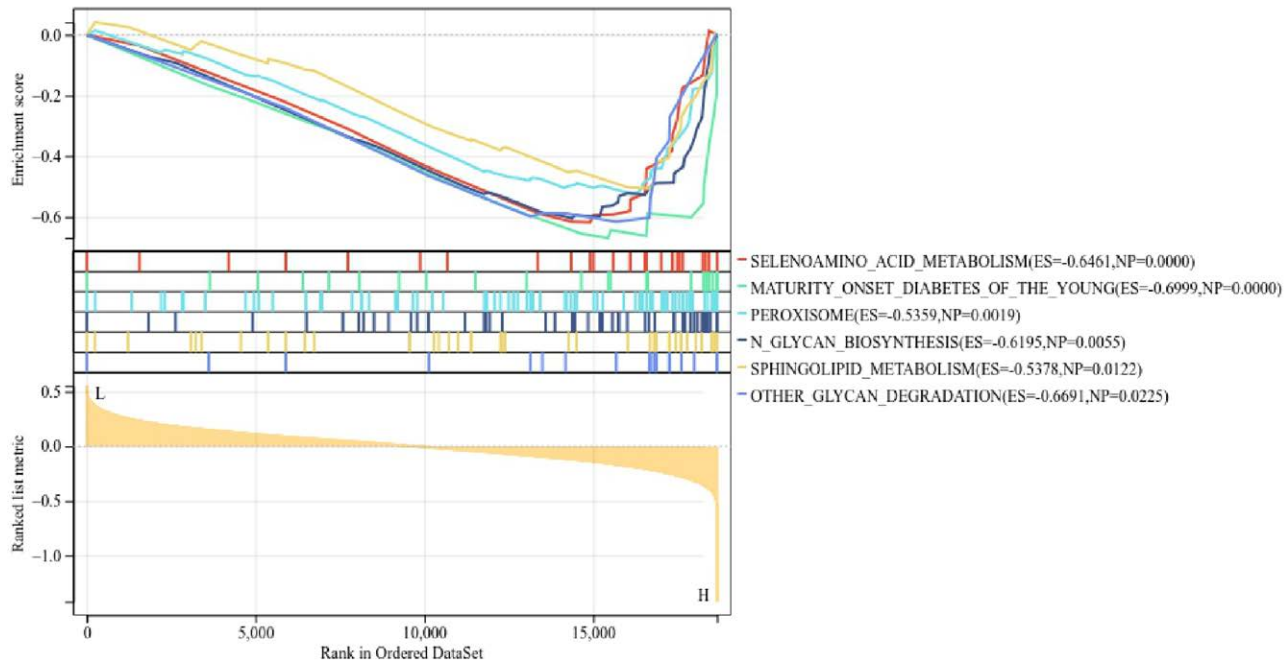


Figure 6. The KEGG pathway of high and low expression groups of HENMT1 in ESCA was analyzed based on GSEA. H denotes high expression group and L denotes low expression group. Gene sets with $P < .05$ and FDR < 0.25 were considered significant. ESCA = esophageal carcinoma, KEGG = Kyoto Encyclopedia of Genes and Genomes.

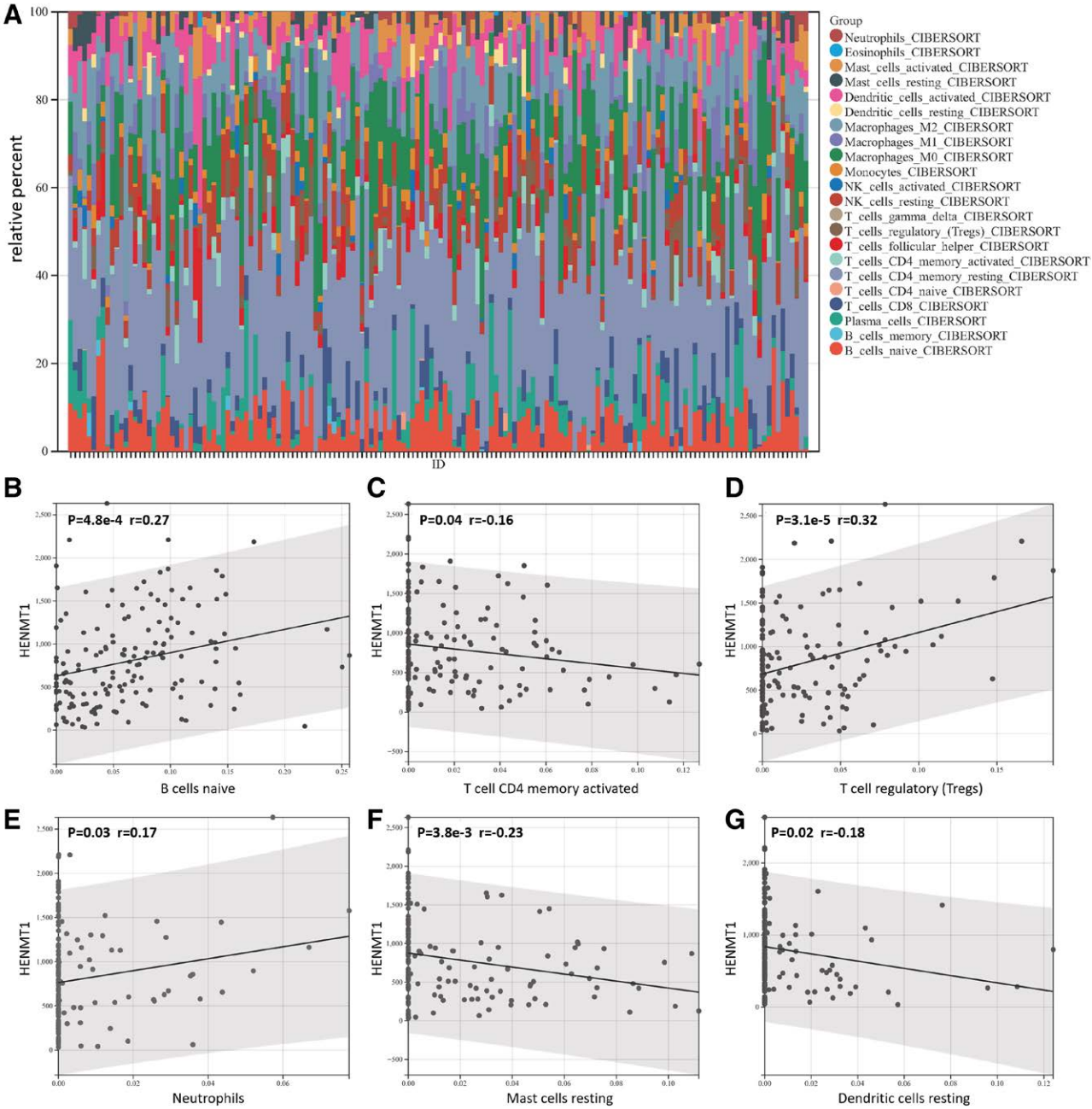


Figure 7. Evaluation of immune cell infiltration. (A) Histogram of the proportion of 22 immune cells in ESCA tissue. Each cell type is represented by a different color. Each column represents an ESCA sample ID. (B–G) Correlation between HENMT1 and the level of immune cell infiltration. R represents correlation coefficient, positive number indicates positive correlation, and negative number indicates negative correlation. ESCA = esophageal carcinoma.

the CytoHubba plug-in of Cytoscape software. One hub target: HENMT1 was obtained by drawing the Wayne map (Fig. 4A). In ESCA, it was found that HENMT1 in tumor samples (TCGA data) was higher than that in normal samples (TCGA data) (Fig. 4B). In combination with GTEx data, HENMT1 was also higher in tumor samples (TCGA data) than in normal samples (TCGA + GTEx data) (Fig. 4C). ROC curve showed that HENMT1 had better diagnostic efficacy for ESCA (AUC = 0.789) (Fig. 4D). According to the best cutoff value of HENMT1 expression obtained by X-tile software analysis (Fig. 4E), ESCA samples were divided into 2 groups (Fig. 4F). Patients with high expression of HENMT1 in ESCA have a poor prognosis (Fig. 4G).

3.3. HENMT1 protein interaction network and functional enrichment

The HENMT1 protein interaction network was constructed using string platform (Fig. 5A). The 10 proteins that most closely interact with HENMT1 are as follows: ASZ1, MAEL, MOV10L1, PIWIL1, PIWIL2, PIWIL4, TDRD1, TDRD12, TDRD9, and TDRKH (Fig. 5B). Subsequently, enrichment analysis of the above genes was carried out. The GO analysis results were divided into biological process, cell components, and molecular function groups (Fig. 5C). The proteins in the interaction network are mainly involved in piRNA metabolic process, DNA methylation involved in game generation, gene silencing by RNA, meiotic cell cycle, chromosome, piRNA

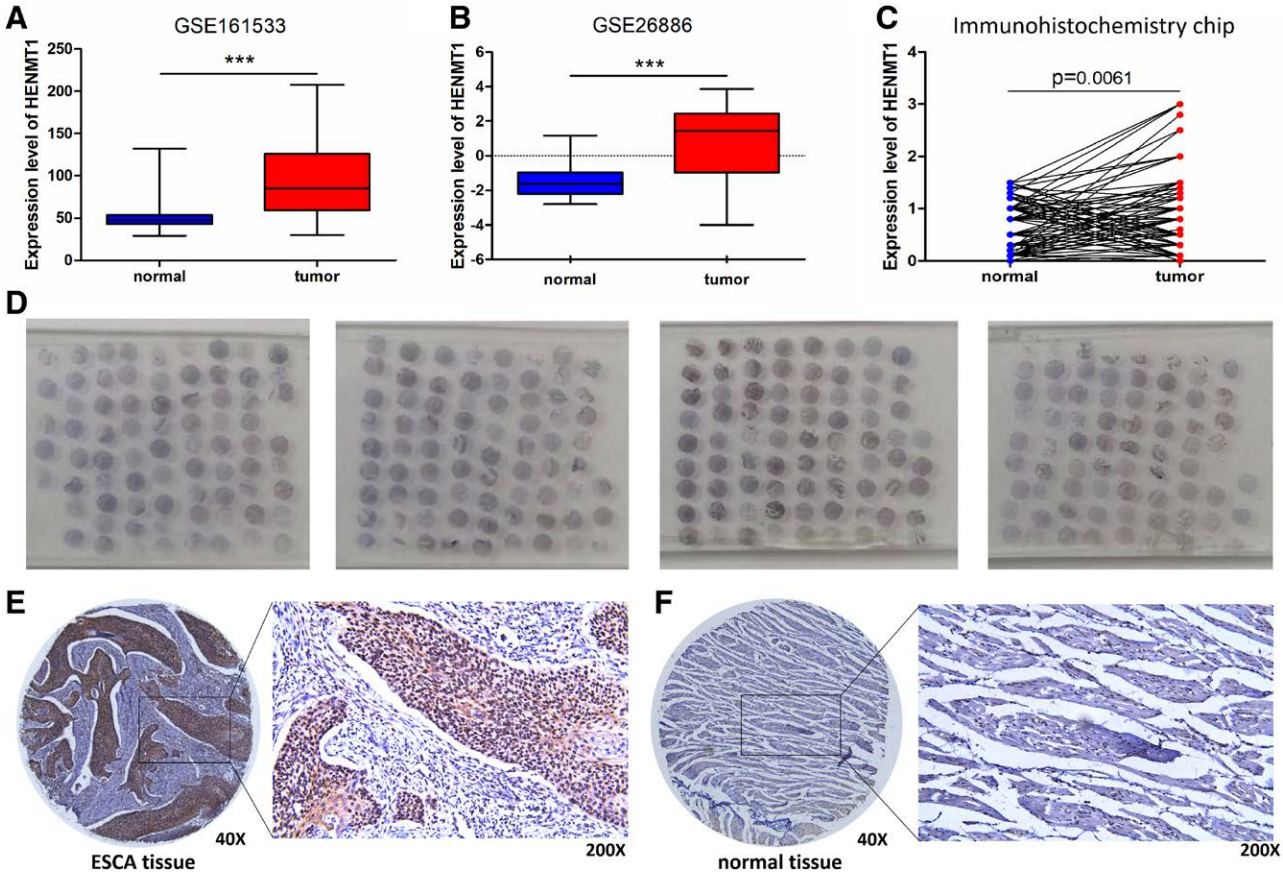


Figure 8. Expression validation of HENMT1 in ESCA. (A) Expression of HENMT1 in esophageal cancer dataset GSE161533. (B) Expression of HENMT1 in esophageal cancer dataset GSE26886. (C) Differential expression analysis of HENMT1 in cancer and adjacent tissues in tissue expression micro-array. Paired sample t-test was used to compare cancer and adjacent samples. (D) A micro-array study on the expression of HENMT1 in 150 patients with esophageal cancer and adjacent tissues. (E) Expression of HENMT1 protein in esophageal carcinoma. (F) Expression of hemmt1 protein in normal tissues. ***$P < .001$. ESCA = esophageal carcinoma.

binding, cell differentiation, RNA binding, nuclear acid binding, RNA helicase activity, RNA phosphodiester bond hydrogenation, endogenous, cytoplast, ATPase activity. The gene distribution in the GO enrichment function is shown by the chord plot (Fig. 5D and 5E). The KEGG signaling pathways involved by HENMT1 in ESCA include selenoamino acid metabolism, maturity onset diabetes of the young, peroxisome, N-glycan biosynthesis, sphingolipid metabolism and other glycan degradation (Fig. 6).

3.4. Analysis of immune cell infiltration

TCGA data were used for analysis, and CIBERSORT algorithm was used to analyze the composition of immune cells in all ESCA sample tissues in the database (Fig. 7A). The expression of HENMT1 was positively correlated with the infiltration of B cells naive ($R = 0.27$, $P = 4.8e\text{-}4$), T cells regulatory (Tregs) ($R = 0.32$, $P = 3.1e\text{-}5$) and neutrophils ($R = 0.17$, $P = .03$). The expression of HENMT1 was negatively correlated with T cells CD4 memory activated ($r = -0.16$, $P = .04$), mast cells resting ($r = -0.23$, $P = 3.8e\text{-}3$) and dendritic cells resting cell infiltration ($r = -0.18$, $P = .02$) (Fig. 7B–G).

3.5. Expression validation of HENMT1 in ESCA

In the data sets of GSE161533 and GSE26886, HENMT1 expression was higher in ESCA tumor tissues (Fig. 8A and 8B). Immunohistochemical expression micro arrays were constructed with 150 pairs of cancer tissues and adjacent tissues (Fig. 8D). The expression of HENMT1 was higher in ESCA tissues than in adjacent tissues (Fig. 8C). The results of immunohistochemistry showed that henmt1 was significantly expressed in ESCA (Fig. 8E) and relatively low in adjacent tissues (Fig. 8F). In addition, GEPIA database analysis showed a positive correlation between HENMT1 and MKI67 expression (Fig. 9C). Immunohistochemical detection showed that MKI67 was highly expressed in ESCA tumor tissues (Fig. 9A and 9B). Consistent with the database results, there was a positive correlation between the expression of HENMT1 and MKI67 (Fig. 9D).

4. Discussion

ESCA is a malignant tumor with high mortality, and the global incidence rate is on the rise.[19] The majority of ESCA patients have occult onset, and most of them have developed into middle and late stage.[20] Based on the gene data set of TCGA database, this study analyzed the gene data set of ESCA and screened 105 RBPs with differential expression. According to the PPI network constructed by the differentially expressed RBPs, HENMT1 may be closely related to the occurrence of ESCA through the cytohubba plug-in. We found that the expression of HENMT1 was up-regulated in ESCA and correlated with poor prognosis. Enrichment analysis showed that HENMT1 was a component of cytoplast and P granule, and played a role in piRNA metabolic process and RNA binding. The pathways involved by HENMT1 in ESCA are selenoamino acid metabolism, maturity onset diabetes of the young, peroxisome, N-glycan biosynthesis, sphingolipid metabolism and other glycan degradation.

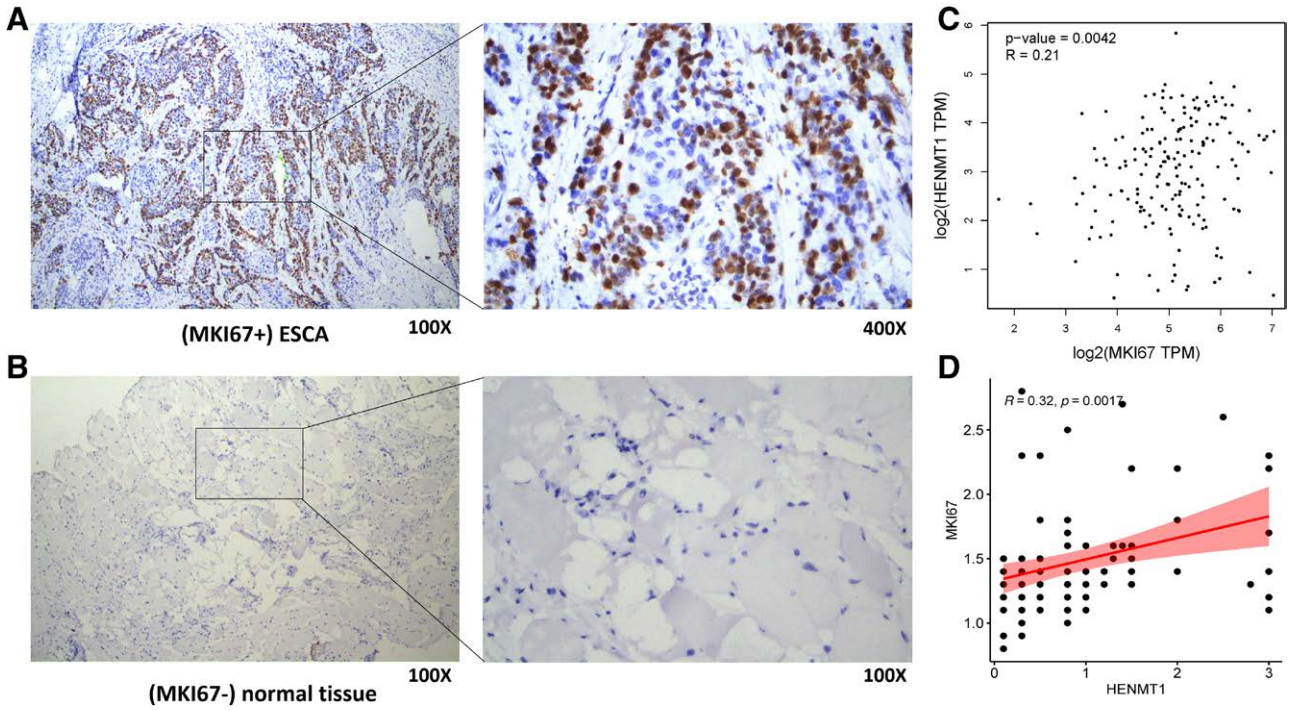


Figure 9. Correlation between HENMT1 and ESCA clinical markers. (A) Expression of clinical marker (MKI67) in ESCA. The brown part represents the target protein. (B) Expression of clinical marker (MKI67) in normal tissues. (C) The correlation between HENMT1 and ESCA clinical marker (MKI67) was analyzed by GEPIA database. R represents the correlation coefficient. (D) The correlation between HENMT1 and clinical marker (MKI67) was analyzed according to the clinical immunohistochemical results. ESCA = esophageal carcinoma.

Tumor microenvironment is an important factor in cancer progression and metastasis, and a large amount of evidence supports its important clinical significance in predicting the survival status and treatment effect of tumor patients.[21] Tumor infiltrating immune cells regulate the immune status of tumors, and immune cells participate in the progression and metastasis of tumors. Exploring the tumor infiltrating immune cells in the tumor microenvironment will help to further understand the immune status of ESCA. In this study, CIBERPORT algorithm was used to analyze the composition and infiltration of immune cells in ESCA tissues in the database. Found B cells naive, T cells Regulation (Tregs) and neutrophils were positively correlated with HENMT1 expression in ESCA. B cells naive is the precursor of mature B cells, and the infiltration of B cells naive can indirectly indicate the severity of the tumor. T cell regulation (Tregs) can inhibit the function of CD8 T cells by secreting cytokines, which indirectly promotes tumor progression.[21] It has been reported that neutrophils are associated with tumor progression. Circulating tumor cells (CTCs), as precursor cells of several types of cancer metastasis, play an important role in cancer metastasis. It has been found that neutrophils interact with CTCs, driving the cell cycle progression in the blood flow and accelerating the spread of tumor metastasis.[22] It suggests that HENMT1 may promote the metastasis and diffusion of HENMT1 through immune related pathways. Therefore, we speculate that the high infiltration of B cells naive, T cells regulatory (Tregs) and neutrophils in patients with high expression of HENMT1 may be related to the decreased survival rate of ESCA. T cells CD4 memory activated, mast cells resetting and dendritic cells resetting cell infection were negatively correlated with the expression of HENMT1 in ESCA. The increased proportion of T cells CD4 memory activated infiltration is related to the improvement of recurrence free survival of esophageal cancer.[22] Due to its unique functional plasticity, mast cells resting plays a key role in maintaining tissue homeostasis and has anticancer effects.[23] High expression of HENMT1 was accompanied by low infiltration of T cells CD4 memory activated and T cells CD4 memory activated, which may be related to the decreased survival rate of ESCA. Results of immune infiltration explain the potential mechanism by which HENMT1 predicts patient prognosis. Inhibiting the regulatory function of cancer promoting immune cells may become a potential therapeutic strategy.

HENMT1 (Small RNA 2 "- O-methyltransferase) is a protein that adds 2" - 0-methyl to the 3 "- end of piRNA, which protects the 3" - end of piRNA from degradation. The expression of HENMT1 was significant in ovarian cancer, and the expression was proportional to the increase of tumor grade and malignancy.[24] HENMT1 is highly expressed in cervical cancer and positively correlated with cell proliferation related protein MKI67.[25] At the same time, we found that the tumor marker (MKI67) was stronger in cancer tissues and had a positive correlation with the expression of HENMT1.

To sum up, this study screened and analyzed the biomarkers of ESCA with the help of bioinformatics database, identified HENMT1 as the key gene, and found that HENMT1 was highly expressed in ESCA and was associated with poor prognosis. In clinic, there was a positive correlation between HENMT1 and the expression of tumor marker MKI67. Further analysis revealed that HENMT1 may play a pivotal role in the occurrence of ESCA by affecting the tumor microenvironment. Therefore, HENMT1 is expected to become a potential biomarker and immunotherapeutic target of ESCA, which needs further research and confirmation.

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