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Analysis

Alcohol consumption and esophageal cancer risk: unveiling DLEU2 as a key immune modulator through Mendelian randomization and transcriptomic analysis

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Abstract

Esophageal cancer (EC) is a leading cause of cancer-related mortality globally, with alcohol consumption being a significant risk factor. However, the genetic and molecular mechanisms linking alcohol intake to EC remain unclear. This study utilized Mendelian randomization analysis to establish a causal relationship between alcohol consumption and EC (OR: 4.11 [95% CI 1.83–9.23]). Transcriptomic analysis identified 83 differentially expressed genes (log_2 fold change > 1, false discovery rate [FDR] < 0.05), among which DLEU2 was uniquely transcribed into a long non-coding RNA (lncRNA). Pan-cancer analysis revealed its association with the tumor immune microenvironment and cancer progression. Single-cell RNA sequencing localized *DLEU2* expression predominantly to T cells, particularly exhausted subpopulations, and pseudo-temporal analysis demonstrated increased *DLEU2* expression during late T cell differentiation stages, co-expressing immune suppression markers, with consistent expression patterns observed across multiple patient-derived samples. Additionally, cell communication analysis suggested that *DLEU2* modulates TNF signaling through TNFRSF1A/B pathways, contributing to immune evasion and poor prognosis. These findings position *DLEU2* as a pivotal regulator of the immune landscape in EC and a potential prognostic biomarker and therapeutic target.

Keywords Mendelian randomization · Esophageal cancer · Alcohol · Single-cell sequencing · Bulk-RNA sequencing · DLEU2

Abbreviations

EC	Esophageal cancer
ESCC	Esophageal squamous cell carcinoma
DLEU2	Deleted in lymphocytic leukemia 2
MR	Mendelian randomization
SNPs	Single nucleotide polymorphisms
GWAS	Genome-wide association studies
TCGA	The Cancer Genome Atlas
GEO	Gene Expression Omnibus

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DEGs	Differentially expressed genes
GSEA	Gene Set Enrichment Analysis
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
GSVA	Gene Set Variation Analysis
scRNA-seq	Single-cell RNA sequencing
Tex	Exhausted T cells
TNF	Tumor necrosis factor
TNFRSF1A/B	Tumor necrosis factor receptor superfamily member 1A/B
UPR	Unfolded protein response
IVW	Inverse variance weighting
LDSC	Linkage disequilibrium score regression
Treg	Regulatory T cells
UMAP	Uniform Manifold Approximation and Projection
t-SNE	T-distributed stochastic neighbor embedding

1 Introduction

Cancer remains a leading global health burden, with approximately 20.2 million new cancer cases and 9.9 million cancer-related deaths estimated worldwide in 2024, according to *Cancer Statistics, 2024*. Esophageal cancer (EC), in particular, continues to pose a significant challenge, ranking eighth in global incidence and sixth in cancer-related mortality. Despite advances in early detection, EC often presents at advanced stages, resulting in a 5-year survival rate of less than 20%. This underscores the urgent need for deeper molecular and mechanistic understanding of EC to inform more effective prevention and treatment strategies [1, 2]. Esophageal cancer (EC) is the most prevalent type of the most prevalent cancer, with its morbidity and mortality ranking in the top 10 among all cancers. EC is the eighth most common cause of cancer morbidity and the sixth most common cause of cancer-related death worldwide [3]. EC has no obvious symptoms in its early stages and is often diagnosed at an advanced stage, typically after lymph node metastasis or surrounding tissue infiltration has occurred, resulting in a poor prognosis and a 5-year survival rate of less than 20% [4]. Alcohol is associated with a multiplied risk of development of esophageal carcinoma [5], particularly esophageal squamous cell carcinoma (ESCC) [6]. Alcohol drinking is a serious public health issue. WHO has estimated that 3.6% of global deaths in 2004 and 4.4% of disability-adjusted life years (DALYs) were attributable to alcohol drinking [1]. According to the results of the International Agency for Research on Cancer (IARC), there is evidence of a causal relationship between alcohol consumption and EC [7]. A clear dose-response relationship was shown between the amount of alcohol drinking and EC risk [1]. Light drinking, including even one alcoholic drink a day, is associated with increased risks of EC [8]. Reducing alcohol is associated with a lower risk of EC [9].

Some discoveries have already been made regarding the mechanism by which alcohol increases the incidence of EC. The carcinogenic mechanism of ethanol is closely related to its metabolism. In the liver, ethanol is converted into acetaldehyde through alcohol dehydrogenase (ADH), and then acetaldehyde is further converted into acetic acid by another enzyme, aldehyde dehydrogenase-2 (ALDH 2). Ethanol is not only associated with increased cell membrane permeability but also promotes the penetration of carcinogens into mucosal epithelial cells due to its solvent properties. Although ethanol itself is not a direct carcinogen, it exerts its carcinogenic effect through downstream metabolic products, which can act as co-carcinogens and/or tumor promoters [10]. High concentrations of acetaldehyde can induce the proliferation of epithelial cells in the upper digestive tract of rats [11]. The inactive ALDH2 enzyme, often resulting from the **ALDH2*1/2 heterozygous genotype*, leads to impaired acetaldehyde metabolism and increased accumulation of this toxic metabolite after alcohol consumption. This genotype is particularly common in East Asian populations and is considered a major genetic risk factor for esophageal cancer due to the heightened carcinogenic effects of acetaldehyde [12, 13].

Another factor that may contribute to the development of EC is the formation of reactive oxygen species (ROS) during ethanol oxidation. Elevated rates of ROS production have been observed in nearly all cancers, where they promote various aspects of tumor development and progression [14]. Additionally, it is hypothesized that disruptions in folate metabolism are related to alcohol-mediated carcinogenesis [15]. The accumulation of ROS and folate deficiency can induce genetic changes closely associated with carcinogenesis. ROS accumulation can increase the levels of monocyte chemoattractant protein-1 and vascular endothelial growth factor, both of which are key mediators of tumor angiogenesis and metastasis

[16]. Folate deficiency can alter DNA methylation and impair DNA integrity and repair. As a result, it may also lead to changes in the expression of key tumor-related genes, such as p53, thereby enhancing carcinogenesis [17]. However, the molecular mechanisms linking alcohol consumption and EC remain unclear.

The abnormal expression of protein-coding mRNA and non-coding RNA, along with their regulatory networks, is closely associated with the occurrence and progression of cancer [18]. Non-coding RNAs include small non-coding RNAs, long non-coding RNAs (lncRNAs), and ultralong non-coding RNAs. MiRNAs are short, endogenously expressed non-coding RNAs that can bind to their target genes in a sequence-specific manner, negatively regulating gene expression by silencing translation and/or catalyzing mRNA destabilization [19]. LncRNAs contain miRNA response elements and can act as miRNA 'sponges', thereby competing with target mRNAs for binding to specific miRNAs as competitive endogenous RNAs (ceRNAs) [19, 20] An increasing number of studies have revealed the significant role of miRNAs/lncRNAs in the pathological progression of EC, including in proliferation, apoptosis, invasion, angiogenesis, metastasis, chemoradiotherapy resistance, and cancer stemness. This indicates that these non-coding RNAs have potential clinical application value as diagnostic and prognostic biomarkers [21, 22] However, research focusing on the RNA regulatory networks involved in alcohol-related EC development is very scarce.

Mendelian Randomization (MR) is a genetic epidemiological method that uses genetic variants as instrumental variables to assess causal relationships between exposures and outcomes, minimizing confounding. Recent MR studies have explored links between traits like sarcopenia and ovarian cancer, and eczema with pan-cancer risk, demonstrating its value in cancer research [23, 24]. By leveraging genetic variants, which are allocated randomly and unaffected by lifestyle factors and chronic conditions, MR studies mitigate bias from both known and unknown confounders. Additionally, as genetic variants act as proxies for the enduring impact of exposure or mediator, MR studies typically remain resilient to non-differential measurement errors. Moreover, the availability of summary statistics from large genome-wide association studies (GWAS) provides a timely and cost-effective opportunity to investigate the causal relationships between alcohol consumption and malignant neoplasm of the esophagus.

To better understand the relationship between alcohol consumption and EC, this study employs Mendelian randomization to re-examine the impact of alcohol consumption on the incidence of EC. By identifying SNPs strongly associated with alcohol consumption through GWAS data and screening genes related to lncRNA expression, the study aims to explore the mechanisms by which alcohol increases the incidence of EC.

2 Materials and methods

2.1 Study design

The present study consisted of two parts. First, we used Mendelian randomization (MR) to analyze the relationship between alcohol consumption and EC, followed by a meta-analysis of the results. We further refined the selection of genes that are differentially expressed between EC and normal tissues. Genes strongly associated with alcohol and capable of being transcribed into lncRNA were identified. Subsequently, single-cell sequencing and bulk-RNA sequencing were employed to investigate the pathways through which lncRNAs contribute to the increased risk of EC associated with alcohol consumption in detail (Fig. 1).

This study adheres to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for MR studies. In a two-sample MR study, summary-level data from genome-wide association studies (GWAS) were used to identify genetic proxies for the exposure and investigate their associations with the outcome. Supplement Table 1A Data extraction was conducted between June 1, 2024, and July 1, 2024.

2.2 Materials

Data sources of alcohol consumption are from GWAS and Sequencing Consortium of Alcohol and Nicotine Use (GSCAN) and IEU OpenGWAS project (https://gwas.mrcieu.ac.uk/), containing 941,280 and 462,346 individuals respectively. The GWAS data of malignant neoplasm of esophagus we extracted are from FinnGen database (release11, https://www.finngen.fi/en/access_results) and IEU OpenGWAS project, containing 345,881 and 476,306 individuals respectively. The additional information and sources of these data and R packages can be found in Supplement Table 1B. To minimize the issue of sample overlap between exposure and outcome, we divided the data into two groups for analysis (Fig. 1).

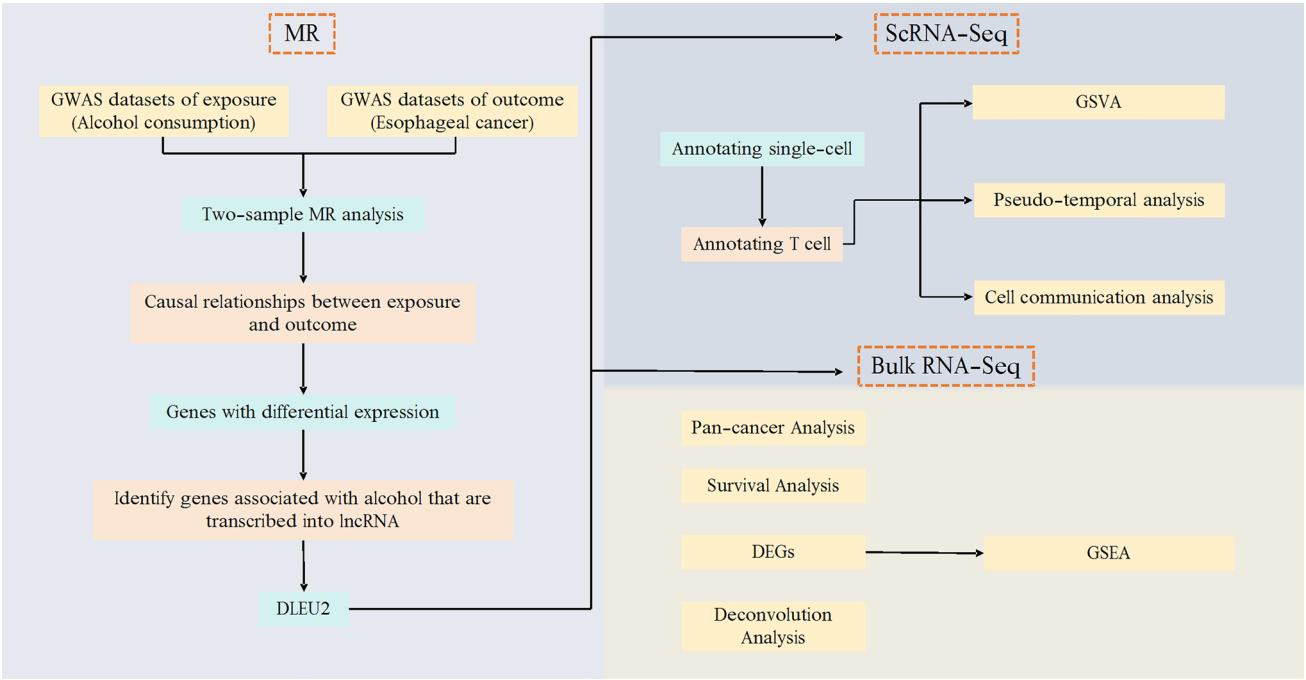

Fig. 1 Study design and experimental workflow. Schematic diagram illustrating the experimental design for investigating the effects of Alcohol Consumption on Esophageal Cancer

Gene expression profiles for Esophageal carcinoma and their corresponding clinical data were obtained from the Cancer Genome Atlas Program (TCGA) database via the UCSC Xena platform [25] (http://xena.ucsc.edu/). We also obtained the raw data of GSE196756 from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) [26, 27].

We extracted SNPs associated with Alcohol consumption. Genome-wide significant ($P < 5 \times 10^{-8}$) SNPs were clumped by linkage disequilibrium (LD) using a threshold of $r^2 < 0.01$ within a 10,000-kb window to ensure the independence of instrumental variables (IVs) and avoid bias due to correlated variants. The strength of correlation between SNPs and the target phenotype was represented by the F statistic, calculated as $F = \beta^2/SE^2$, where β is the effect size and SE is the standard error of the SNP–exposure association. Generally, an F statistic > 10 indicates a strong correlation between instrumental variables (IVs) and the target phenotype. We estimated the genetic correlation between alcohol consumption and EC using LDSC. The LDSC examines the association between test statistics and linkage disequilibrium to quantify the contribution of inflation from a true polygenic signal or bias [28]. This method can evaluate genetic correlation from GWAS summary statistics and is not biased by sample overlap [29]. The detailed steps can be found in Supplement Table 1C. We computed the Wald ratio for each SNP and used the Inverse Variance Weighting (IVW) analysis to summarize the associations of all SNPs, which emphasizes estimates with the smallest variance and demonstrates markedly higher statistical power compared to other MR techniques, particularly MR-Egger. Therefore, IVW is typically used as the primary method for identifying potentially important results in MR analysis. However, for IVW estimates to be unbiased, all SNPs included in the analysis were validated (Supplement Table 1D, E).

2.3 Sensitivity analysis

We conducted the following sensitivity analyses—including Weighted Median, Weighted Mode, MR-Egger regression, and MR-PRESSO—to ensure the robustness of our results, following Mendelian Randomization best practices as outlined by Burgess et al. [30], which provide unbiased estimates even in the presence of some invalid instruments: Weighted Median, Weighted Mode, and MR Egger regression [31]. We also applied the MR-PRESSO method [32] to remove outliers and conducted heterogeneity tests (Cochran's Q test) and pleiotropy tests [30]. If horizontal pleiotropy exists, IVW estimates may be biased. In such cases, MR-Egger estimates should be considered as a reference, as MR-Egger can adjust the IVW analysis by allowing the horizontal pleiotropy effects of all SNPs to be unbalanced or oriented. We generated leave-one-out plots, funnel plots, and density plots to help evaluate the robustness of the results. Additionally, we conducted a meta-analysis of the MR results.

2.4 Gene selection

To further explore the relationship between alcohol consumption and EC, we adopted a refined localization strategy to map specific SNPs to genes. Specifically, each selected SNP was positioned according to the GRCh37 reference genome version. To comprehensively consider potential long-range regulatory effects, we extended the localization range to include the regions 100 kb upstream and downstream of the SNP site. This approach allows us to capture genetic variations that may not only be limited to the gene coding regions but also potentially impact gene expression and function. Through this systematic analysis method, we can more accurately understand how SNPs are associated with specific genes and how they may influence biological processes and disease risk. Through GEPIA (http://gepia.cancer-pku.cn/), we further refined the selection of genes that are differentially expressed between EC and normal tissues. Genes strongly associated with alcohol and capable of being transcribed into lncRNA were identified.

2.5 Expression and survival analysis

We used the GEPIA (http://gepia.cancer-pku.cn/) database to conduct a pan-cancer analysis of the DLEU2 [33]. Then we focused on Esophageal carcinoma to examine the differential expression of the DLEU2 in tumor tissues and adjacent normal tissues. We then analyzed the relationship between the expression of DLEU2 and overall survival time in Esophageal carcinoma. Clinical variable analysis was assessed statistically using Log-rank tests, with a p-value (FDR) < 0.05 considered statistically significant.

2.6 Differential gene analysis and functional enrichment analysis

Using the DESeq2 R package, we performed differential gene expression analysis on high and low expression groups of DLEU2, followed by visualization using a volcano plot. We conducted functional enrichment analysis of this gene set using Gene Set Enrichment Analysis (GSEA) and visualized enriched pathways in Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and hallmark gene sets [34–36]. Pathways with FDR q-values < 0.05 were considered significant.

2.7 Deconvolution analysis

The "ssGSEA" deconvolution algorithm was employed to identify the composition of immune cell types in the Esophageal carcinoma immune microenvironment from TCGA data [37]. The statistical significance was determined using the Spearman test. The "ggpubr" package was used to visualize the differences in immune microenvironment composition between high and low DLEU2 expression groups in samples, and the Wilcox test was used to detect its statistical significance.

2.8 The dimensionality reduction, clustering, and cell annotation of single-cell data

After obtaining single-cell data, we used the Seurat package for data loading, followed by steps including doublet removal, dimensionality reduction, clustering, and batch effect correction using MNN integration. Subsequently, we annotated 26 Seurat clusters comprising 26,295 cells into 10 major cell types and subtypes: "Epithelial/Tumor cell," "Mast cell," "B cell," "T cell," "Fibroblast cell," "Neutrophil cell," "Myeloid cell," "NK cell," "Plasma cell," and "Endothelial cell" [30]. Subsequently, the extracted 6267T cells were further subjected to dimensionality reduction clustering and annotated as "CD4+ Tn," "CD8+ Ttox," "CD4+ Th17," "CD4+ Treg," "CD4+ Tfh," and "CD8+ Tex".

2.9 Gene set variation analysis

Single-cell data was processed using the GSVA package to compute Gene Set Variation Analysis (GSVA) scores based on the Hallmark gene set database. First, the expression matrix was prepared where each row represents a gene and each column represents a cell. The Hallmark gene sets were obtained from the org.Hs.eg.db package. Subsequently, GSVA scores were calculated using the gsva function, applying the GSVA method. These scores reflect the relative enrichment of gene sets within individual cells. Visualization of the GSVA scores was achieved through heatmaps.

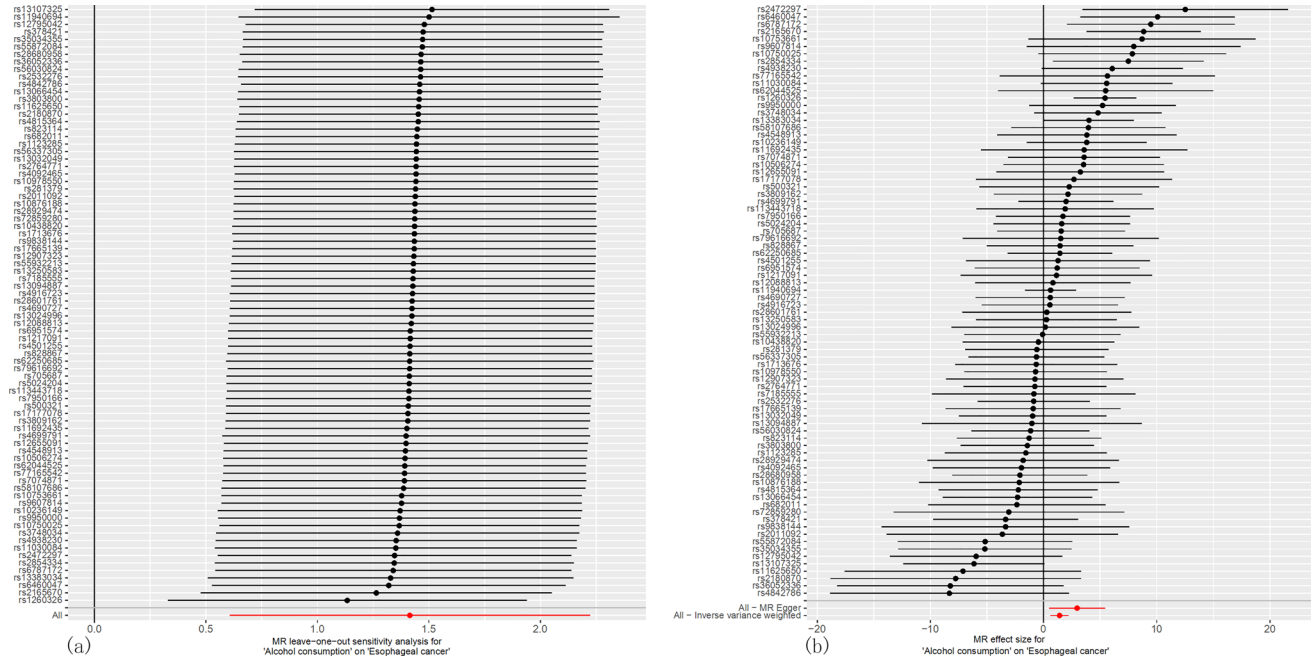

Fig. 2 MR Analysis results for Group 1: leave-one-out plot and forest plot. Group 1: GWAS data of alcohol consumption from GSCAN as exposure, and GWAS data of esophageal cancer from IEU database. *Mendelian randomization (MR) analysis results showing the causal association between alcohol consumption and esophageal cancer. The leave-one-out plot assesses the robustness of individual genetic variants, while the forest plot summarizes causal estimates from different single nucleotide polymorphisms (SNPs)*

2.10 Pseudo-time lineage trajectory

The lineage trajectory analysis of re-annotated T cells was performed using the R package "Monocle2". We employed the dpFeature method to identify genes associated with biological processes. Subsequently, "Monocle2" utilized machine learning techniques to order cells along trajectories in a two-dimensional space [38]. Concurrently, we assessed the expression of genes related to immune suppression over the temporal axis in T cells.

2.11 Cell communication network construction

We employed the "CellChat" R package to identify overexpressed genes and significant ligand-receptor pairs from single-cell data. Communication probabilities were calculated based on the ligand-receptor pairs, and the communication network was derived using permutation test-based p values.

3 Results

The F-statistics for all SNPs were greater than 10, detailed information can be found in Supplement Table 1F.

3.1 Mendelian randomization analysis

Alcohol consumption is positively associated with EC (OR: 4.11; 95% CI 1.83–9.23, $p < 0.001$), where the odds ratio reflects the effect per genetically predicted one-unit increase in alcohol consumption (measured as drinks per day). No directional pleiotropy was found in the MR-Egger regression (egger_intercept = − 0.019, $p = 0.199$). Only a slight heterogeneity ($p = 0.041$) was observed in Cochran's Q test. However, we primarily adopted the results of the IVW method, which inherently operates as a random effects model. No outlier was identified by the MR-PRESSO global test ($p > 0.05$), indicating no significant distortion due to horizontal pleiotropy. The robustness of result was confirmed by the leave-one-out sensitivity

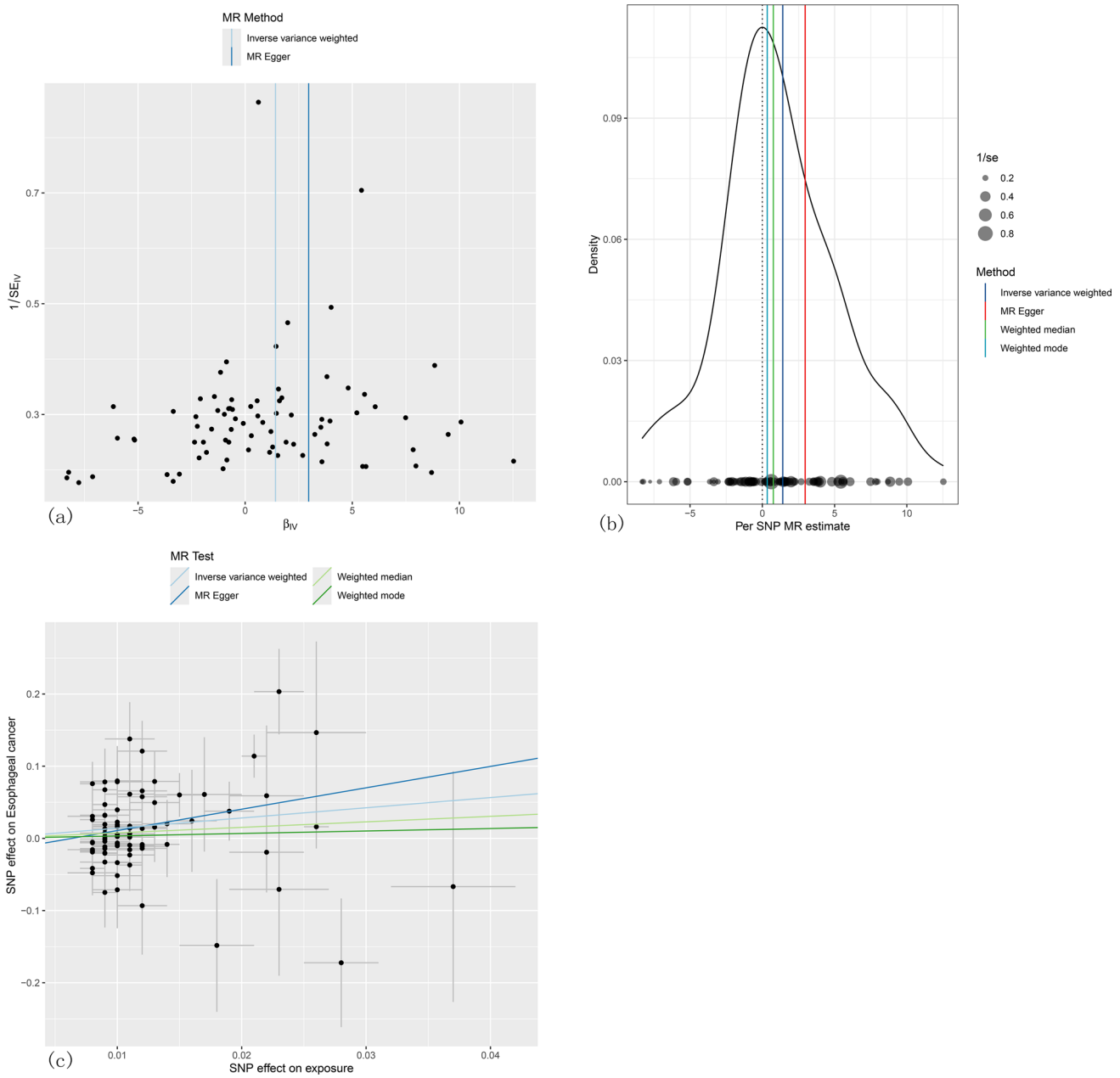

Fig. 3 MR analysis results for Group 1: scatterplot, funnel plot and density plot. Group 1: GWAS data of alcohol consumption from GSCAN as exposure, and GWAS data of esophageal cancer from IEU database

test and the forest plot and scatter plot of causal relationships between genetically predicted alcohol consumption and the risk of E are shown in Fig. 2, and the other details of sensitivity analyses are shown in Fig. 3 (Supplement Table 1F).

We performed an LDSC regression analysis to evaluate the genetic correlation between alcohol consumption and EC. Consistent with previous MR results, the LDSC results show a positive correlation between alcohol consumption and EC (rg = 0.155, p = 0.196) Moreover, the MR estimates are not confounded by shared genetic components. In group 2, we did not find a correlation between alcohol consumption and EC. We utilized a random effects model to conduct a meta-analysis of the results, but not found statistical significance ($p > 0.05$) (Fig. 4). It could be influenced by the sample size of the data. Nevertheless, to further explore the relationship between alcohol consumption and EC, using the SNPs strongly associated with alcohol consumption, we adopted a refined localization strategy to map specific SNPs to genes. Ultimately, we identified 1000 genes and determined that 83 genes exhibited differential expression between normal tissue and EC (Supplement Table 1G). After validation, we found that DLEU2 (chr:13,

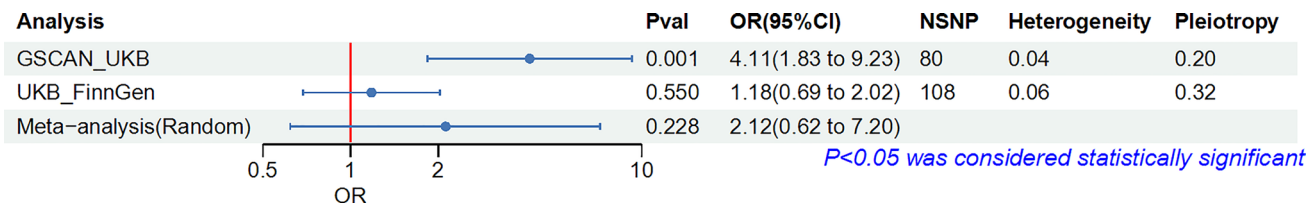

Fig. 4 Meta-analysis results for Group 1 and Group 2. Using a Random-Effects Model. Group 1: GWAS data of alcohol consumption from GSCAN as exposure, and GWAS data of esophageal cancer from IEU database. Group 2: GWAS data of alcohol consumption from IEU database as exposure, and GWAS data of esophageal cancer from UK Biobank

pos:49956670–50125720) can transcribe and express lncRNA, and the SNP (rs7330939, chr:13, pos:49971400) is associated with the DLUE2 gene. DLEU2 was identified based on SNPs strongly associated with alcohol consumption, and the Wald ratio results (β = 3.32) for these SNPs also support the conclusion that alcohol increases the incidence of esophageal cancer. Therefore, we continued to explore DLEU2 as a bridge connecting alcohol consumption and esophageal cancer (Supplement Table 1H).

3.2 Pan-cancer and RNA analysis revealed that high expression of DLEU2 in EC correlates with poor prognosis

We analyzed the expression of DLEU2 across pan-cancer profiles using the GEPIA database. We found that DLEU2 is highly expressed in various environmentally stimulated cancers including esophageal carcinoma, cervical cancer, and glioma (Fig. 5A). In particular, compared to normal tissues, DLEU2 is significantly upregulated in EC, suggesting its potential relevance to carcinogenesis (Fig. 5B). Further survival analysis of EC patients stratified by DLEU2 expression levels indicates poorer prognosis in those with relatively higher DLEU2 expression (Fig. 5C). We conducted the differential analysis to explore the impact of high DLEU2 expression in EC and visualized the results using volcano plots. Our findings revealed significant upregulation of genes associated with cell cycle and proliferation, such as TOP2A and NUF2, and environmental stress response, including COL9A3, in the high DLEU2 expression group (Fig. 5D). Using GSEA, we further investigated pathways enriched by differentially expressed genes (DEGs), confirming significant upregulation of cell cycle, MYC Pathway, and UPR pathways in the high DLEU2 group, consistent with our DEG analysis (Fig. 5E–G). Additionally, Immune deconvolution analysis revealed that in the high DLEU2 expression group, the proportion of immune cytotoxic cells like γδT cells decreased, while immune escape-related cells such as Th2 cells significantly increased. These findings suggest a role for DLEU2 in immune evasion in EC (Fig. 5H).

3.3 Single-cell sequencing reveals specific expression of DLEU2 in exhausted T cells

Due to the limitations of bulk sequencing in assessing DLEU2 expression across whole tumor tissues, we employed single-cell sequencing for further analysis of EC. This dataset included three pairs of EC and adjacent normal tissues. Following doublet removal, quality control, and batch correction, we annotated 26,295 cells into 10 distinct cell subtypes (Fig. 6A). We observed a higher proportion of T cells in tumor tissues compared to normal tissues, suggesting EC is characterized by enriched T cell infiltration (Fig. 6B).

Furthermore, we analyzed DLEU2 expression and distribution. Violin plots demonstrated significantly higher expression of DLEU2 in tumor tissues compared to normal tissues, consistent with our bulk sequencing analysis (Fig. 6C). Additionally, using feature plots, we observed pronounced expression of DLEU2 within T cell subtypes in EC, suggesting its potential role in promoting tumorigenesis through modulation of T cell function (Fig. 6D). GSVA analysis revealed that the main pathway activity differences between EC and normal tissues lie in cell cycle and UPR pathways, with significant upregulation observed particularly in T cells within these pathways (Fig. 6E, F). Therefore, we further isolated T cells and performed additional dimensionality reduction clustering and cell annotation, resulting in the identification of 6T cell subtypes: Tex, Tfh, Treg, Th17, Ttox, and Tn (Fig. 7A–C). Fig Interestingly, DLEU2 showed significantly high expression specifically in the Tex subtype, which exhibited a notable increase in proportion within tumor tissues (Fig. 7D). Moreover, pathways like UPR were also expressed in this T cell subtype, suggesting Tex cells play an essential pro-tumorigenic role

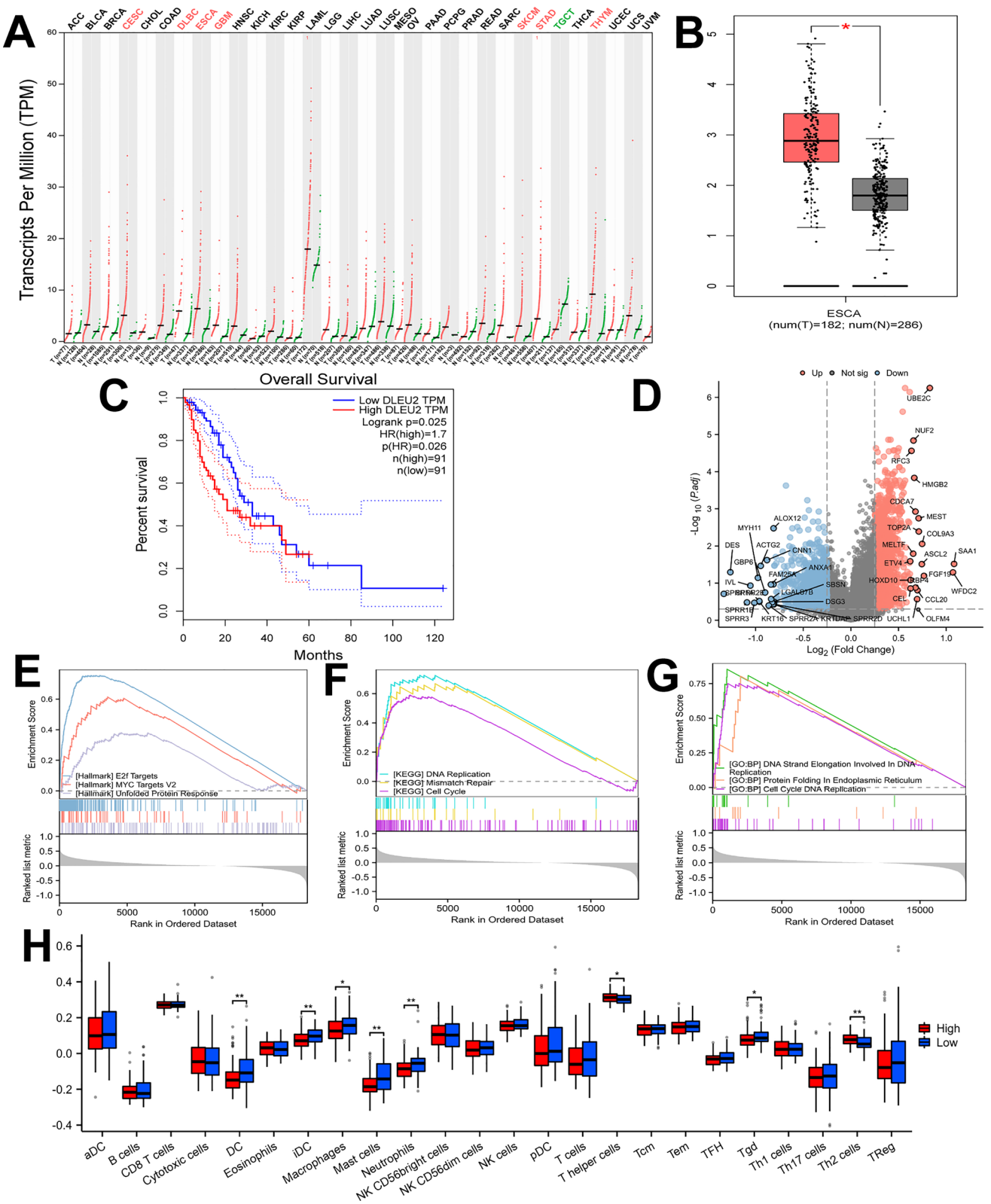

Fig. 5 Landscape of DLEU2 in bulk transcriptomics. **A** Pan-cancer analysis of DLEU2 expression. **B** Expression differences of DLEU2 between esophageal squamous carcinoma and normal tissue. *DLEU2 is significantly upregulated in endometrial carcinoma* (*EC*). The data represent a comparison of DLEU2 expression levels in EC versus normal tissues. Statistical significance was determined using an unpaired t-test, with a p-value of 0.002 (effect size: Cohen's d = 1.5). Error bars represent the standard error of the mean (SEM). **C** Survival analysis between high and low DLEU2 expression groups in the TCGA ESCA database. **D** Differential gene expression analysis (DEGs) between high and low DLEU2 expression groups in esophageal cancer. **E**–**G** Gene Set Enrichment Analysis (GSEA) results for hallmark, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO) pathways in high versus low DLEU2 expression groups. **H** Immune infiltration differences between high and low DLEU2 expression groups based on deconvolution analysis

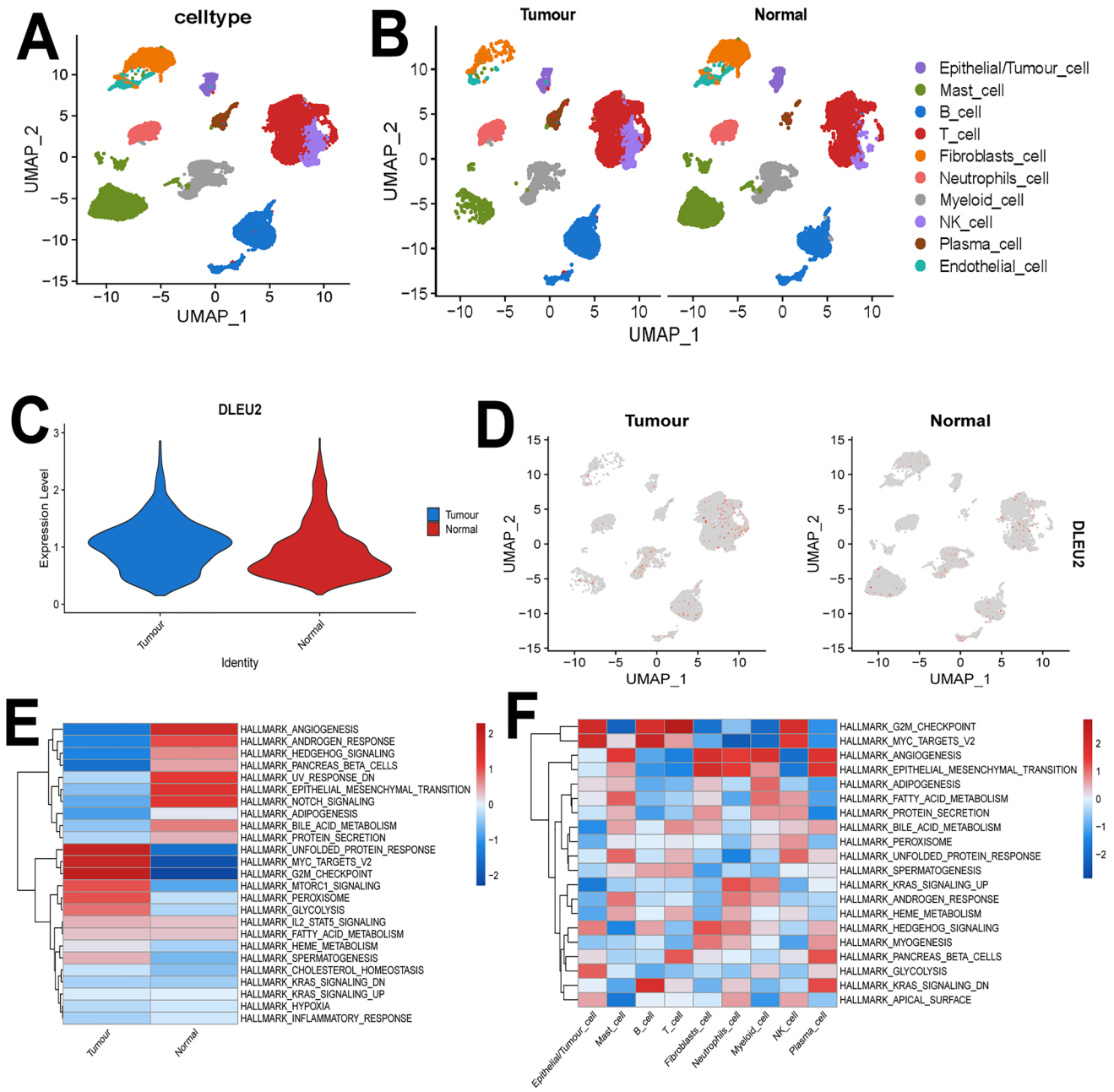

Fig. 6 Distribution landscape of DLEU2 in esophageal cancer and matched normal tissues. **A** Uniform Manifold Approximation and Projection (UMAP) plot of single cells from esophageal cancer and normal tissues. **B** UMAP plot of single cells in esophageal cancer and normal tissues, highlighting T cell populations. **C** Violin plot showing differential expression of DLEU2 in tumor versus normal tissues. **D** Feature plot depicting spatial expression patterns of DLEU2 in tumor and normal tissues. **E** Gene Set Variation Analysis (GSVA) score heatmap comparing pathway activity in esophageal cancer and normal tissues. **F** GSVA score heatmap for different immune cell subpopulations in esophageal cancer and normal tissues

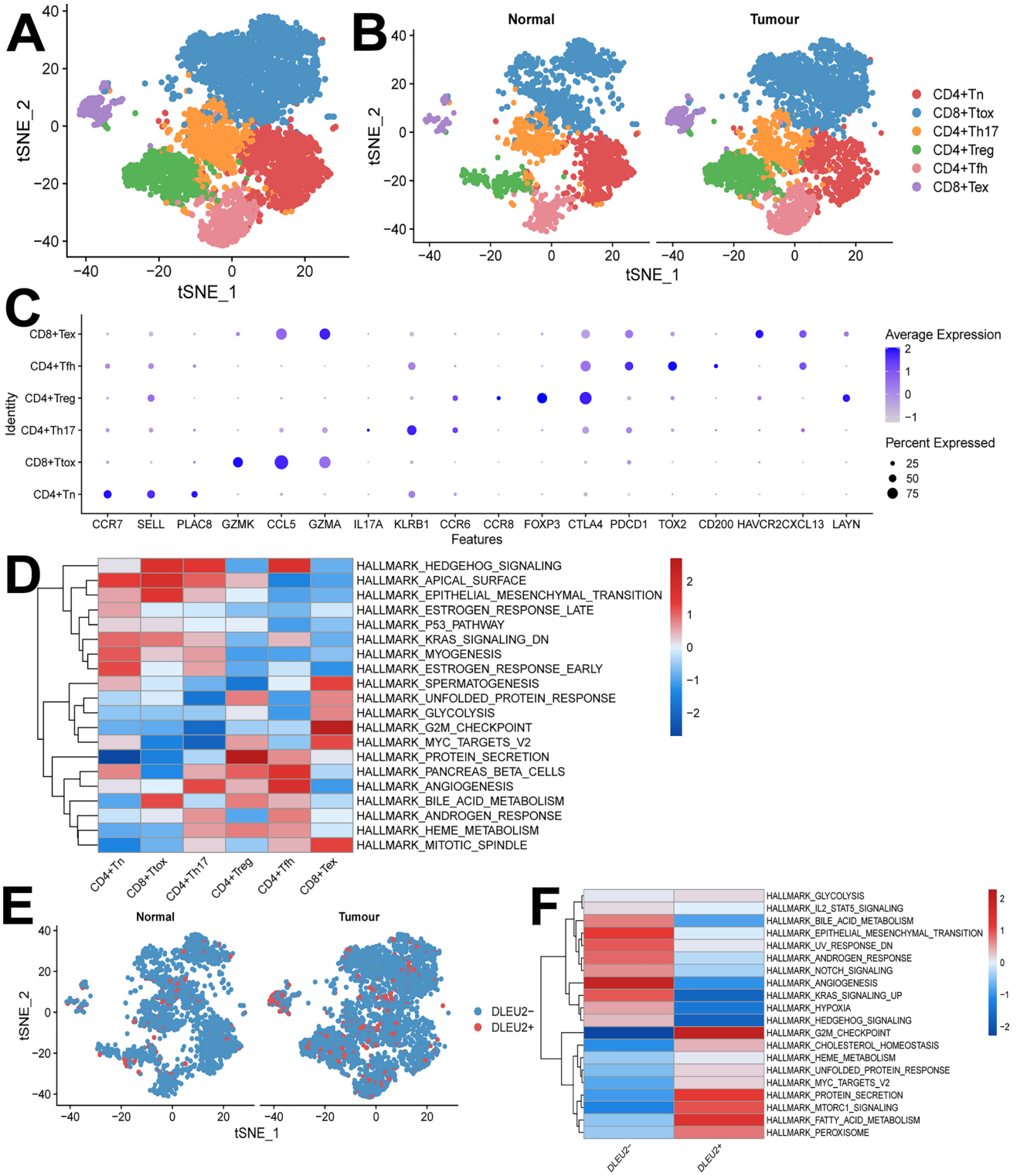

Fig. 7 Distribution landscape of DLEU2 in T cells from esophageal cancer. **A** t-SNE plot of T cells from esophageal cancer and normal tissues. **B** t-SNE plot showing T cell clusters in esophageal cancer and normal tissues. **C** Bubble plot of annotated T cells in esophageal cancer and normal tissues. **D** Functional scoring of T cell subpopulations based on the HALLMARK gene set. **E** Distribution of DLEU2 + T cells in esophageal cancer and normal tissues. **F** Functional scoring of DLEU2 + T cell subpopulations based on the HALLMARK gene set

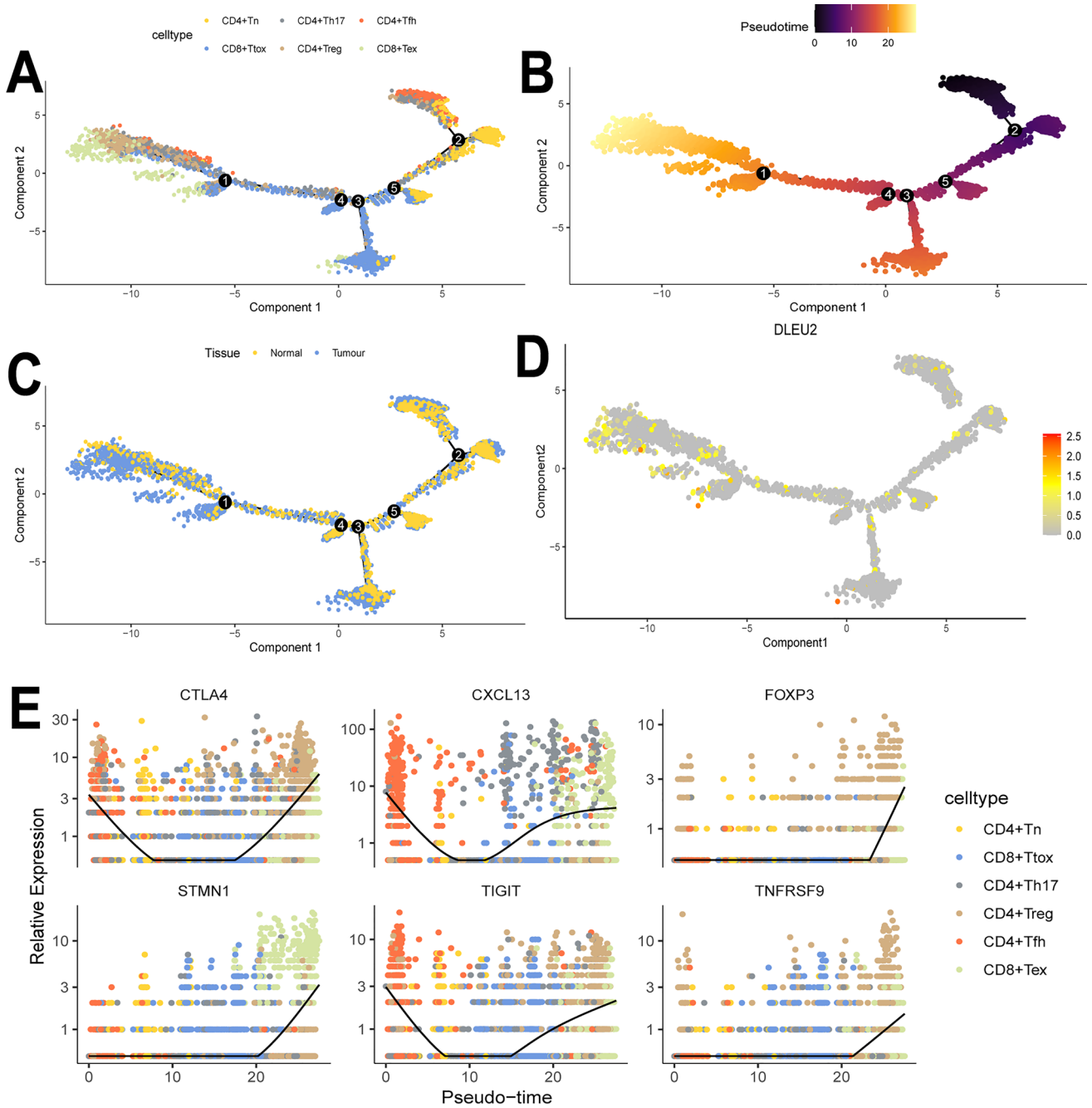

Fig. 8 Pseudotime analysis reveals the transcriptional trajectory of DLEU2. **A** Developmental trajectory states of different T cell subsets. **B** Pseudotime developmental axis of T cells. **C** Developmental states of T cells from different tissue origins. **D** Pseudotime trajectory of DLEU2 transcriptional levels. **E** Transcriptional changes of genes associated with T cell dysfunction along the developmental trajectory

in EC tissues (Fig. 7E). The UPR, glycolysis, and MYC signaling pathways are highly activated in T cells with high expression of DLEU2 (Fig. 7F).

3.4 Pseudo-temporal analysis identifies DLEU2-linked exhausted T cells at the differentiation terminus in EC, associated with immune suppression

We first observed that Tex cells are positioned at the terminal end of the developmental axis, which aligns with known biological progression (Fig. 8A–C). Subsequently, we also found that DLEU2 expression is located at the terminal

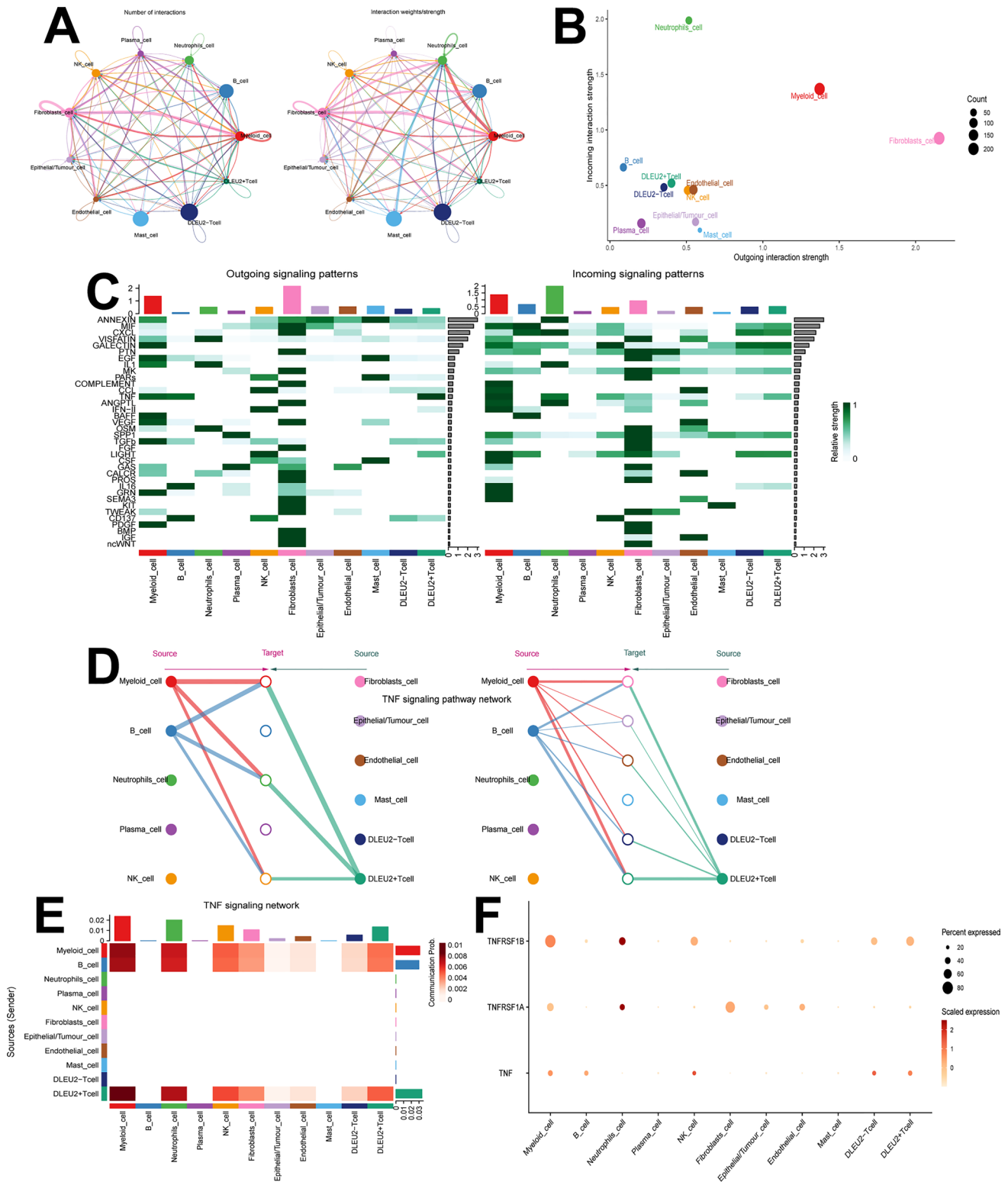

Fig. 9 Cell communication landscape of DLEU2+T cells. **A** Overview of cell–cell communication in esophageal cancer. **B** Dot plot of communication strength between cell subpopulations in esophageal cancer tissue. **C** Overall cell–cell communication heatmap in esophageal cancer tissue. **D** Cell communication interaction map of the TNF signaling pathway. **E** Cell communication heatmap of the TNF signaling pathway in esophageal cancer. **F** Feature dot plot of cell communication

end of the developmental axis, further validating the biological relevance of our analysis (Fig. 8D). Additionally, we found that genes associated with immune suppression, such as CTLA4, CXCL13, FOXP3, STMN1, TIGIT, and TNFRSF9, exhibit expression trajectories similar to DLEU2 (Fig. 8E). This suggests that DLEU2 plays an immunosuppressive role in T cell subtypes in EC.

3.5 Cell communication analysis suggests that DLEU2 may exert its effects through the TNF pathway

To explore how DLEU2+ T cells exert immune suppression, we conducted cell communication analysis. Compared to DLEU2- T cells, DLEU2+ T cells exhibited stronger input and output signals (Fig. 9A, B). Heatmap analysis revealed higher cell signal intensities for pathways like PTN, SPP1, TNF, and LIGHT in DLEU2+ T cells (Fig. 9C). Particularly, the TNF pathway showed significantly higher input and output signals in DLEU2+ T cells compared to DLEU2− T cells. Further analysis demonstrated that DLEU2+ T cells interact significantly with myeloid cells, NK cells, and fibroblasts through the TNF pathway (Fig. 9D, E). Bubble plots illustrated higher expression levels of TNFRSF1A/B in DLEU2+ T cells compared to DLEU2− T cells. Therefore, DLEU2 may contribute to poor prognosis in EC patients by modulating TNFRSF1A/B through the TNF pathway (Fig. 9F).

4 Discussion

Non-coding RNAs, such as exosomal miR-92b-5p, play a critical role in cancer drug resistance. Li et al. [39] showed that miR-92b-5p enhances PTEN mono-ubiquitination in doxorubicin-resistant AML. Liquid biopsy techniques, such as ctDNA detection, offer a non-invasive method to monitor these RNA-related resistance mechanisms [39]. Jahangiri [40] highlights the utility of liquid biopsies in neuroblastoma for treatment monitoring, while Ohyama et al. [41] improved detection sensitivity with molecular barcode systems for pancreaticobiliary malignancies. Additionally, DNA methylation signatures [41], as reviewed by Gonzalez et al. [42], can also serve as biomarkers for early cancer detection and may complement liquid biopsy technologies in tracking non-coding RNA changes linked to drug resistance.

The present MR findings are consistent with those of observational studies [43, 44] indicating that alcohol consumption increases the risk of cancers of the esophagus, although our estimates had low precision and did not reach statistical significance. Compared with observational studies, MR analysis can reduce the impact of confounding bias because genetic alleles are randomly assigned at conception and, thus not correlated with environmental factors. Furthermore, we used the SNP to bridge MR and GEO data analysis. Our findings suggest that DLEU2 plays a pivotal role in shaping the tumor immune microenvironment of esophageal cancer (EC), particularly through its impact on T cell function and TNF signaling. Single-cell RNA sequencing revealed that DLEU2 is highly expressed in exhausted T cells (Tex), a subset known for immune dysfunction and reduced anti-tumor activity. The co-expression of DLEU2 with immunosuppressive markers (CTLA4, FOXP3, TIGIT, and TNFRSF9), hypothesize how DLEU2 might regulate these markers (e.g., via ceRNA networks).

Further, cell–cell communication analysis demonstrated that DLEU2+ T cells exhibit increased interactions with other immune and stromal components via the TNF-TNFRSF1A/B axis, a pathway known to regulate inflammation, apoptosis, and immune evasion. The upregulation of TNFRSF1A/B in DLEU2+ T cells indicates that DLEU2 may drive a pro-tumorigenic feedback loop, wherein TNF signaling contributes to the maintenance of T cell exhaustion and facilitates tumor progression. This aligns with previous studies showing that chronic TNF exposure can impair cytotoxic T cell function while enhancing immune suppressive pathways, further reinforcing DLEU2's role in modulating tumor-immune interactions.

These findings highlight DLEU2 as a key mediator of immune evasion in EC, potentially making it a therapeutic target for modulating T cell exhaustion and restoring anti-tumor immunity. Future studies should investigate whether DLEU2 inhibition can reverse T cell exhaustion and enhance responses to immune checkpoint inhibitors, providing a new avenue for therapeutic intervention in esophageal cancer. Single-cell RNA sequencing localized DLEU2 expression predominantly to T cells, particularly exhausted subpopulations, a finding that was consistent across three paired esophageal cancer and adjacent normal tissue samples. This localization approach is supported by previous studies that leverage single-cell transcriptomics to map lncRNA expression to specific immune subsets, such as the identification of lncRNA GAS5 targets in renal fibrosis [45].

LncRNA DLEU2 plays crucial roles in various cancers, impacting tumor growth and invasion through intricate molecular pathways. For instance, DLEU2 promotes progression in oral cancers and glioma via distinct miRNA-mediated mechanisms and enhances invasiveness by promoting lymphovascular invasion in cervical cancer [45–47]. However, its involvement in EC remains underexplored. In our study, we observed elevated DLEU2 expression in EC tissues compared to

normal tissues. Notably, high DLEU2 expression correlated with shorter overall survival in our patient cohort, suggesting its potential as a prognostic marker for EC. Alcohol exposure is a known stressor that induces a state of stress across multiple organs, including the pancreas and liver, where activation of the unfolded protein response (UPR) pathway has been implicated [48, 49]. Moreover, alcohol exposure alters the immune microenvironment in the liver [50]. In our study, Gene Set Enrichment Analysis (GSEA) revealed that the high DLEU2 expression group in EC is enriched for pathways related to UPR and cell cycle, indicative of a stressed intracellular environment. Additionally, within the subgroup of EC patients with elevated DLEU2 expression, there was an increase in immunosuppressive Th2 cells and a decrease in immune-activating γδT cells. These findings contribute to a better understanding of how DLEU2 expression alters the microenvironment in EC, potentially fostering immune evasion and creating a niche favorable for tumor growth.

Previous studies have highlighted the role of DLEU2 in exerting anti-inflammatory effects within T cell populations involved in autoimmune conditions like autoimmune encephalomyelitis, closely linked to regulatory T cell (Treg) differentiation [51]. However, transcriptomic sequencing alone cannot provide insights into the specific expression of DLEU2 across distinct cellular subgroups. In our study, we employed single-cell sequencing for the first time to investigate the distribution of DLEU2 at the single-cell level in EC. We found that within the T cell subset, which constitutes a significantly increased proportion within the tumor microenvironment, DLEU2 expression levels were elevated compared to T cells from normal tissues. Further dimensionality reduction clustering identified that DLEU2 exhibited the highest expression in exhausted T cells. These findings contribute to a deeper understanding of how DLEU2 expression contributes to the transformation of the immune-suppressive microenvironment in EC.

Pseudo-temporal analysis provides a visual depiction of the expression dynamics of DLEU2 during T cell development within EC. In our study, we observed that multiple immune-suppressive T-cell markers associated with tumor promotion were upregulated in branches exhibiting high DLEU2 expression. This suggests that DLEU2, as a lncRNA, may facilitate tumor progression by modulating these pro-tumorigenic signals. To explore the intercellular signaling interactions of DLEU2-expressing T cells with other components of the tumor microenvironment, we employed cell communication analysis methods. Our findings revealed that DLEU2-positive T cells exhibit stronger intercellular communication with various microenvironmental cells compared to DLEU2-negative T cells. Notably, pathways such as SPP1, MIF, and TNF signaling were significantly enhanced in DLEU2-positive T cells, particularly demonstrating heightened TNF signaling in both input and output signals. SPP1 has been implicated in immune checkpoint inhibition of T cell activation and immune tolerance in colorectal cancer [52]. MIF has been shown to regulate EC through interactions with Treg cells, while TNF contributes to EC progression by inducing cell migration through cytoskeletal remodeling and impairing NK cell function via TIM-3 induction [53, 54]. These results enhance our understanding of the evolution and immune-suppressive role of DLEU2-positive T cells within the EC immune microenvironment. Identifying DLEU2 as a potential target gene could pave the way for diagnostic and therapeutic strategies aimed at improving outcomes for patients with EC. By delineating the intricate cellular interactions mediated by DLEU2 in the immune context of EC, our study provides insights that may guide the development of targeted therapies to restore anti-tumor immunity and mitigate immune evasion mechanisms in this challenging disease. Our findings not only reinforce the association between alcohol consumption and an increased risk of esophageal cancer but also provide new insights into the role of DLEU2 in the tumor immune microenvironment. The significant upregulation of DLEU2 in exhausted T cell subpopulations suggests that it may contribute to immune suppression and tumor progression. Notably, its co-expression with key immunosuppressive markers (CTLA4, FOXP3, TIGIT, and TNFRSF9) indicates that DLEU2-positive T cells play an active role in dampening anti-tumor immunity.

These findings have important implications for understanding esophageal cancer progression and potential therapeutic strategies. The observed interaction between DLEU2+ T cells and immune components via the TNF-TNFRSF1A/B pathway suggests that DLEU2 may contribute to immune evasion mechanisms. Given that TNF signaling plays a crucial role in tumor-associated inflammation and T cell dysfunction, targeting DLEU2-mediated TNFRSF1A/B signaling may provide a novel approach to reinvigorating anti-tumor immunity.

Furthermore, our study highlights the potential of DLEU2 as a prognostic biomarker in esophageal cancer. Patients with high DLEU2 expression exhibited poorer survival outcomes, reinforcing the need for further research into its clinical utility in patient stratification. Future studies should explore DLEU2 inhibition using CRISPR interference (CRISPRi) to repress its expression or antisense oligonucleotides (ASOs) to specifically degrade DLEU2 RNA, enabling functional validation of its role in tumor progression and immune modulation. By elucidating the mechanistic role of DLEU2 in immune modulation, this study provides a foundation for developing novel immunotherapeutic strategies aimed at counteracting tumor-induced immunosuppression and improving patient outcomes.

Although our study provides strong evidence linking DLEU2 expression to esophageal cancer, the direct causal relationship between alcohol consumption and DLEU2 upregulation remains uncertain. To address the causal relationship

between alcohol and DLEU2 upregulation, in vitro experiments could involve exposing cancer cell lines to varying alcohol concentrations and measuring DLEU2 expression via qPCR or RNA sequencing to assess dose- and time-dependent effects. Additionally, a luciferase reporter assay could be used to evaluate alcohol's impact on DLEU2 promoter activity. Sample selection bias, with a predominance of European ancestry, may limit the generalizability of findings to other populations with different genetic backgrounds, potentially overlooking important genetic variations or environmental factors. Studies like "The Diversity of Human Genetic Variation in the 1000 Genomes Project" (2015) and "Global diversity in human genomic studies" (2020) highlight the importance of including diverse populations to improve the applicability and accuracy of research conclusions across ethnic groups. Additionally, transcriptomic data constraints and batch effects could influence gene expression results. While DLEU2 emerges as a potential biomarker, further functional validation and clinical studies are needed to confirm its mechanistic role and therapeutic potential in esophageal cancer. The analysis of alcohol consumption specifically exhibited low precision due to the limited variance explained by the SNPs, despite an F-statistic exceeding the conventional threshold of 10. The absence of a statistically significant link between genetically predicted alcohol consumption and EC could be attributed to insufficient statistical power. Another limitation is the binary nature of the alcohol consumption variable, making it challenging to interpret the Mendelian randomization estimate as a direct causal effect when both the exposure and outcome are binary [55]. Additionally, since our study focused on individuals of European ancestry, the findings may not be applicable to other populations.

5 Conclusion

This study establishes DLEU2 as a critical immune regulator in esophageal cancer and suggests its potential role in mediating the link between alcohol consumption and tumor progression. Our findings indicate that DLEU2 is highly expressed in exhausted T cells, co-expresses with immunosuppressive markers, and modulates TNF-TNFRSF1A/B signaling, contributing to immune evasion and poor prognosis. These insights provide a foundation for exploring DLEU2 as a biomarker for patient stratification and a potential therapeutic target in esophageal cancer. Future studies should focus on functional validation of DLEU2's role in immune suppression, investigate its predictive value for immunotherapy response, and assess whether targeting DLEU2 or its associated pathways could enhance anti-tumor immunity and improve treatment outcomes.

5.1 Future directions

Given the potential role of DLEU2 in esophageal cancer, future research should focus on functional validation through in vitro and in vivo models to elucidate its precise mechanistic role in immune regulation and tumor progression. Additionally, clinical studies are needed to assess DLEU2's potential as a prognostic and predictive biomarker, particularly in relation to immunotherapy response. Exploring therapeutic strategies targeting DLEU2, such as RNA-based interventions or pathway inhibitors, could provide new avenues for precision medicine in esophageal cancer treatment. Moreover, future studies should investigate how drinking patterns—including frequency, quantity, and type of alcohol consumed—affect DLEU2 expression and esophageal cancer risk, offering deeper insights into the interplay between alcohol exposure and tumor biology.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate The summary-level data and RNA-seq data we utilized are publicly available and ethically approved. Each individual study within the GWAS was approved by the appropriate Institutional Review Board, and the participants or their authorized representatives provided informed consent.

Competing interests The authors declare no competing interests.

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