Original Article

Pan-cancer bioinformatics analysis of hepatic leukemia factor and further validation in colorectal cancer

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Background: Hepatic leukemia factor (HLF) is associated with cancer onset, growth, and progression; however, little is known regarding its biological role in pan-cancer. In order to further evaluate the diagnostic and prognostic value of HLF in pan-cancer and colorectal cancer (CRC), we performed comprehensive bioinformatics analyses of the molecular mechanism of HLF in pan-cancer, with subsequent verification in CRC.

Methods: We downloaded data (gene expression, clinical data, follow-up duration, and immune-related data) related to 33 solid tumor types from UCSC Xena (University of California Santa Cruz cancer database, https://xena.ucsc.edu/). HLF expression was analyzed in pan-cancer, and its diagnostic efficacy, prognostic value, and correlation with pathological stage and cancer immunity were determined. We also analyzed gene alterations in HLF and biological processes involved in its regulation in pan-cancer. Using CRC data in The Cancer Genome Atlas (TCGA), we assessed correlations between HLF and CRC diagnosis, prognosis, and drug sensitivity and performed functional enrichment analyses. Moreover, we constructed an HLF-related ceRNA regulatory network. Finally, we externally validated HLF expression and diagnostic and prognostic value in CRC using Gene Expression Omnibus (GEO) database, as well as by performing *in vitro* experiments.

Results: HLF expression was downregulated in most tumors, and HLF showed good predictive potential for pan-cancer diagnosis and prognosis. It was closely related to the clinicopathological stages of pan-cancer. Further, HLF was associated with tumor microenvironment and immune cell infiltration in many tumors. Analyses involving cBioPortal revealed changes in HLF amplifications and mutations in most tumors. HLF was also closely associated with microsatellite instability and tumor mutational burden in pan-cancer and involved in regulating various tumor-related pathways and biological processes. In CRC, HLF expression was similarly downregulated, with implications for CRC diagnosis and prognosis. Functional enrichment analysis indicated the association of HLF with many cancer-related pathways. Further, HLF was associated with drug (e.g., oxaliplatin) sensitivity in CRC. The ceRNA regulatory network showed multigene regulation of HLF in CRC. External validation involving GEO databases and quantitative real-time polymerase chain reaction (qRT-PCR) data substantiated these findings.

Conclusions: HLF expression generally exhibited downregulation in pan-cancer, contributing to tumor occurrence and development by regulating various biological processes and affecting tumor immune characteristics. HLF was also closely related to CRC occurrence and development. We believe HLF can serve as a reliable diagnostic, prognostic, and immune biomarker for pan-cancer.

Keywords: Hepatic leukemia factor (HLF); pan-cancer; diagnosis; prognosis; cancer immunity

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Introduction

Cancer continues to remain a major public health concern, ranking among the leading causes of death worldwide. The incidence and mortality of cancer are rapidly increasing across the globe, with an estimated annual occurrence of >19.3 million new cases and 10 million new death (1). Our current treatment strategies for cancer primarily include surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. These approaches have exhibited substantial advancements over the past few decades, enabling the cure of numerous cases previously considered fatal. However, challenges such as drug resistance and adverse effects persist, resulting in unsatisfactory patient prognoses and survival rates. The incidence of various types of tumors, as well as the mortality associated with them, continue to increase, marked by pronounced disparities between different cancer types (2,3). In addition, many patients with cancer develop distant metastases during treatment, which significantly impacts prognosis (4). Hence, the quest for more precise early detection methods for cancer, and also for improved treatments, represents a pivotal strategy to alleviate the global cancer burden. Achieving this goal necessitates comprehensive research to elucidate the molecular mechanisms of carcinogenic and tumor suppressor genes within the human body. This can ultimately promote the development of precise and personalized diagnostic and therapeutic methods (5).

Transcription factors (TFs) are DNA-binding proteins that specifically interact with cis-regulatory elements of eukaryotic genes to either activate or inhibit gene transcription. They generally include a DNA binding domain, transcriptional regulatory domain, oligomerization site, and nuclear localization signal. Approximately 8% of all human genes encode TFs. TFs affect gene expression by directly recruiting RNA polymerase or recruiting cofactors that facilitate specific transcription stages. Notably, TFs are implicated in diverse diseases and phenotypes (6), and several TF family members have been found to be dysregulated in tumor tissues, contributing to tumor initiation and progression (7,8). Zhang *et al.* reported that FOXO1 participates in limiting the progression of non-small cell lung cancer by positively regulating NM23H1, a metastasis suppressor gene (9). Further, Ye *et al.* suggested that FOXO6 overexpression inhibits breast cancer cell migration and invasion (10). The overexpression of ETS-related gene, a member of the ETS TF family, is reportedly a key driver of prostate cancer (11). Besides, the TFs RUNX1–RUNX3 are strongly associated with epithelial cancer progression (12). These investigations highlight the influence of TF activity in the development of new anticancer drugs (13). Collectively, these findings indicate that TFs play multifaceted roles in carcinogenesis and anticancer functions, emphasizing the importance of continued exploration into gene regulation mechanisms mediated by TFs to comprehensively understand tumor genesis and development.

Hepatic leukemia factor (HLF), a member of the proline and acid-rich protein family within the bZIP TF family, forms homologous dimers or heterodimers with other proline and acid-rich protein family members. These dimers then bind to sequence-specific promoter elements to activate transcription (14). Originally, the involvement of HLF was recognized when its chromosome translocation fused part of the gene within the E2A gene, causing childhood B-line acute lymphoblastic leukemia (15). HLF is also evidently involved in regulating tumor occurrence and development. For example, Li *et al.* indicated that transforming growth factor-β1, secreted by tumor-associated macrophages, regulates HLF expression. This, in turn, transactivates γ-glutamyltransferase 1 to enhance cellular resistance to iron-mediated cell death, eventually promoting triple-negative breast cancer cell proliferation, metastasis, and

Highlight box

Key findings

- Hepatic leukemia factor (HLF) expression generally exhibited downregulation in pan-cancer, contributing to tumor occurrence and development by regulating various biological processes and affecting tumor immune characteristics. HLF was also closely related to colorectal cancer (CRC) occurrence and development.

What is known and what is new?

- HLF is associated with cancer onset, growth, and progression; However, existing research on the function of HLF in cancer is mostly limited to specific cancer types.
- Our findings reveal the multifaceted role of HLF in pan-cancer and provide new insights into the diagnosis and treatment of HLF in CRC.

What is the implication, and what should change now?

- We believe HLF can serve as a potential beneficial diagnostic, prognostic, and immune biomarker for pan-cancer. However, our study was mainly based on bioinformatics analysis and the *in vitro* experimental part only verified the expression trends of HLF in CRC via quantitative real-time polymerase chain reaction. In the future, based on the present results, we will conduct in-depth research of the expression of HLF related proteins and their interaction mechanisms in CRC and other cancers.

cisplatin resistance (16). In hepatocellular carcinoma, HLF promotes the generation of tumor-initiating cells by activating c-JUN and enhances the tumor-initiating cell-like properties of hepatocellular carcinoma cells, ultimately promoting the occurrence and progression (17) of hepatocellular carcinoma. A *vivo* study has revealed that upregulating HLF inhibition enhances the growth and bone, liver, and brain metastases of non-small cell lung cancer cells, whereas silencing HLF promotes growth and metastases (18). However, existing research on the function of HLF in cancer is mostly limited to specific cancer types. Therefore, it is particularly important to study the regulatory functions and molecular mechanisms of HLF in pan-cancer contexts to identify new directions and devise strategies for clinical cancer therapy.

Herein we systematically describe the molecular mechanisms underlying HLF in pan-cancer. We combined data from different databases, including UCSC Xena (University of California Santa Cruz cancer database, https://xena.ucsc.edu/), The Cancer Genome Atlas (TCGA), and Gene Expression Omnibus (GEO), to investigate the significance of HLF in diagnosis, prognosis, and immune response prediction in pan-cancer. We assessed gene mutations in HLF and explored potential associations between HLF expression and tumor mutational burden (TMB), microsatellite instability (MSI), tumor microenvironment, and immune infiltration in multiple cancer types. Moreover, we explored the biological functions and pathways of HLF, and validated its diagnostic and prognostic value, as well as molecular regulatory network, in colorectal cancer (CRC).

Currently, various studies have confirmed the crucial role of HLF in cancer, but the occurrence and development of cancer is the result of the combined influence of numerous factors. Due to the complexity of gene interactions, the role of HLF remains to be further investigated. Our study is the first to confirm the role of HLF in CRC and fills a gap in the mechanism of divergent transcription in the occurrence and development of CRC. It provides a potentially significant direction for future research in the diagnosis and treatment of CRC. We present this article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2274/rc).

Methods

Data download and collation

We downloaded data related to 33 types of solid tumors from UCSC Xena (19) (https://xena.ucsc.edu/), which encompassed clinical data and information related to gene expression, follow-up duration, tumor immune-related indicators, and tumor stem index. A summary of all gene expression data samples was generated using Strawberry Perl (v5.32.1.1, http://strawberryperl.com). Complete CRC data were obtained by downloading and integrating colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) gene expression and clinical data from TCGA. In addition, CRC-related datasets were downloaded from GEO (20) for external validation.

Analysis of HLF expression profile in pan-cancer

HLF expression profiles of all samples were obtained from the data downloaded from UCSC Xena (https://xena.ucsc.edu/), which were initially in Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) format and subsequently converted to log2 standard format. To analyze HLF expression in pan-cancer and evaluate differences in HLF expression between normal and tumor tissues in pan-cancer, we utilized the R software package "limma".

Gene activity, reflecting the extent to which genes are transcribed and translated into proteins within cells, influences individual phenotypic characteristics and structural and functional changes. We employed the single-sample gene set enrichment analysis (GSEA) algorithm to calculate HLF activity across all samples and also analyzed differences in HLF activity between normal and tumor tissues.

Clinical significance analysis of HLF expression in pan-cancer

To evaluate the clinical relevance of HLF in pan-cancer, we integrated the clinicopathological staging data for 33 tumors with HLF expression data. We conducted an analysis to discern variations in HLF expression across different tumor stages and evaluated the relationship between HLF and clinicopathological staging. In addition, we applied the R software package "pROC" to calculate and plot receiver operating characteristic (ROC) curves for HLF diagnostics in each tumor. The area under the curve (AUC) was calculated to evaluate the diagnostic efficacy of HLF in pan-cancer.

Further, we evaluated the association of HLF with the prognosis of pan-cancer. We retrieved data on overall survival, disease-specific survival, disease-free survival,

and progression-free survival for all tumor patients from UCSC. Patients were categorized into high and low HLF expression groups depending on the median HLF expression value. Using the R software packages "survival", "survminer", and "forestplot", we evaluated differences in four prognostic indices between the groups employing the Kaplan-Meier survival curve method. The log-rank P value and hazard ratio, and 95% confidence interval were calculated.

Correlation analysis of HLF and tumor immunity in pan-cancer

Tumor tissues contain not only tumor cells but also surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, signaling molecules, and extracellular matrix, which collectively constitute the tumor micro-environment. The tumor microenvironment substantially influences the immune characteristics of tumors. To estimate the stromal and immune cell content, we used the ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) algorithm, which derives stromal and immune scores for pan-cancer based on tumor gene expression data. These scores were summed to obtain the ESTIMATE score, which also provided a calculation for tumor purity. Spearman correlation analysis was then performed to evaluate the relationship between HLF and these four scores.

The CIBERSORT algorithm was employed to quantitatively assess the proportion of different immune cell subsets in pan-cancer, leveraging gene expression data to estimate the relative abundance of each immune cell subset within mixed cell samples from pan-cancer tissues. Spearman correlation analysis was performed to determine the relationship between HLF and immune cell infiltration.

Genetic variations of HLF in pan-cancer and correlation analysis with mutations

We assessed HLF using cBioPortal (21,22) (http://www.cbioportal.org/) to investigate genetic variations in carcinoma, including mutations, structural variants, amplifications, deep deletions, and multiple alterations.

TMB is calculated as the total count of somatic non-synonymous mutations within the coding region. TMB has emerged as a biomarker for predicting the efficacy of immunotherapy. MSI is the insertion or deletion of nucleotides in microsatellite loci. We obtained TMB and MSI scores for all tumor samples from the UCSC database and performed Spearman correlation analysis to examine the correlation of HLF with TMB and MSI.

HLF biological functions and pathways in pan-cancer

To understand the biological functions and pathways of HLF, we compiled a gene set that considered gene location, function, and metabolic pathways, which was saved in the Molecular Signatures Database (23-25) (Broad Institute, USA, http://www.gsea-msigdb.org/gsea/). We downloaded the "c5.go.symbols.gmt" and "c2.cp.kegg.symbols.gmt" files, which facilitated Gene Ontology (GO) (26) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (27) pathway enrichment analyses. Based on HLF expression in tumor tissues, the samples were classified into high and low expression groups. The GSEA algorithm was used to analyze GO- and KEGG-related differences between these groups and to evaluate biological processes and enrichment pathways involved in HLF regulation.

GeneMANIA (http://www.genemania.org) (28) is an effective tool for gene function based on several networks collected from diverse genomic/proteomic data. We used GeneMANIA to identify genes with expression patterns similar to those of HLF, and analyzed their network of interactions and biological processes that they collectively influence.

Expression profile analysis, clinical relevance, and external validation of HLF in CRC

We examined HLF expression profile in CRC using data from TCGA, analyzed differences in HLF expression between normal colorectal and CRC tissues, and explored the relationship between HLF expression and CRC diagnosis and prognosis. Further, we performed Pearson correlation analysis to identify HLF-related genes in CRC. Genes with correlation coefficient absolute value >0.4 and P<0.001 were included. Subsequently, we conducted GO and KEGG pathway enrichment analyses using the clusterProfiler (29) R package to evaluate the biological processes and pathways associated with these genes in CRC. The findings were further verified using the GSEA algorithm.

To evaluate the relationship between HLF and CRC drug sensitivity, we acquired gene expression and tumor cell data from Genomics of Drug Sensitivity in Cancer (http://www.cancerrxgene.org/) (30). We used Genomics

of Drug Sensitivity in Cancer results as the training group and TCGA data as the validation group. OncoPredict R was used to calculate the drug sensitivity score for each CRC sample, and differences in drug sensitivity were then determined between high and low HLF expression groups.

Besides, we employed external validation groups, including GSE87211, GSE106582, GSE17536, and GSE39582 from the GEO database to further evaluate the relationship between HLF and CRC diagnosis and prognosis.

Construction of ceRNA regulatory network

To decipher the molecular mechanisms of HLF in CRC, we searched for HLF-related miRNAs through StarBase (31), miRDB (32), mirTarBase (33), TargetScan (34), and mirWalk (35). miRNAs predicted in at least three of these databases simultaneously were included in the analysis. The intersection of predicted miRNAs and differentially expressed miRNAs was used to identify HLF-related miRNAs. Further, HLF-related miRNAs were searched in StarBase to predict relevant lncRNAs. The intersection of predicted lncRNAs and differentially expressed lncRNAs was used to identify HLF-related lncRNAs. We then compared the predicted miRNA-lncRNA relationship to construct a lncRNA-miRNA-HLF ceRNA regulatory network.

Quantitative real-time polymerase chain reaction (qRT-PCR)

CRC tissues and corresponding normal colorectal tissues were collected from patients with CRC who underwent radical surgical treatment at The First Affiliated Hospital of Guangxi Medical University from April 2022 to July 2023. In total, we collected CRC and normal colorectal tissues from 59 patients. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University (No. 2023-E628-01). Written informed consent was obtained from all patients prior to tissue sampling.

All samples were stored at −80 ℃ until use. Total RNA was extracted using TRIzol (B511321; Biotechnology, Shanghai, China). cDNA was synthesized using the SweScript RT II First Strand cDNA Synthesis Kit (G3333, Servicebio, Wuhan, China). Gene expression was detected using 2× Universal Blue SYBR Green qRT-PCR Master Mix (G3326, Servicebio). The expression levels of gene pairs were normalized to those of GAPDH, and HLF expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Differences in HLF expression were compared via pairing analysis.

The following primer sequences were used: GAPDH-F: GGAAGCTTGTCATCAATGGAAATC, GAPDH-R: TGATGACCCTTTTGGCTCCC; HLF-F: CCCTCGGTCATGGACCTCA, and HLF-R: ACTTGGTGTATTGCGGTTTGC.

Statistical analysis

Data were statistically analyzed using R (v4.2.2). Wilcoxon rank-sum test was used for intergroup comparisons, with $P<0.05$ indicating statistically significant differences. Spearman or Pearson correlation analysis was performed to evaluate correlations among variables; strong correlations were considered when correlation coefficient absolute value was >0.4 and P was <0.001. * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$.

Results

HLF expression in human normal and tumor tissues

HLF expression was the highest in liver hepatocellular carcinoma (LIHC) and lowest in acute myeloid leukemia (LAML) (*Figure 1A*). In comparison with normal tissues, HLF expression was downregulated in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), COAD, glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), LIHC, lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), READ, sarcoma (SARC), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), and other tumor tissues ($P<0.05$) (*Figure 1B*).

HLF gene activity was the highest in pheochromocytoma and paraganglioma (PCPG) and lowest in esophageal carcinoma (ESCA) (*Figure 1C*). As with HLF gene expression, relative to normal tissues, HLF gene activity was lower in most tumor tissues, including BLCA, BRCA, CESC, CHOL, COAD, ESCA, GBM, HNSC, KICH, KIRC, kidney renal papillary cell carcinoma (KIRP), LIHC,

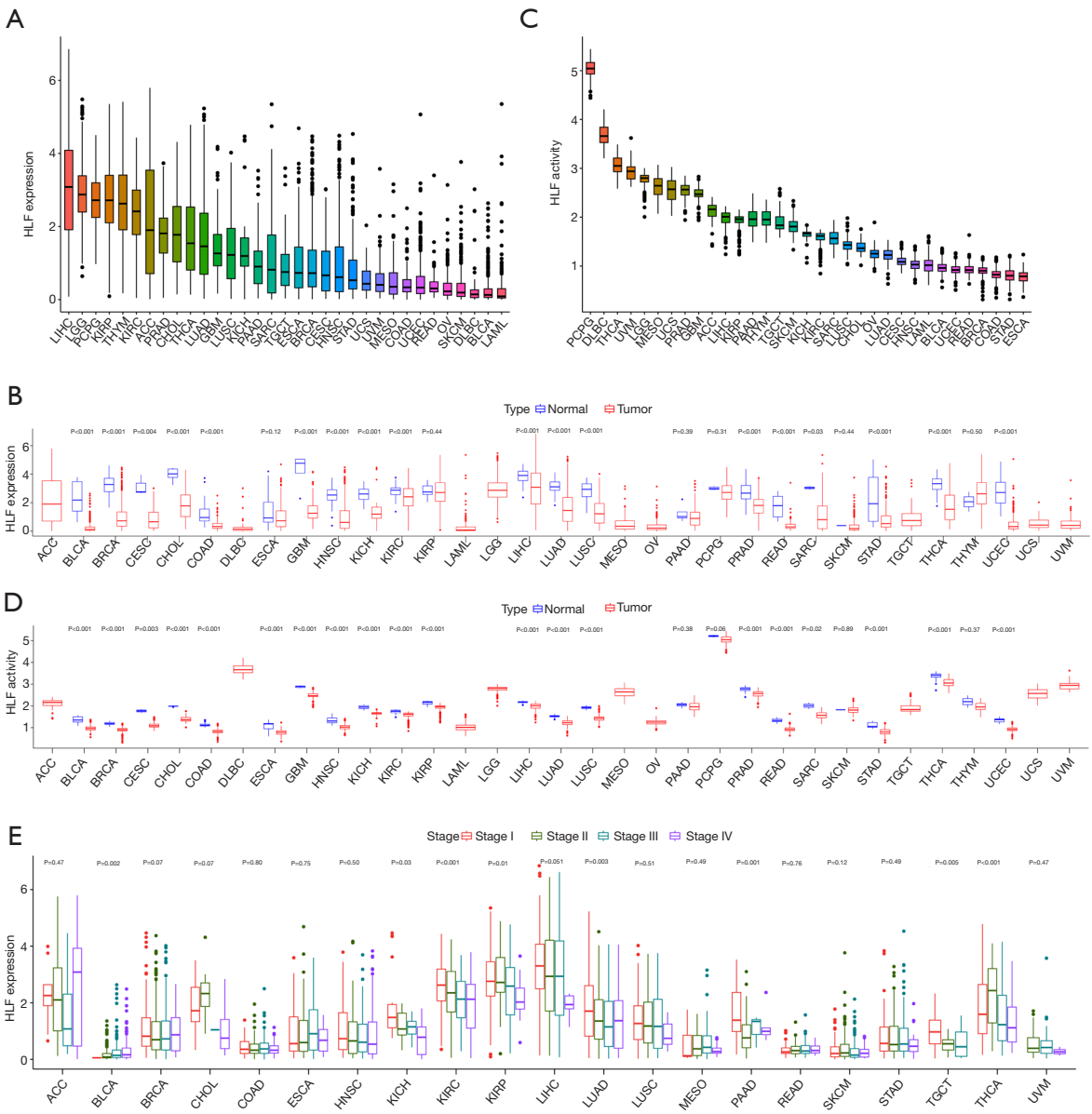

Figure 1 HLF gene expression and activity. Relationship between HLF expression and clinicopathological stage. (A) HLF gene expression in tumor tissues. (B) HLF gene expression between human normal and tumor tissues. (C) HLF activity in tumor tissues. (D) HLF activity between human normal and tumor tissues. (E) Relationship between HLF expression and clinicopathological stage. HLF, hepatic leukemia factor; LIHC, liver hepatocellular carcinoma; LGG, brain lower grade glioma; PCPG, pheochromocytoma and paraganglioma; KIRP, kidney renal papillary cell carcinoma; THYM, thymoma; KIRC, kidney renal clear cell carcinoma; ACC, adrenocortical carcinoma; PRAD, prostate adenocarcinoma; CHOL, cholangiocarcinoma; THCA, thyroid carcinoma; LUAD, lung adenocarcinoma; GBM, glioblastoma multiforme; LUSC, lung squamous cell carcinoma; KICH, kidney chromophobe; PAAD, pancreatic adenocarcinoma; SARC, sarcoma; TGCT, testicular germ cell tumors; ESCA, esophageal carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; HNSC, head and neck squamous cell carcinoma; STAD, stomach adenocarcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; MESO, mesothelioma; COAD, colon adenocarcinoma; UCEC, uterine corpus endometrial carcinoma; READ, rectum adenocarcinoma; OV, ovarian serous cyst adenocarcinoma; SKCM, skin cutaneous melanoma; DLBC, lymphoid neoplasm diffuse large B cell lymphoma; BLCA, bladder urothelial carcinoma; LAML, acute myeloid leukemia.

LUAD, LUSC, PRAD, READ, SARC, STAD, THCA, and UCEC (*Figure 1D*).

Correlation analysis of HLF expression with clinicopathological stage, diagnosis, and prognosis in pan-cancer

HLF was related to the clinicopathological stages of BLCA, KICH, KIRC, LUAD, PAAD, testicular germ cell tumors (TGCT), and THCA. HLF expression gradually decreased with an increase in tumor stage in most cases, but in BLCA, HLF expression increased with advancing clinicopathological stages (*Figure 1E*).

In terms of pan-cancer diagnostic capability, HLF showed good diagnostic value for many tumors. Notably, it exhibited superior diagnostic efficacy for BLCA, BRCA, CESC, CHOL, COAD, GBM, HNSC, LUAD, LUSC, READ, SARC, THCA, UCEC, and other tumors, with diagnostic ROC AUC values exceeding 0.85 (*Figure 2A-2N*).

Across many tumors, there was a significant difference in prognosis between high and low HLF expression groups. In several cases, patients with low HLF expression faced a worse prognosis. For example, in COAD, HNSC, and KIRC, overall survival in the low HLF expression group was worse than that in the high HLF expression group (*Figure 3A*). In mesothelioma (MESO), PAAD, and SARC, disease-free survival in the low HLF expression group was worse than that in the high HLF expression group (*Figure 3B*). Further, in COAD, HNSC, KIRC, and other tumors, disease-specific survival in the low HLF expression group was worse than that in the high HLF expression group (*Figure 3C*). In HNSC, KIRC, brain lower grade glioma (LGG), and other tumors, progression-free survival in the low HLF expression group was worse than that in the high expression group (*Figure 3D*).

Correlation analysis of HLF and pan-cancer tumor immunity

HLF demonstrated associations with tumor matrix and immune scores, indicating its role in shaping the tumor microenvironment and immune infiltration level in various cancers. Notable correlations included the positive association of HLF with ESTIMATE and stromal scores of BLCA, along with a negative correlation with tumor purity (*Figure 4A*). Moreover, HLF displayed a negative correlation with ESTIMATE and immune scores of LGG and positive correlation with tumor purity (*Figure 4B*). HLF was positively correlated with ESTIMATE and stromal scores of PRAD and negatively correlated with tumor purity (*Figure 4C*). In THCA, HLF exhibited a negative correlation with ESTIMATE and immune scores and positive correlation with tumor purity (*Figure 4D*). Finally, HLF displayed a negative correlation with immune score in THYM (*Figure 4E*).

In terms of the correlation between HLF and immune infiltration, we found that HLF showed the strongest correlation with mast cells resting in LUAD, macrophages M0 in PAAD, and B cells naive and NK cells in TGCT (*Figure 5*).

Correlation of HLF with genetic alterations in pan-cancer

Analyses involving cBioPortal revealed that HLF was the most mutated in breast cancer, primarily via amplifications and mutations. In addition, HLF was associated with TMB in BRCA, lymphoid neoplasm diffuse large B cell lymphoma (DLBC), ESCA, KIRP, LAML, LGG, LIHC, LUAD, PAAD, PRAD, STAD, THCA, and THYM and with MSI in DLBC, ESCA, PRAD, and STAD (*Figure 6*).

Functional enrichment analysis

GO and KEGG pathway enrichment analysis, based on the GSEA algorithm, indicated the involvement of HLF in several core biological processes in tumors and in the regulation of various tumor-related pathways. For instance, in ACC, HLF was associated with ascorbate and aldarate metabolism, porphyrin and chlorophyll metabolism, RNA degradation, starch and sucrose metabolism, and steroid hormone biosynthesis, and also in processes such as chromatin disassembly, positive regulation of gluconeogenesis, positive regulation of necrotic cell death, protein-DNA complex disassembly, and regulation of smooth muscle cell differentiation. In STAD, HLF was observed to promote various biological processes such as regulation of cardiac conduction and glial cell projection. HLF also participated in regulation of amyotrophic lateral sclerosis, neuroactive ligand receptor interaction, renin angiotensin system, and other pathways (*Figure 7A-7D*).

GeneMANIA results revealed a relationship between HLF and ANXA2, DBP, TEF, and other genes and the involvement of HLF in RNA polymerase II transcription regulator complex, myeloid leukocyte differentiation, and

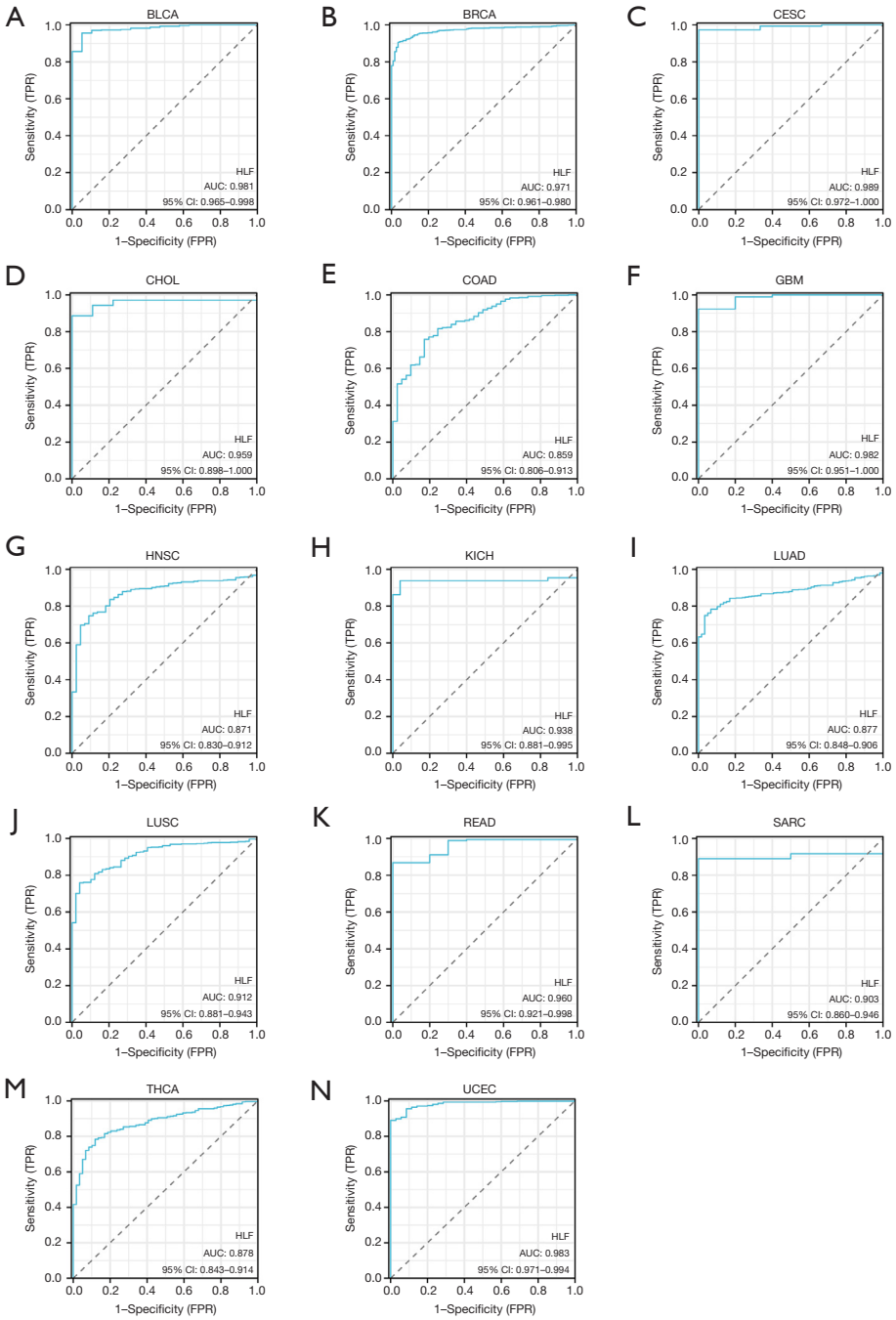

Figure 2 Diagnostic capability of HLF. (A) Diagnostic capability of HLF in BLCA. (B) Diagnostic capability of HLF in BRCA. (C) Diagnostic capability of HLF in CESC. (D) Diagnostic capability of HLF in CHOL. (E) Diagnostic capability of HLF in COAD. (F) Diagnostic capability of HLF in GBM. (G) Diagnostic capability of HLF in HNSC. (H) Diagnostic capability of HLF in KICH. (I) Diagnostic capability of HLF in LUAD. (J) Diagnostic capability of HLF in LUSC. (K) Diagnostic capability of HLF in READ. (L) Diagnostic capability of HLF in SARC. (M) Diagnostic capability of HLF in THCA. (N) Diagnostic capability of HLF in UCEC. HLF, hepatic leukemia factor; AUC, area under the curve; CI, confidence interval; FPR, false positive rate; TPR, true positive rate; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma.

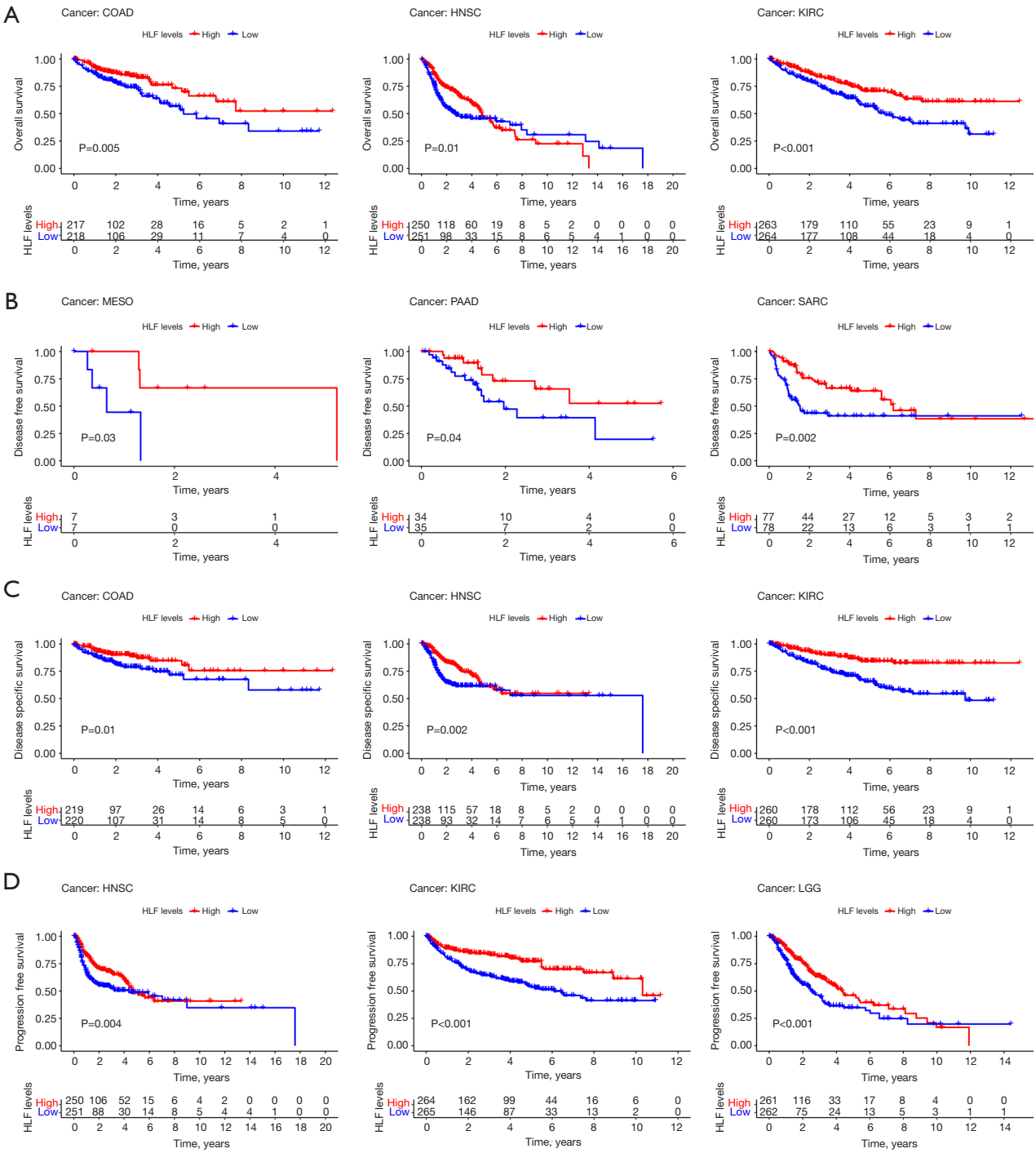

Figure 3 Relationship between HLF expression in pan-cancer and its implications for prognosis. (A) In COAD, HNSC, and KIRC, overall survival between the low HLF expression group and the high HLF expression group. (B) In MESO, PAAD, and SARC, disease-free survival between the low HLF expression group and the high HLF expression group. (C) In COAD, HNSC, and KIRC, disease-specific survival between the low HLF expression group and the high HLF expression group. (D) In HNSC, KIRC, and LGG, progression-free survival between the low HLF expression group and the high HLF expression group. HLF, hepatic leukemia factor; COAD, colon adenocarcinoma; HNSC, head and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; MESO, mesothelioma; PAAD, pancreatic adenocarcinoma; SARC, sarcoma; LGG, brain lower grade glioma.

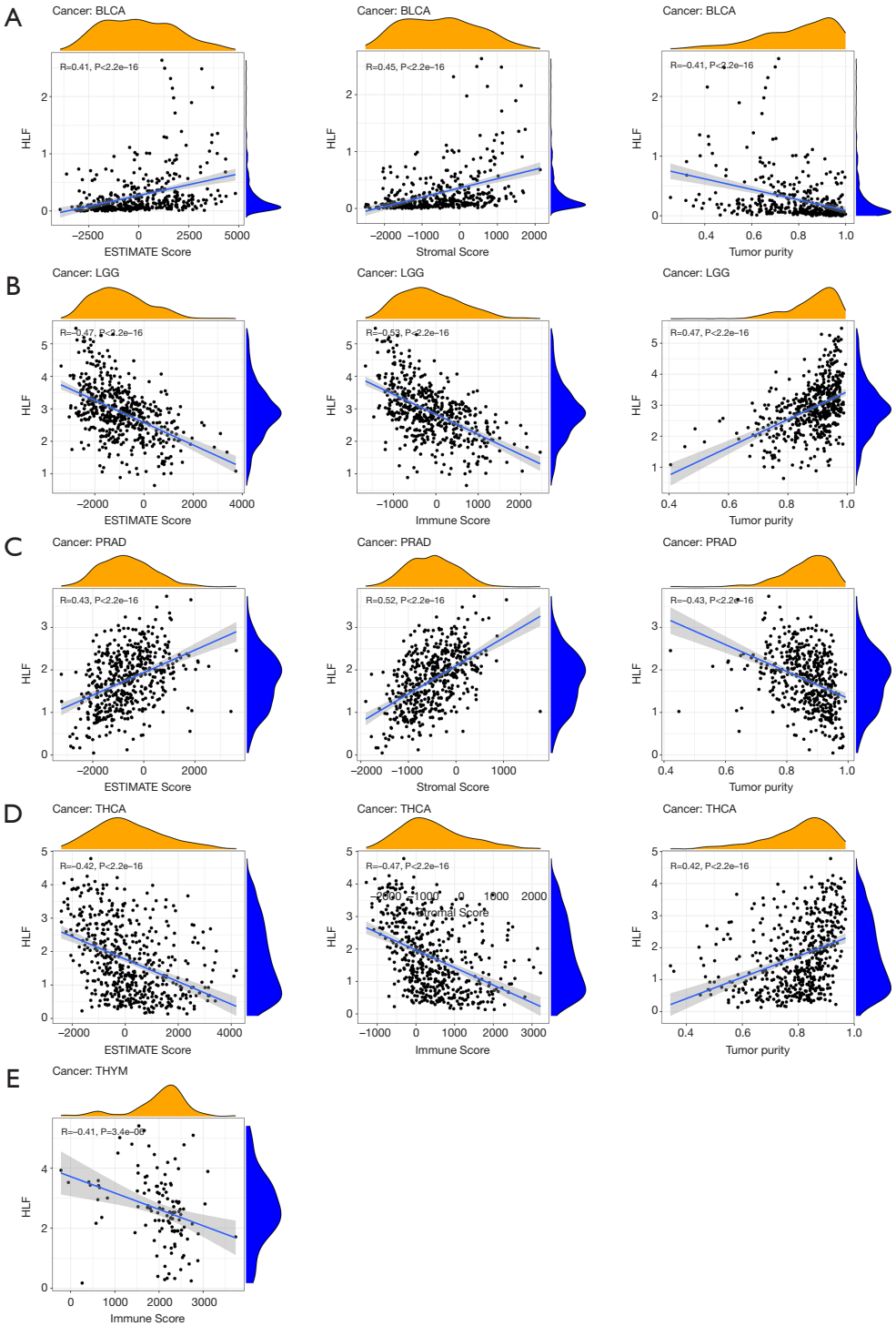

Figure 4 Correlation of HLF with ESTIMATE, immune, and stromal scores and with tumor purity. (A) Correlation of HLF with ESTIMATE score, stromal score and tumor purity in BLCA. (B) Correlation of HLF with ESTIMATE score, immune score and tumor purity in LGG. (C) Correlation of HLF with ESTIMATE score, stromal score and tumor purity in PRAD. (D) Correlation of HLF with ESTIMATE score, immune score and tumor purity in THCA. (E) Correlation of HLF with immune score in THYM. HLF, hepatic leukemia factor; ESTIMATE, Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data; BLCA, bladder urothelial carcinoma; LGG, brain lower grade glioma; PRAD, prostate adenocarcinoma; THCA, thyroid carcinoma; THYM, thymoma.

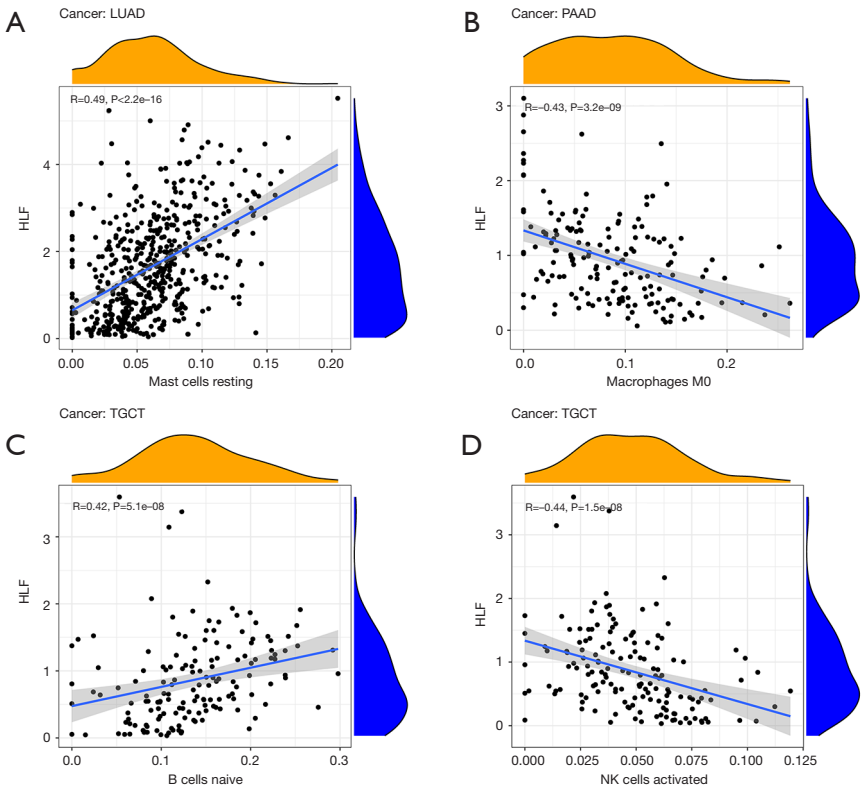

Figure 5 Correlation of HLF with immune infiltration. (A) Correlation of HLF with mast cells resting in LUAD. (B) Correlation of HLF with macrophages M0 in PAAD. (C) Correlation of HLF with B cells naive in TGCT. (D) Correlation of HLF with NK cells activated in TGCT. HLF, hepatic leukemia factor; LUAD, lung adenocarcinoma; PAAD, pancreatic adenocarcinoma; TGCT, testicular germ cell tumors.

RNA polymerase II cis-regulatory region sequence-specific regulation of biological processes such as DNA binding (*Figure 7E*).

Correlation analysis between HLF and CRC

HLF expression was significantly downregulated in CRC than in normal colon tissue (P<0.001). The AUC of ROC reached 0.887 (95% confidence interval: 0.842–0.927), indicating good diagnostic efficacy of HLF for CRC. Cox regression analysis also indicated a general prognostic correlation, with AUC values of 0.462, 0.458, and 0.530 for HLF pairs in predicting 1-, 3-, and 5-year outcomes for CRC, respectively (*Figure 8A-8C*).

In the context of CRC, HLF exhibited significant correlations with 123 genes, with AKAP6, LDB3, PDZRN4, SCN7A, SYNM, ATP1A2, FILIP1, GNAO1, CHRM2, and SYNPO2 showing the strongest associations (*Figure 8D*). GO analysis indicated that HLF-related genes were mainly located in "contractile fiber", "myofibril", "cell-cell junction", amongst others, and they were involved in processes such as "muscle system process", "regulation of heart contraction", and "regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum". Moreover, these genes were involved in functions such as actin binding, metal ion transmembrane transporter activity, and protein-macromolecule adaptor activity. KEGG pathway enrichment analysis revealed that HLF-related genes were principally involved in the regulation of calcium signaling pathway, cGMP-PKG signaling pathway, and regulation of actin cytoskeleton (*Figure 8E,8F*). The results obtained from GO and KEGG pathway enrichment analysis, based on the GSEA algorithm, were similar (*Figure 8G,8H*).

Furthermore, drug sensitivity analysis showed that HLF was associated with the drug sensitivity of several common chemotherapy agents, such as 5-fluorouracil, irinotecan, and oxaliplatin, which are commonly used in CRC. Patients with high HLF expression exhibited higher drug sensitivity (*Figure 8I-8K*).

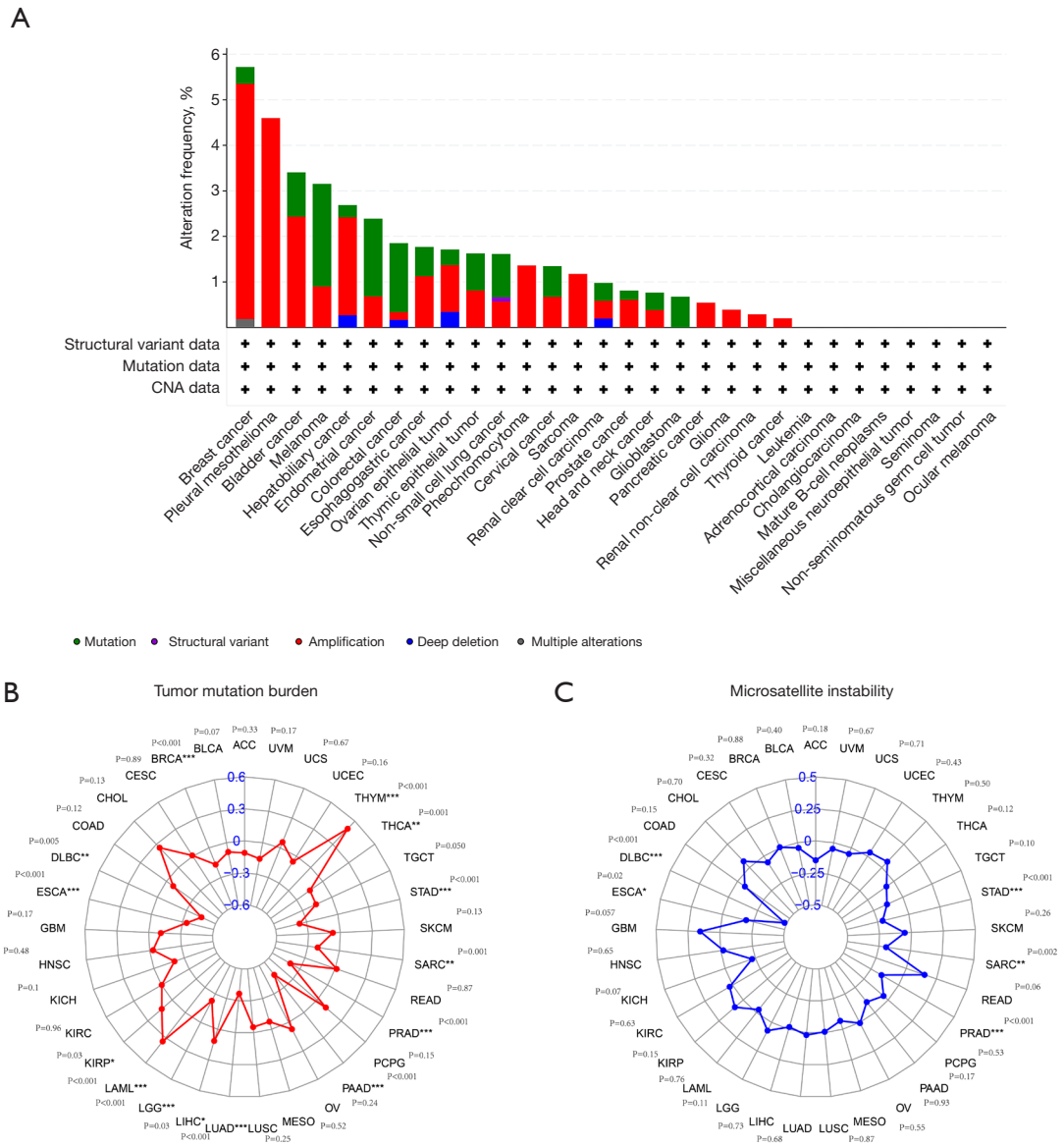

Figure 6 Correlation of HLF with TMB and MSI. cBioPortal analysis of HLF. (A) cBioPortal analysis of HLF. (B) Correlation of HLF with TMB. (C) Correlation of HLF with TMB and MSI. *, P<0.05; **, P<0.01; ***, P<0.001. CNA, copy number alteration; BLCA, bladder urothelial carcinoma; ACC, adrenocortical carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cyst adenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; HLF, hepatic leukemia factor; TMB, tumor mutation burden; MSI, microsatellite instability.

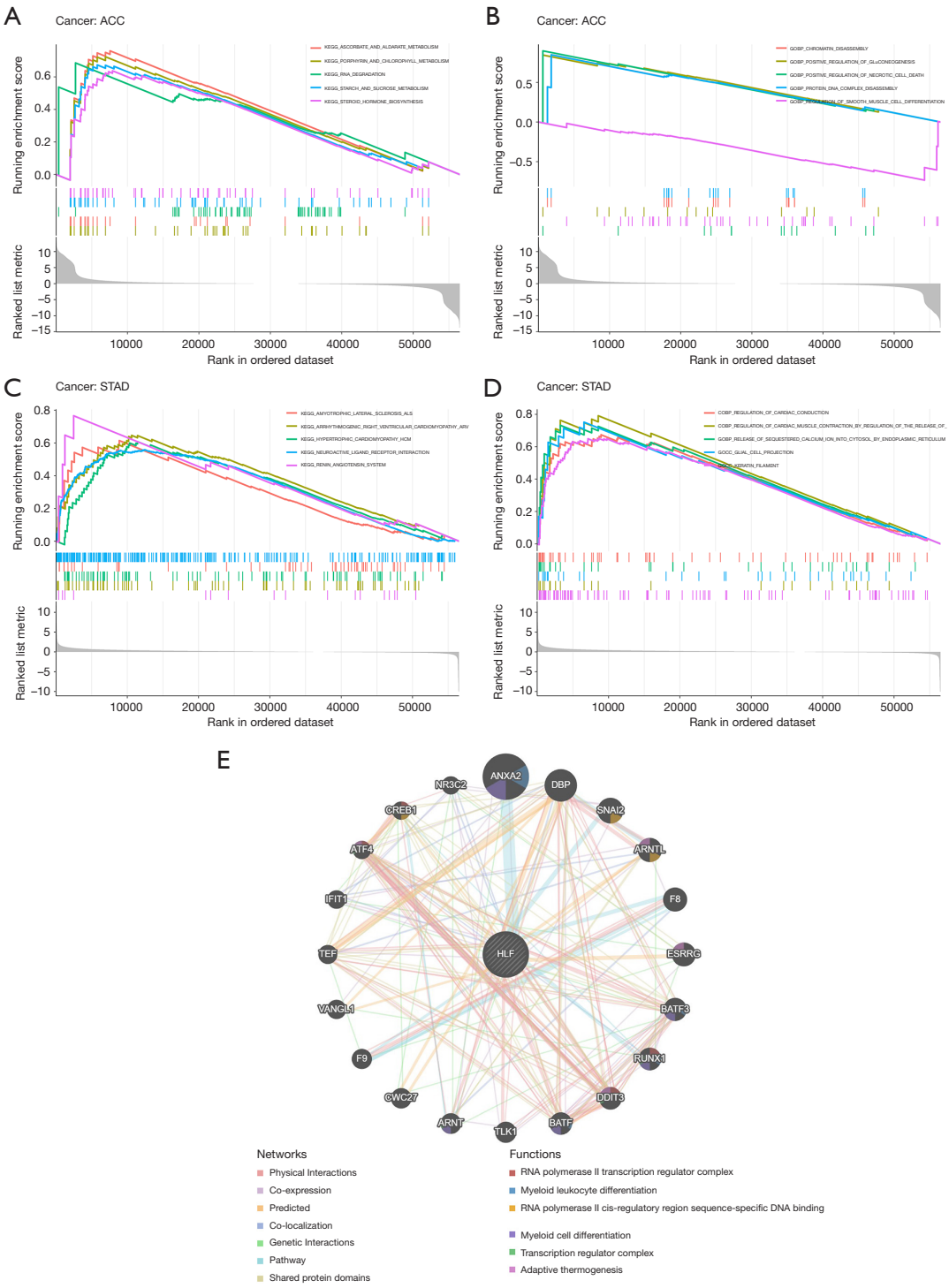

Figure 7 Functional enrichment analysis of HLF in pan-cancer. (A) KEGG pathway enrichment analysis of HLF in ACC. (B) GO pathway enrichment analysis of HLF in ACC. (C) KEGG pathway enrichment analysis of HLF in STAD. (D) GO pathway enrichment analysis of HLF in STAD. (E) GeneMANIA results of HLF. ACC, adrenocortical carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; STAD, stomach adenocarcinoma; HLF, hepatic leukemia factor.

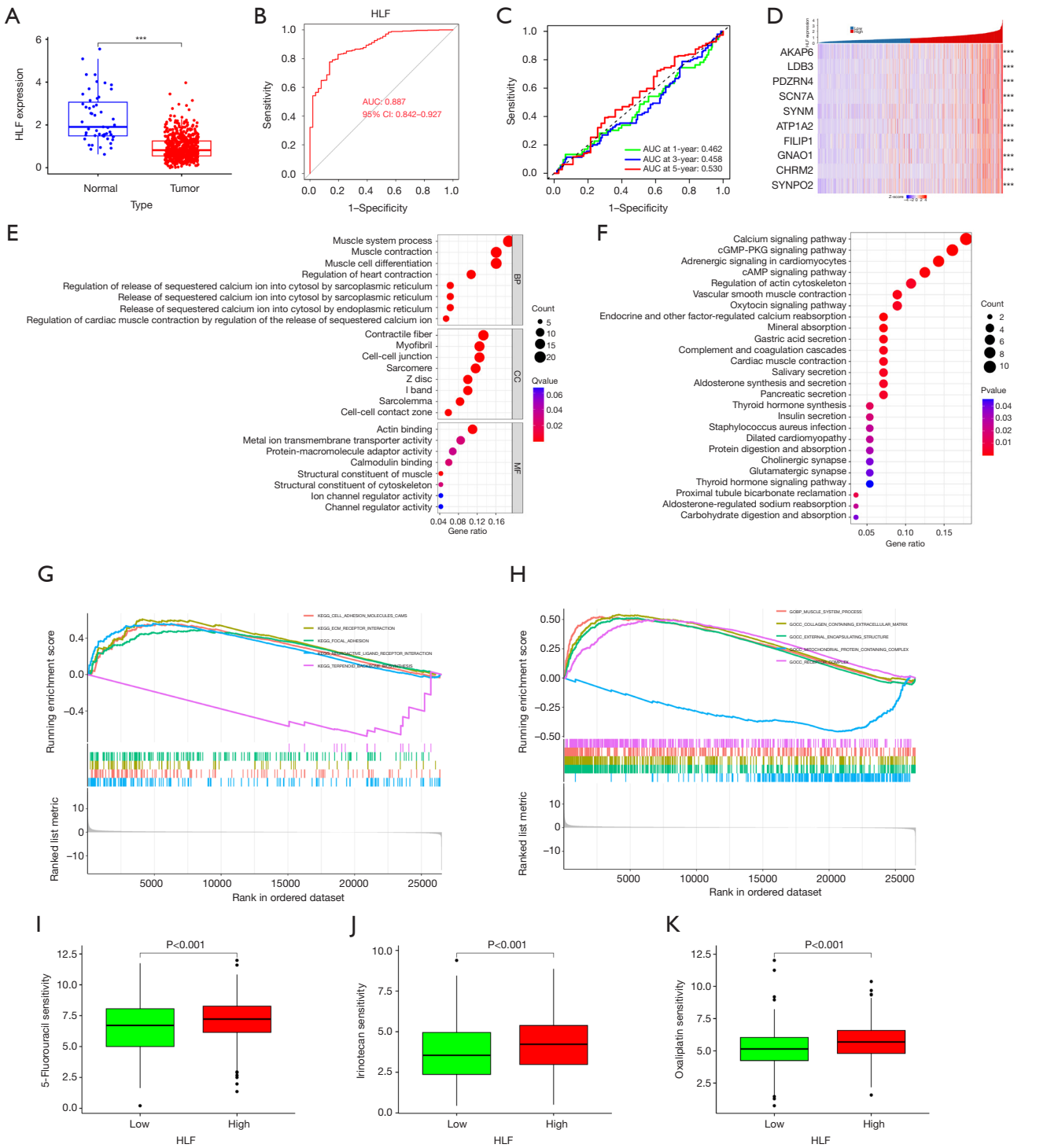

Figure 8 Correlation analysis between HLF and CRC in TCGA. Clinical relative analysis between HLF and CRC (A-C). The top ten genes associated with HLF (D). GO, KEGG, GSEA analysis of HLF (E-H). Drug sensitivity of 5-fluorouracil, irinotecan, and oxaliplatin (I-K). ***, P<0.001. HLF, hepatic leukemia factor; AUC, area under the curve; CI, confidence interval; BP, biological process; CC, cell component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas; GSEA, gene set enrichment analysis.

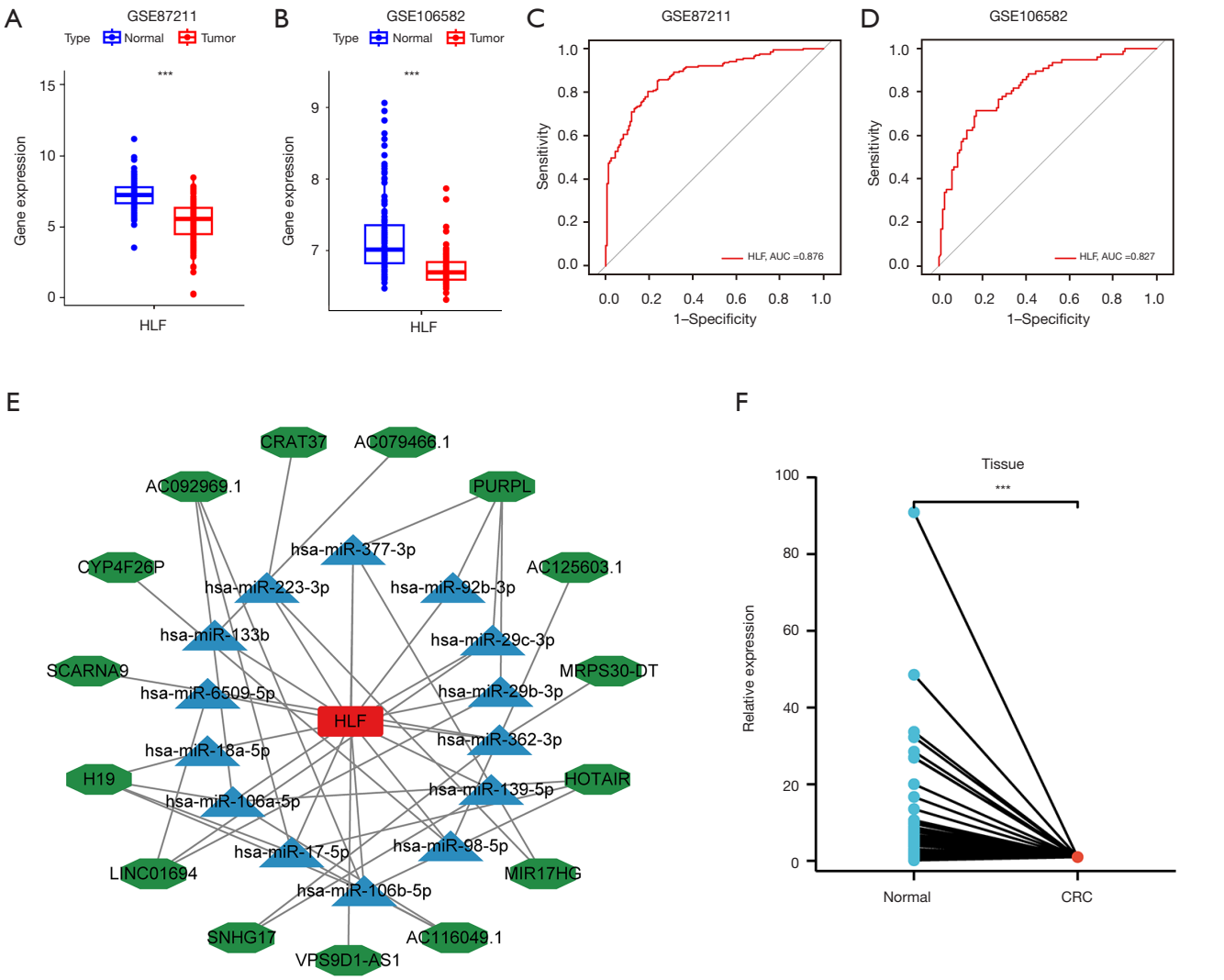

Figure 9 Expression differences and diagnostic ability of HLF in GSE87211 and GSE106582; ceRNA regulatory network of HLF; qRT-PCR results showing HLF expression in CRC and adjacent normal colorectal tissues. (A) In GSE87211, HLF gene expression between human normal and tumor tissues. (B) In GSE106582, HLF gene expression between human normal and tumor tissues. (C) Diagnostic ability of HLF in GSE87211. (D) Diagnostic ability of HLF in GSE106582. (E) ceRNA regulatory network of HLF. (F) qRT-PCR results showing HLF expression being downregulated in CRC tissues. ***, P<0.001. HLF, hepatic leukemia factor; AUC, area under the curve; CRC, colorectal cancer; ceRNA, competing endogenous ribonucleic acids; qRT-PCR, quantitative real-time polymerase chain reaction.

In GSE87211 and GSE106582, HLF expression was downregulated in tumor tissues, and the AUC of diagnostic ROC for CRC was >0.8 (*Figure 9A-9D*). In GSE39582 and GSE17536, patients with low HLF expression displayed worse AUC results than those with high HLF expression.

Construction of ceRNA regulatory network

By integrating data from multiple databases (StarBase, miRDB, mirTarBase, TargetScan, and mirWalk), 22 miRNAs were predicted to participate after intersection with differentially expressed miRNAs the regulation of HLF. According to StarBase and differential analysis, 15 lncRNAs were associated with HLF-related miRNAs. A ceRNA regulatory network of 15 lncRNAs, 14 miRNAs, and HLF was established. The core components in this regulatory network, as determined the degree algorithm, included HLF, hsa-miR-17-5p, hsa-miR-106a-5p, hsa-miR-

106b-5p, hsa-miR-362-3p, hsa-miR-377-3p, H19, PURPL, hsa-miR-29b-3p, and AC116049.1 (*Figure 9E*).

Analysis of HLF expression in tissues

qRT-PCR results revealed differences in HLF expression between normal colorectal and CRC tissues, with HLF expression being downregulated in CRC tissues (*Figure 9F*).

Discussion

Cancer poses a serious threat to human health due to its high incidence and mortality. At present, breast, lung, and colon (1) cancers are the three most common cancers worldwide. Standard cancer treatment modalities include surgical resection, radiotherapy, adjuvant chemotherapy, and immunotherapy, but their effectiveness remains limited (2). Early detection and effective treatment strategies are important prerequisites for improving the prognosis of patients with cancer. Pan-cancer analysis has the potential to reveal similarities and differences between different cancers and provide profound insights for developing tailored cancer prevention and personalized treatment approaches.

Herein we found a consistent decrease in HLF expression across various tumors, including BLCA, BRCA, and CESC. This downregulation in HLF expression was mostly associated with worse overall, disease-specific, disease-free, or progression-free survival in patients. In some cancers, HLF expression was also closely related to clinicopathological stage. These results suggest that HLF functions as a tumor suppressor gene in multiple malignancies, which is consistent with previous findings. Chen *et al.* reported that HLF enhances miR-132 expression by directly binding to the miR-132 promoter, leading to direct inhibition of the expression of the downstream target gene TTK. This, in turn, hinders glioma cell proliferation, metastasis, and radioresistance (36). Huang *et al.* identified a significant association between HLF polymorphisms and renal cell carcinoma risk; lower HLF expression was associated with more advanced renal cell carcinoma, and elevated HLF expression was associated with improved patient prognosis (37). Xue *et al.* also found that HLF expression was downregulated in pancreatic cancer and that elevated HLF expression was associated with a lower rate of distant metastasis (38).

While genes in normal cells typically replicate in a 1:1 ratio, cancer cells may exhibit ratios of 1:2 or higher, often indicating gene amplification. Mutation, on the other hand, denotes changes in base pair structure or sequence of a gene. Our genetic alteration analysis revealed that HLF was most frequently amplified in many tumors, followed by instances of mutation. These alterations may contribute to the dysregulation of HLF expression in tumors and play a role in tumor progression. Gene amplifications and mutations are closely related to tumor occurrence and development. For example, HER2, a member of the epidermal growth factor receptor family, overexpression and gene amplification have been reported in various solid tumor types (39). HER2 amplification in esophageal cancer is reportedly associated with a more favorable prognosis, earlier tumor stage, and lower lymphatic metastasis rate (40). Besides, programmed death ligand-1 (PD-L1) amplification occurs in some malignancies; more focal amplification is linked to increased PD-L1 expression at different ploidy thresholds, and this amplification can predict responsiveness to anti-PD-1/PD-L1 immunotherapy (41). Although studies on HLF amplification and mutation in pan-cancer remain limited, further investigations may lead to the development of new targeted anticancer drugs for HLF in the future.

Cancer immunity is a pivotal component in tumor initiation and progression, with cancer immunotherapy representing a key cancer treatment method. The tumor microenvironment and immune cell composition are inextricably related to the efficacy of cancer immunotherapy (42,43). Our analysis underscores the association between HLF and tumor microenvironment composition in BLCA, LGG, PRAD, THCA, and THYM. HLF was also found to be linked to the degree of immune cell infiltration in LUAD, PAAD, and TGCT and to TMB and MSI in multiple tumors. This suggests that HLF expression plays a crucial role in cancer immunity. To date, our understanding of the role of HLF in the human immune system is limited, and the involvement of HLF in the tumor immune microenvironment remains an important gap in our knowledge, necessitating further investigation.

We attempted to elucidate the mechanism via which HLF influences CRC and found that HLF was involved in the regulation of calcium signaling pathway, cGMP-PKG signaling pathway, and regulation of actin cytoskeleton. Furthermore, HLF demonstrated sensitivity to numerous common chemotherapeutic agents. Kania *et al.* showed that calcium homeostasis is a major factor affecting autophagy, and some calcium channels, such as voltage-gated T- and L-type channels, IP3 receptors, or Ca^{2+} release-activated Ca^{2+} channel (CRAC), are involved in

autophagy regulation (44). Boo *et al.* found that autophagy is closely related (45) to oxaliplatin resistance. Zhang *et al.* also indicated that calcium signaling plays a vital role in physiological and pathological conditions, profoundly affecting the melanoma microenvironment, including immune cells, extracellular matrix, blood vessel network, and chemical and physical environments (46). Piazza *et al.* reported that the cGMP-PKG signaling pathway acts on the downstream Wnt/β-catenin pathway to influence cancer cell growth and tumor immunity (47). Browning *et al.* reported cGMP/PKG to be a potential therapeutic target for CRC (48). Dysregulation of the actin cytoskeleton pathway has been observed during the progression of normal cells through dysplasia to inflammatory bowel disease-related CRC (49). Altering the status and content of actin cytoskeleton can also evidently affect the ability of CRC cells to metastasize (50). Collectively, these findings indicate that HLF is closely related to the occurrence, development, and distant metastasis of CRC.

Conclusions

This study represents the first expression validation of HLF in CRC and comprehensive system analysis of the expression and molecular mechanism of HLF in pan-cancer. Our pan-cancer analysis systematically demonstrated the various facets of HLF, including its expression patterns and mutations, and also its associations with MSI, TMB, tumor microenvironment, immune infiltration, and signaling pathways. The potential of HLF as a therapeutic target for cancer is evident, gives its consistent dysregulation across numerous carcinomas and its robust diagnostic and prognostic value. In addition, aberrant expression of HLF is closely related to CRC occurrence and development, and HLF is regulated by multiple genes in CRC. Our findings reveal the multifaceted role of HLF in pan-cancer and provide new insights into the diagnosis and treatment of HLF in CRC. However, our study was mainly based on bioinformatics analysis and the *in vitro* experimental part only verified the expression trends of HLF in CRC via qRT-PCR. In the future, based on the present results, we will conduct in-depth research of the expression of HLF related proteins and their interaction mechanisms in CRC and other cancers.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2274/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2274/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2274/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The research protocol was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University (No. 2023-E628-01), and written informed consent was provided by all participants.

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