The Establishment and Experimental Verification of an lncRNA-Derived CD8+ T Cell Infiltration ceRNA Network in Colorectal Cancer

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ABSTRACT

BACKGROUND: Long noncoding RNAs (LncRNA) lead a vital role in colorectal cancer (CRC) development. The infiltrating CD8+ T cell is the main target of immunotherapy. Our study aimed to figure out the potential mechanism of lncRNAs regulating the function of CD8+ T cells in CRC.

METHODS: We collected bulk RNA-seq, miRNA-seq, and single-cell RNA-seq (scRNA-seq) data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) database. The cibersort algorithm and correlation analysis were used to estimate the abundance of CD8+ T cells and screened out the most relevant lncRNAs. We used scRNA-seq data to identify the main cell lncRNA expressed. Furthermore, one competing endogenous RNA (ceRNA) network focusing on the potential mechanism of lncRNA-derived CD8+ T cell infiltration was constructed. We established a co-culture system to assess the immunosuppressive function of the lncRNA. And we evaluated the effects of the lncRNA on CD8+ T cell cytotoxicity by flow cytometry, qPCR, and clone formation assay.

RESULTS: Three CD8+ T cell infiltration-related lncRNAs were identified, and LINC00657 was expressed mainly in tumor cells, negatively associated with CD8+ T cell infiltration. Hsa-miRNA-1224-3p and hsa-miRNA-338-5p and SCD, ETS2, UBE2H, and YY1 were identified to construct the ceRNA network. Immunosuppression-related tumor marker CD155 was proved to be positively correlated with LINC00657 and mRNAs in the ceRNA network. In addition, we proved that LINC00657 could impair the cytotoxicity of CD8+ T cells, and its expression was positively associated with CD155 in vitro.

CONCLUSIONS: We successfully constructed an lncRNA-derived CD8+ T cell infiltration ceRNA network in CRC. LINC00657 may play a leading role in the CRC immune escape and could be a novel immunotherapy target.

KEYWORDS: Colorectal cancer, LncRNA, LINC00657, CD8+ T cell, immunotherapy

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world. The incidence of CRC ranks third among all types of cancers, and the mortality rate ranks second.[1] CRC could be divided into colon cancer (COAD) and rectal cancer (READ) according to the location of the tumor. Early colon cancer can be treated with surgery, and the prognosis is relatively good. But most patients with colon cancer have no apparent symptoms at the time of diagnosis. The advanced disease usually contributes to a poor prognosis, the 5-year survival of which is less than

40%.[2,3] For advanced CRC, chemotherapy ± molecular-targeted agents were the standard regimens. Although great promotion in CRC treatment, drug resistance remains an intractable problem. Therefore, the development of novel targets and therapies for the treatment of CRC is significant.

Immunotherapy was proven effective in several types of advanced cancers. Even when other drugs do not work, immunotherapy showed remarkable effectiveness, becoming a pillar for refractory cancers.[4] Pembrolizumab and nivolumab were 2 monoclonal checkpoint antibodies targeting T cells.[5] An accredited responsive and prognostic biomarker was microsatellite instability (MSI) status in CRC. DNA mismatch repair (MMR)

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protein (MLH1, MSH2, MSH6, or PMS2) defects could contribute to the formation of MSI status.[6] In CRC, 12% to 15% of patients can be identified as microsatellite instability-high (MSI-H).[7] MSI-H patients were usually combined with increased tumor mutation burden (TMB) and increased CD8+ T cell function and infiltration, 85% of which could respond to the PD-1-based immunotherapy.[8] There are many clinical trials of immunotherapy for MSI-H type CRC that are currently underway or have been completed.[9] Although some PD-1 combination treatments for MSI-stability (MSS) patients are emerging, most MSS patients and 15% of MSI-H patients could not benefit from PD-1 immunotherapy.[10,11] Thus, the identification of novel effective targets for CRC immunotherapy is urgent.

Long noncoding RNA (lncRNA) was usually considered one type of particular RNA which is longer than 200 nucleotides. LncRNAs are widely distributed in cells. They can maintain RNA stability, regulate mRNA translation and protein modification, function as miRNA precursors, and participate in competitive endogenous RNA (ceRNA).[12] In addition, lncRNAs can function via cis- and trans-acting in the nucleus.[13,14] ceRNA was one of the most critical regulatory biological processes of lncRNAs, forming a complicated interaction regulatory network in the progression of diseases.[15] Abnormally expressed lncRNA in tumor cells could promote the formation of the immunosuppressive microenvironment and the drug resistance of tumors.[16-18] In addition, the infiltration of immune cells could also be regulated by lncRNA,[19] which is a vital reference for the selection of potential immunotherapy targets.

Our research comprehensively used bioinformatics algorithms, qPCR, colony formation assay, and flow cytometry to find potential immunotherapy targets on CRC tumor cells. The cibersort algorithm was applied to evaluate the abundance of CD8+ T cell infiltration. CD8+ T cell infiltration-related lncRNAs were then screened out to construct the ceRNA network. In addition, we used a tumor cell and CD8+ T cell coculture system to evaluate the immunosuppressive features of the selected lncRNA. Finally, we constructed an lncRNA-derived CD8+ T cell infiltration ceRNA network and identified one lncRNA which could be a novel immunotherapy target in CRC.

Materials and Methods

Research ethics and patient consent

This study was strictly conducted according to the World Medical Association Declaration of Helsinki and approved by the Medical Ethics Committee of Zhongshan Hospital, Fudan University (B2017-166R). All the participants provided informed consent.

Data resources and data preprocessing

The RNA-seq (FKPM), miRNA-seq (mature), and clinical information (MSI status) of the TCGA-COAD cohort and TCGA-READ cohort were downloaded from the website

(https://portal.gdc.cancer.gov). Cell lines RNA-seq data was downloaded from Broad Institute Cancer Cell Line Encyclopedia (CCLE).

R package sva (version 3.13) was applied to combine and remove the batch effect. In general, RNA-seq data comprised 644 colorectal tumor tissue and 51 normal colon and rectum tissue; miRNA-seq data contained 601 colorectal tumor tissue and 11 normal colons and rectum tissue. The extraction of lncRNA expression from RNA-seq expression was carried out according to the annotation file from GENCODE (version 27). Two scRNA-seq datasets were collected from GEO (GSE132456, GSE146771). Unique molecular identifiers (UMIs) between 101 and 6000 and ⩽10% UMI from the mitochondrial genome were set as the criteria to include cells for further research. The scRNA-seq data was then normalized by log2(UMI + 1). R package Seurat (version 3.1.2) was used for scRNA-seq data analysis. OmniBrowser (https://omnibrowser.abiosciences.com/#/home/index) is a powerful online tool for scRNA-seq data visualization and analysis. We applied it to generate an mRNA expression heatmap and analyze the mRNA correlation in specific cell clusters.

Differential mRNA, lncRNA, and microRNA analysis

We performed differential expression of mRNA (DEmRNA), lncRNA (DElncRNA), and microRNA (DEmicroRNA) between tumor tissue and normal tissue based on the R package limma (version 3.13), the P value < .01 and $|\text{logFC}| > 1$ was set as the threshold value.

CD8+ T cell infiltration and correlation analysis

The cibersort (http://cibersort.stanford.edu/) algorithm was used to evaluate the CD8+ T cell infiltration in the tumor tissue. Next, we used the Pearson correlation analysis to calculate the relevance between target mRNA, microRNA, lncRNA, and CD8+ T cell infiltration. Correlation absolute value was >0.2, and P value < 0.01 was considered significant.

Dimension reduction and the identification of tumor cells

Dimension reduction analysis was applied for scRNA-seq data, including t-distributed stochastic neighbor embedding (tSNE) and uniform manifold approximation and projection (UMAP). We can divide cells into different clusters through dimension reduction analysis, including the CD8+ T cluster. R package inferCNV (version 3.14) was used to distinguish malignant and benign cells.

CeRNA network construction

LncRNA-microRNA interaction prediction was performed using the online database DIANA-LncBase (LncBase

Experimental v.2), which contains experimental supporting evidence and bioinformatic prediction.

MicroRNA-mRNA interaction prediction was verified by multi-database: miRDB (http://mirdb.org/), targetscan (http://www.targetscan.org/vert_71/), and miTarbase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2019/php/index.php). We extracted negatively regulated DElncRNA-DEmRNA pairs and DEmicroRNA-DEmRNA pairs to construct the ceRNA network. R package ggplot2 was used to visualize the network.

The establishment of T cell–tumor cell co-culture system

According to the manufacturer's instructions, human T cells were obtained from 3 healthy volunteers' PBMCs (07851, Stemcell). And we used EasySep™ Human CD8+ T Cell Isolation Kit (17953, Stemcell) to isolate the CD8+ T cells from PBMCs. CD8+ T cells were cultured in the Human ImmunoCult-XF T Cell Expansion medium (10981, Stemcell) with 1% Penicillin-Streptomycin (15140122, Gibco). The CD8+ T cells were cultured in the medium at 1×10^6/mL. To activate T cells, $2\,\mu g$/mL of anti-CD3 (02121-25-500, Peprotech), $1\,\mu g$/mL of anti-CD28 (10311-25-500, Peprotech), and $200\,U$/mL of IL-2 (200-02-10, Peprotech) were added to the cell suspension for 3 days. HCT116 cell line was purchased from the Chinese Academy of Sciences (Shanghai, China). DMEM (11995065, Gibco) with 10% fetal bovine serum (SA101, Cellmax) and 1% Penicillin-Streptomycin (15140122, Gibco) were used to culture tumor cells. After being prepared as described above, CD8+ T cells were directly added to tumor cells at a 5:1 ratio to establish the CD8+ T cell-tumor cell co-culture system.

Flow cytometry assay

Flow cytometry assay was applied to evaluate the number of positively stained cells. The Annexin V-APC/7-ADD (40309ES2, Yeasen), Brilliant Violet 421™ anti-human Perforin BV421 (308122, BioLegend), and FITC anti-human/mouse Granzyme B Antibody (515403, BioLegend) were used to stain the cells. The stained cells were counted by flow cytometer (Becton Dickinson) and analyzed using FlowJo-V10 software.

Colony formation assay

We used a 6-cell plate to establish the co-culture system for colony formation assay. After 7 days, we removed suspended cells and stained adherent tumor cell colonies with crystal violet staining solution (C0121, Beyotime). We counted the formed clones directly using the naked eye and using the Graphpad software (version 8.3) for statistical analysis, using the *t* test to compare the clone number of the 2 groups, and $P < 0.05$ was considered statistically significant.

Table 1. The sequence of RNAi plasmid.

ID	SEQUENCE
sh1(101392-11)	GCGAATGCAGACTATTCATTG
sh2(101393-13)	GCGGTTGGTCTTCATTCTACC
sh3(101394-11)	GCATCTCTTGGCTGTACTATA
NC(CON313)	TTCTCCGAACGTGTCACGT

Table 2. The sequence of primers.

ID		SEQUENCE
IFNG	Forward primer	TCGGTAACTGACTTGAATGTCCA
	Reverse primer	TCGCTTCCCTGTTTTAGCTGC
GZMB	Forward primer	CCCTGGGAAAACACTCACACA
	Reverse primer	GCACAACTCAATGGTACTGTCG
PRF1	Forward primer	GGCTGGACGTGACTCCTAAG
	Reverse primer	CTGGGTGGAGGCGTTGAAG
CD155(PVR)	Forward primer	TGGAGGTGACGCATGTGTC
	Reverse primer	GTTTGGACTCCGAATAGCTGG
GAPDH	Forward primer	GGAGCGAGATCCCTCCAAAAT
	Reverse primer	GGCTGTTGTCATACTTCTCATGG

Transfection

RNAi plasmid targeting LINC00657 and normal control were designed and synthesized by Shanghai Genechem (hU6-MCS-CBh-gcGFP-IRES-puromycin). The target sequences are shown in Table 1. According to the manufacturer's instructions, we applied Lipofectamine 3000 to transfect the plasmid into tumor cells (L3000015, Thermo Fisher Scientific). Quantitative real-time polymerase chain reactions (qRT-PCR) were performed to verify transfection efficiency. We selected the most efficient target sh2 (101393-13) for further research.

RNA extraction and qRT-PCR

We used RNAeasy™ Animal RNA Extraction Kit (R0026, Beyotime, China) to extract the total RNA. The primers of mRNAs are shown in Table 2. The reverse transcription process was completed using Goldenstar™ RT6 cDNA Synthesis Kit Ver.2 (Tsingke, China). Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (YEASEN, China) was applied to perform qRT-PCR.

Results

The screening of tumor CD8+ T cell infiltration–related lncRNA

First, the CD8+ T cell infiltration of each sample in TCGA was calculated by cibersort. In total, 33 most significantly
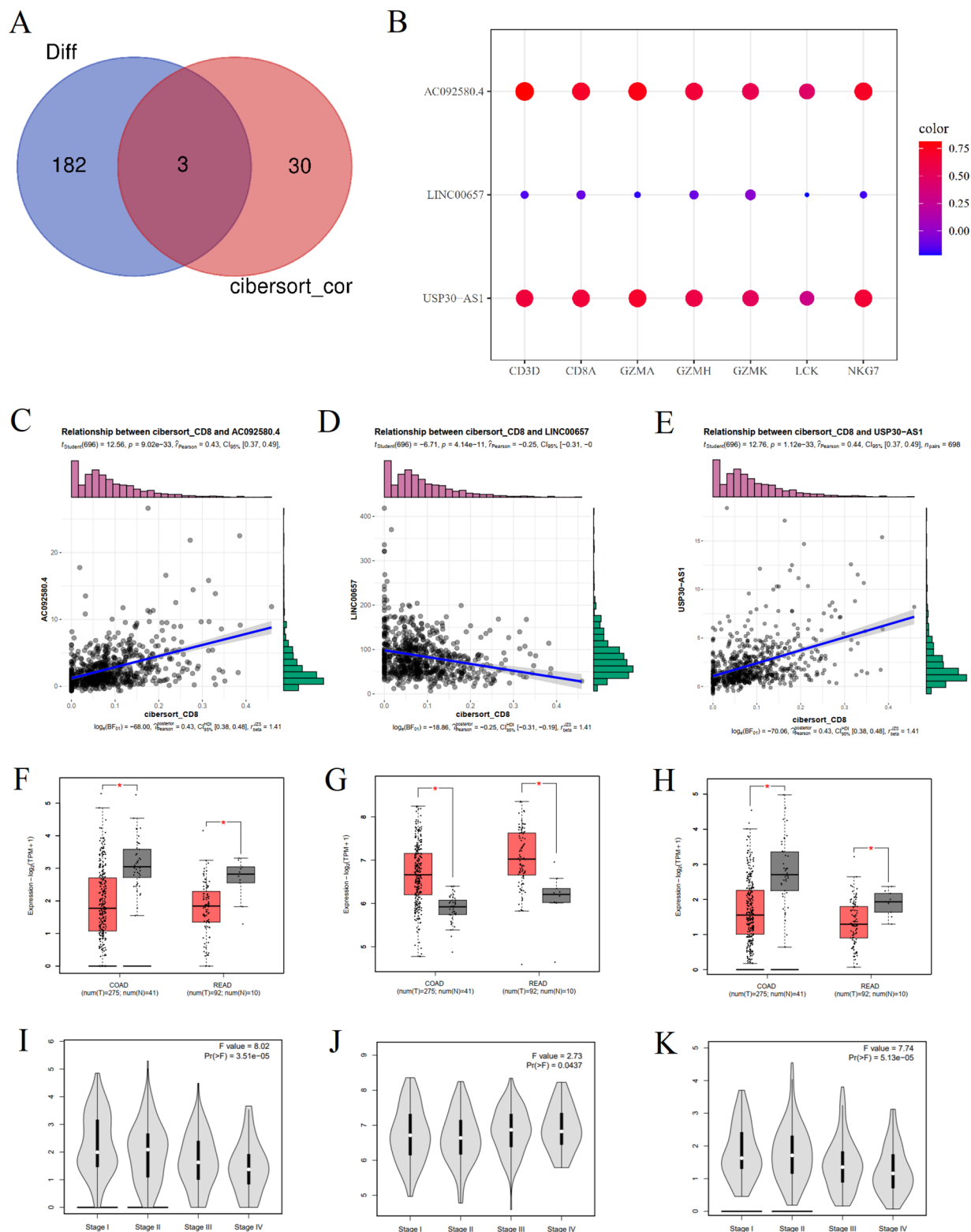
Figure 1. The identification of CD8+ T cell infiltration–related lncRNAs. (A) The Venn plot showed the intersection of DElncRNAs and CD8+ T cell infiltration-related lncRNAs. (B) The dot plot showed the correlation coefficient of the expression of known CD8+ T cell marker and selected lncRNAs. (C-E) The correlation analysis of CD8+ T cell infiltration and the expression of selected lncRNAs, respectively. (F-H) The lncRNAs differential expression analysis in colon cancer and rectal cancer via gepia. Red was for tumor group, and gray was for normal group. *$P < .05$ was considered statistically significant. (I-K) The violin plot showed the expression of selected lncRNAs in different stages of CRC. Pr(>F)$<$0.05 was considered statistically significant.
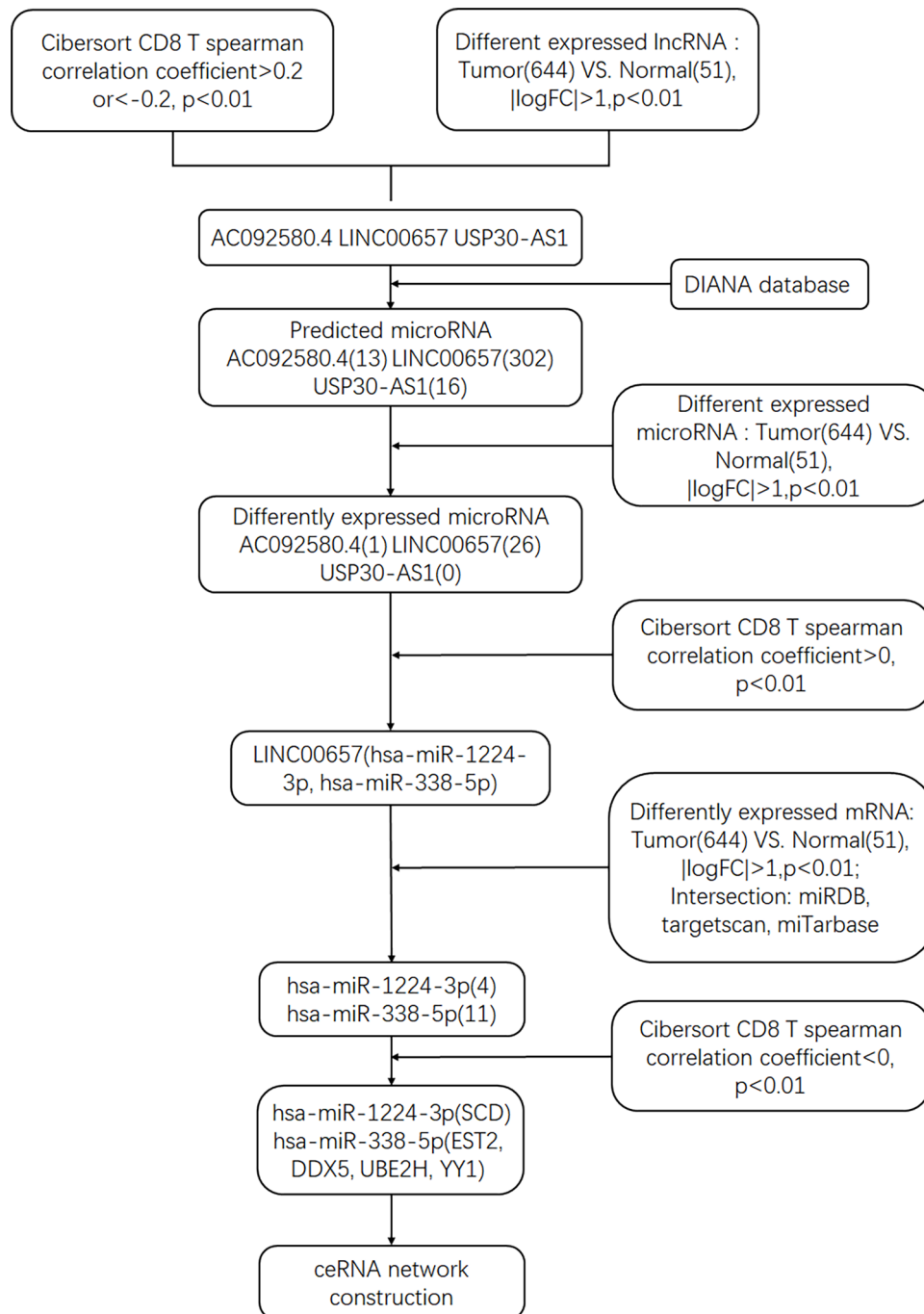
Figure 2. The flow chart of ceRNA network construction.

related lncRNAs were generated. The DElncRNAs between tumor and normal tissue were then figured out and intersected with lncRNAs associated considerably with CD8+ T cell infiltration (Figure 1A). In total, the 3 most potential lncRNAs related to CD8+ T cell infiltration were selected: AC092580.4, LINC00657, and USP30-AS1 (Figure 2). To verify the veracity of the screening method, we performed the correlation analysis between the 3 lncRNAs and several CD8+ T cell markers (CD3D, CD8A, GZMA, GZMH, GZMK, LCK, and NKG7) (Figure 1B, single-gene scatter

plot was shown in Supplementary Figure 1). We found that CD8+ T cell infiltration positively associated lncRNAs (AC092580.4, USP30-AS1) (Figure 1B, C and E), which were positively relevant to CD8+ T cell markers, while CD8+ T cell infiltration negatively associated lncRNA LINC00657 and LINC00657 was negatively relevant to CD8+ T cell markers (Figure 1B and D). Interestingly, apart from different expressions between tumor and normal tissue (Figure 1F to H), these 3 lncRNAs presented trending expression along with the tumor stage increasing (Figure 1I to K).
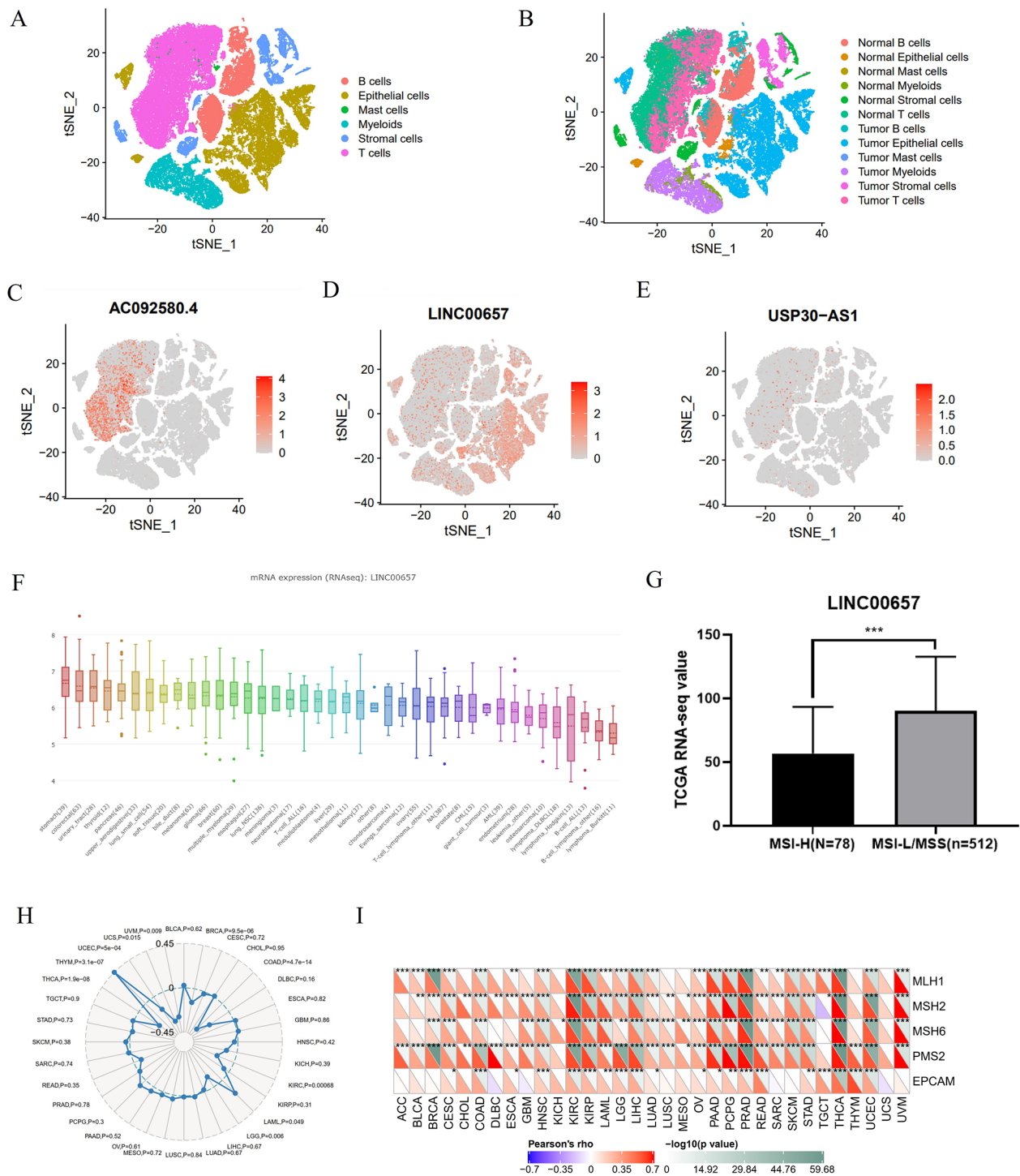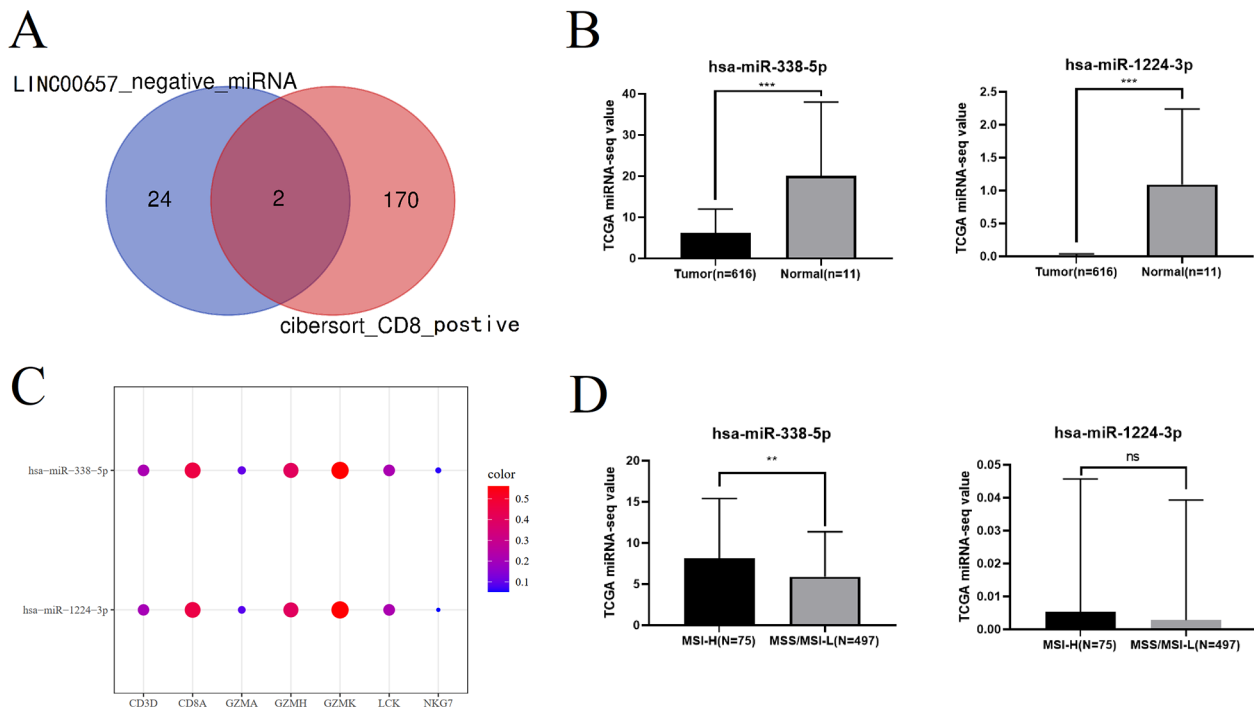
Figure 3. The selection of LINC00657 and the correlation analysis of the expression of LINC00657 and MSI status. (A) Dimensionality reduction analysis (tSNE) of scRNA-seq dataset GSE132456. (B) The tSNE plot showed the distinction between benign and malignant cells by inferCNV package (dataset GSE132456). (C-E) The expression status of lncRNAs in different cells. (F) The expression of LINC00657 in different types of cell lines by CCLE database. (G) The expression of LINC00657 in MSI-H CRC patients and MSI-L/MSS CRC patients. (H) The radar plot showed the correlation of expression of LINC00657 and MSI status in 32 types of cancers. (I) The correlation of expression of LINC00657 and MMR proteins in 32 types of cancers.

LINC00657 was mainly expressed in tumor cells and associated with MSI status

The cibersort algorithm is based on bulk-seq, inevitably producing false-positive results. Thus, we used scRNA-seq data to

verify the expression model of the 3 lncRNAs. To identify the tumor cells in the sample, we used R package inferCNV to distinguish malignant cells from normal cells. As shown in Figure 3B, tumor epithelial cells (light blue) made up most of

Figure 4. The selection of LINC00657-associated microRNA. (A) The Venn plot showed the intersection of the microRNAs negatively associated with LINC00657 and CD8+ T cell infiltration-related microRNAs. (B) The expression of hsa-miR-338-5p and hsa-miR-1224-3p in tumor and normal colon tissue. ***P < .001. (C) The dot plot showed the correlation coefficient of the expression of known CD8+ T cell markers and microRNAs. (D) The expression of hsa-miR-338-5p and hsa-miR-1224-3p in MSI-H CRC patients and MSI-L/MSS CRC patients.

the epithelial cells (brown) compared with Figure 3A. Finally, AC092580.4 and USP30-AS1 were proved expressed mainly in T cells, while LINC00657 was primarily expressed in tumor cells (Figure 3C to E).

Furthermore, we used the CCLE database to explore the expression of LINC00657 in different tumor cell lines. Interestingly, we found that the expression of LINC00657 in CRC cell lines was higher than most of the other tumor cells (Figure 3F). In addition, high LINC00657 expression was positively correlated with MSI-L/MSS status, while low LINC00657 expression was negatively correlated with MSI-L/MSS status (Figure 3G). In addition, the pan-cancer analysis showed a similar correlation trend in some other cancers (Figure 3H). The loss of MMR protein (MLH1, MSH2, MSH6, or PMS2) was defined as MSI status, otherwise MSS status. We found that the expression of LINC00657 was positively correlated with MLH1, MSH2, MSH6, and PMS2 (Figure 3I).

The screening of tumor CD8+ T cell infiltration-related microRNA

We used the DIANA database to identify the LINC00657 associated differently expressed microRNAs (Figure 2). In total, 26 microRNAs were identified, and two of them (hsa-miR-338-5p and hsa-1224-3p) were negatively related to the expression of LINC00657 and positively associated with CD8+ T cell infiltration (Figure 4A). Table 3 shows the

information of hsa-miR-338-5p and hsa-1224-3p in database DIANA. Figure 4B shows hsa-miR-338-5p and hsa-miR-1224-3p were lower in tumor tissue than normal tissue based on TCGA data. We evaluated the correlation between the expression of CD8+ T cell markers and the expression of microRNAs. The dot plot showed that the correlations were all positive (Figure 4C). In addition, TCGA data showed that hsa-miR-338-5p was significantly highly expressed in MSI-H patients, and hsa-1224-3p had the trend of high expression in MSI-H patients (Figure 4D).

The construction of CD8+ T cell infiltration ceRNA network and the correlation analysis of CD155

Hsa-miR-338-5p and hsa-miR-1224-3p were selected hub microRNAs in the T cell infiltration ceRNA network. To find the downstream mRNAs of the 2 microRNAs, we took the intersection of the significantly expressed mRNAs and the results of 3 databases (miRDB, targetscan, and miTarbase) (Figure 5A). The mRNAs negatively correlated with CD8+ T cell infiltration were screened out (Figure 5C-G). scRNA-seq was applied to verify the mRNAs' primary expressed cells, and the heatmap (Figure 5H) showed that YY1, UBE2H, ETS2, and SCD were primarily expressed in malignant cells (Supplementary Figure 2). Thus, these 4 mRNAs were selected to construct the ceRNA network (Figure 6A). CD155 (PVR) was a novel immunosuppressive protein

Table 3. The information of has-miR-338-5p and has-miR-1224-3p in DIANA database.

MICRORNA	PREDICTED. SOCRE	PUBLICATION	TISSUE	CELL TYPE	LOCATION	REGION	METHODS
hsa-miR-338-5p	0.849	Xue Y et al, 2013[20]	Cervix	HELA	20:36048019-36048047	Exon	HITS-CLIP
					20:36048310-36048338	Exon	HITS-CLIP
					20:36045764-36045792	Exon	HITS-CLIP
					20:36048389-36048417	Exon	HITS-CLIP
		Pillai MM et al, 2014[21]	Mammary gland	BT474	20:36045764-36045792	Exon	HITS-CLIP
					20:36048310-36048338	Exon	HITS-CLIP
		Balakrishnan I et al, 2014[22]	Bone marrow	HS5	20:36048310-36048338	Exon	HITS-CLIP
					20:36045764-36045792	Exon	HITS-CLIP
					20:36048019-36048047	Exon	HITS-CLIP
hsa-miR-1224-3p	0.746	Pillai MM et al, 2014[21]	Mammary gland	BT474	20:36050593-36050621	Exon	HITS-CLIP

predominantly expressed on tumor cells, including CRC.[23,24] Interestingly, we found the expression of LINC00657 was positively correlated with CD155 (Figure 6B). And the same is true for YY1, UBE2H, ETS2, and SCD (Figure 6C-F). In addition, the mRNA expression signature constructed by OmniBrowser (YY1, UBE2H, ETS2, SCD) has shown a consistent positive correlation with the expression of CD155 in CRC (Figure 6G).

The co-culture system verified the immunosuppressive ability of LINC00657 and the potential association with CD155

In tumor and CD8+ T cells co-culture system, we found that knockdown of the expression of LINC00657 in tumor cells could decrease the apoptosis rate of CD8+ T cells and increase the apoptosis rate of tumor cells (Figure 7A-D). Colony formation assay showed that knockdown of the expression of LINC00657 in tumor cells in the co-culture system could significantly decrease tumor cell colony formation (Figure 7E and F). In addition, we collected the CD8+ T cells to detect the cytotoxicity markers (IFNG, GZMB, and PRF1). After downregulating LINC00657 in tumor cells, the expression of IFNG, GZMB, and PRF1 in CD8+ T cells were significantly upregulated (Figure 7G-I). We further verified PRF1 and GZMB by flow cytometry and got almost consistent results (Figure 7K and L). In addition, we surprisingly found that the expression of CD155 was downregulated by knockdown of the expression of LINC00657 (Figure 7J).

Discussion

Immunotherapy has been the research focus for CRC in recent years. For MSI-H CRC, several immunotherapy drugs have been approved by FDA, and relevant clinical trials are underway or have been completed. In 2020, the KEYNOTE-177 clinical trial proved that pembrolizumab first-line therapy could significantly prolong progression-free survival (PFS) in patients with MSI-H CRC compared with standard therapy.[25] At 36 months, patients in the pembrolizumab group had an 11% improvement in overall survival (OS) compared with the control group.[26] In the CHECKMATE-142 clinical trial, first-line nivolumab plus low-dose ipilimumab showed a powerful and enduring clinical benefit for MSI-H CRC with a 69% objective response rate (ORR) and 84% disease control rate (DCR).[27] Dostarlimab is a humanized PD-1 mAb recently approved by the FDA. In the GARNET clinical trial, the ORR of dostarlimab for advanced MSI-H CRC was 36.2%.[28]

However, most patients with CRC were MSS "cold tumors" (>85%), with poor response to immunotherapy drugs. Interestingly, researchers found that some MSS patients could still respond to immunotherapy when combined with other inhibitors or chemotherapy,[29,30] which indicates unknown molecular regulatory mechanisms exist. As an acknowledged biomarker of immunotherapy, the ability of MMR to discriminate the efficacy of immunotherapy is still unsatisfactory. Therefore, discovering new targets that can increase CD8+ T cell infiltration and promote CD8+ T cell function is of great significance for the immunotherapy of CRC. Using bioinformatics analysis and a co-culture system, we screened out an lncRNA-derived CD8+ T cell infiltration ceRNA network and verified the anti-immune function of LINC00657, which could be a novel immunotherapy target.

LncRNAs were reported regulating tumor cell development, including lung cancer,[31] pancreatic cancer,[32] CRC,[33] and so on. In the tumor microenvironment, lncRNAs can regulate the upgrowth and function of immune cells and help tumor cells escape from immune surveillance.[34]
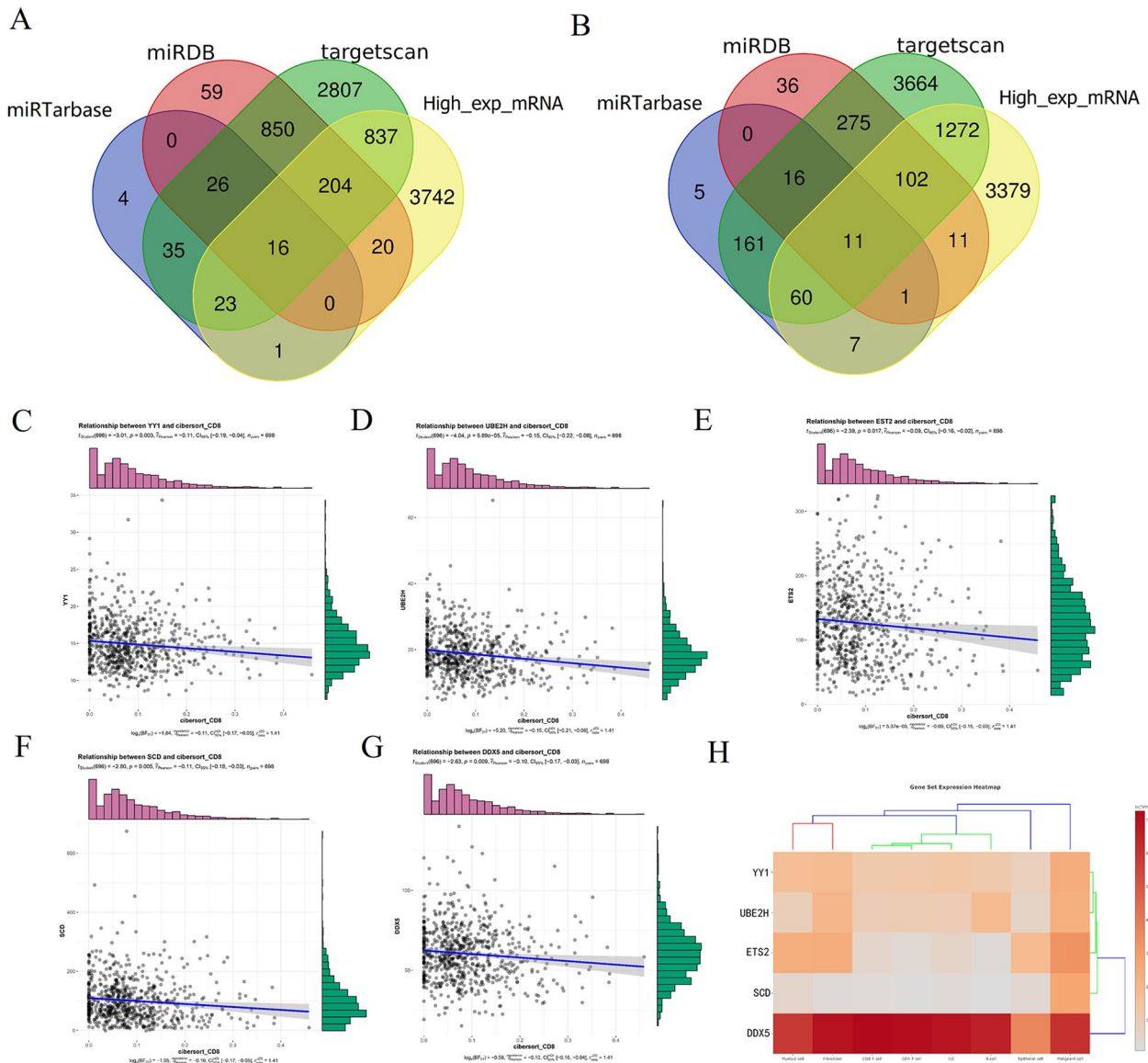
Figure 5. The selection and evaluation of mRNAs. (A, B) The Venn plots showed the selection of microRNA downstream mRNA. The right plot is for hsa-miR-338-5p, and the left plot is for hsa-miR-1224-3p. (C-G) The correlation analysis of CD8+ T cell infiltration and the expression of mRNAs (YY1, UBE2H, ETS2, SCD, DDX5). (H) The heatmap displayed the expression of mRNAs on different types of cells. YY1, UBE2H, ETS2, and SCD were highly expressed in malignant cells (tumor cells).

In addition, immunotherapy and gut microbiome composition had complex interactions.[35] The expression profiles of lncRNAs can effectively distinguish the types of gut microbes.[36] Therefore, lncRNAs have broad and promising application prospects in immunotherapy.

In the current study, we identified three CD8+ T cell infiltration-related lncRNAs: AC092580.4, LINC00657, and USP30-AS1. AC092580.4 and USP30-AS1 showed low expression in CRC and positively associated with CD8+ T cell infiltration, suggesting an anti-tumor effect. In contrast, LINC00657 suggested a tumor promotor effect. In ovarian cancer, the "rs34631313-AC092580.4" pair was related to CD8+ T cell and M1 macrophage infiltration,[37] indicating a tumor suppressor function. There is no research on the function of AC092580.4 in CRC, which is a promising research

direction. USP30-AS1 was reported to play a role in various tumors, such as glioblastoma,[38] acute myeloid leukemia (AML),[39] and so on. USP30-AS1 was upregulated in glioblastoma and could promote the development of glioblastoma by affecting mitochondrial homeostasis.[38] In AML, USP30-AS1 could facilitate the viability and suppress the apoptosis of AML cells via cis-regulating USP30 and ANKRD13A.[39] In addition, in a variety of cancers, USP30-AS1 has been confirmed to participate in the construction of various prognostic signatures, suggesting its potential as a prognostic biomarker in cancers.[14,40,41] In CRC, USP30-AS1 was downregulated and linked to a better prognosis. USP30-AS1 could restrain the progression of CRC via regulating miR-765.[42] Due to the limitation of bulk-seq, the cibersort algorithm could not perfectly identify
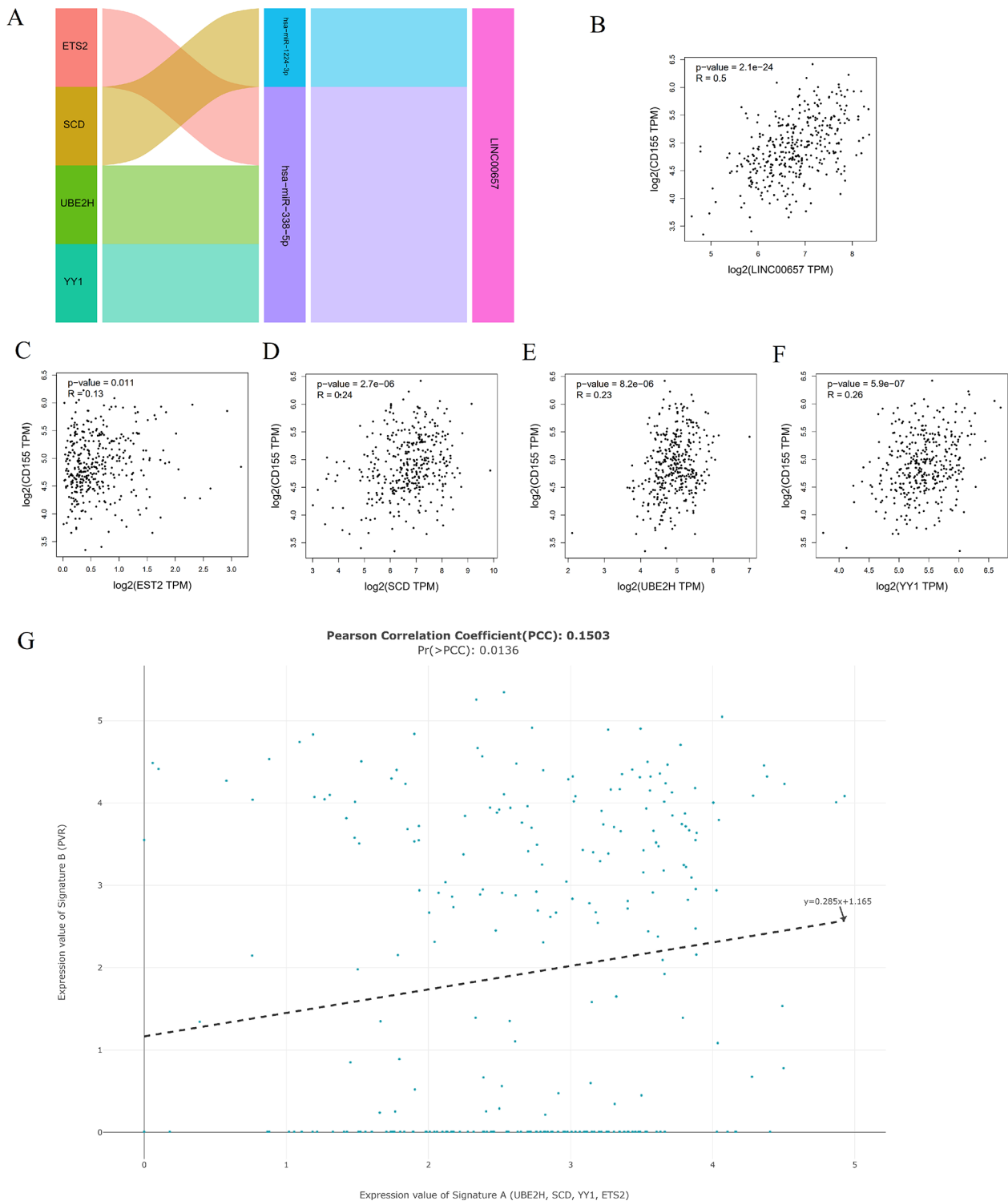
Figure 6. The construction of the ceRNA network. (A) The Sankey diagram showed the interaction of the ceRNA network. (B) The correlation analysis showed the correlation of CD155 and LINC00657. (C-F) The correlation analysis showed the correlation of CD155 and mRNAs in the ceRNA network. (G) The correlation analysis of CD155 and mRNAs signature (YY1, UBE2H, ETS2, SCD) indicated a significant positive correlation.

the expression abundance of lncRNAs in different types of cells in the tumor microenvironment. We used scRNA-seq data to locate the expression of lncRNA accurately. We found that USP30-AS1 and AC092580.4 were primarily expressed on CD8+ T cells, while LINC00657 was mainly expressed on tumor cells. Considering lncRNAs expressed on CD8+ T

cells could result in the false positive of CD8+ T cell infiltration correlation, in current research, we mainly focused on the lncRNA expressed on tumor cells. LINC00657 was also known as NORAD (noncoding RNA activated by DNA damage), which can maintain the stability of genomic.[43] LINC00657 was identified as an oncogene or tumor
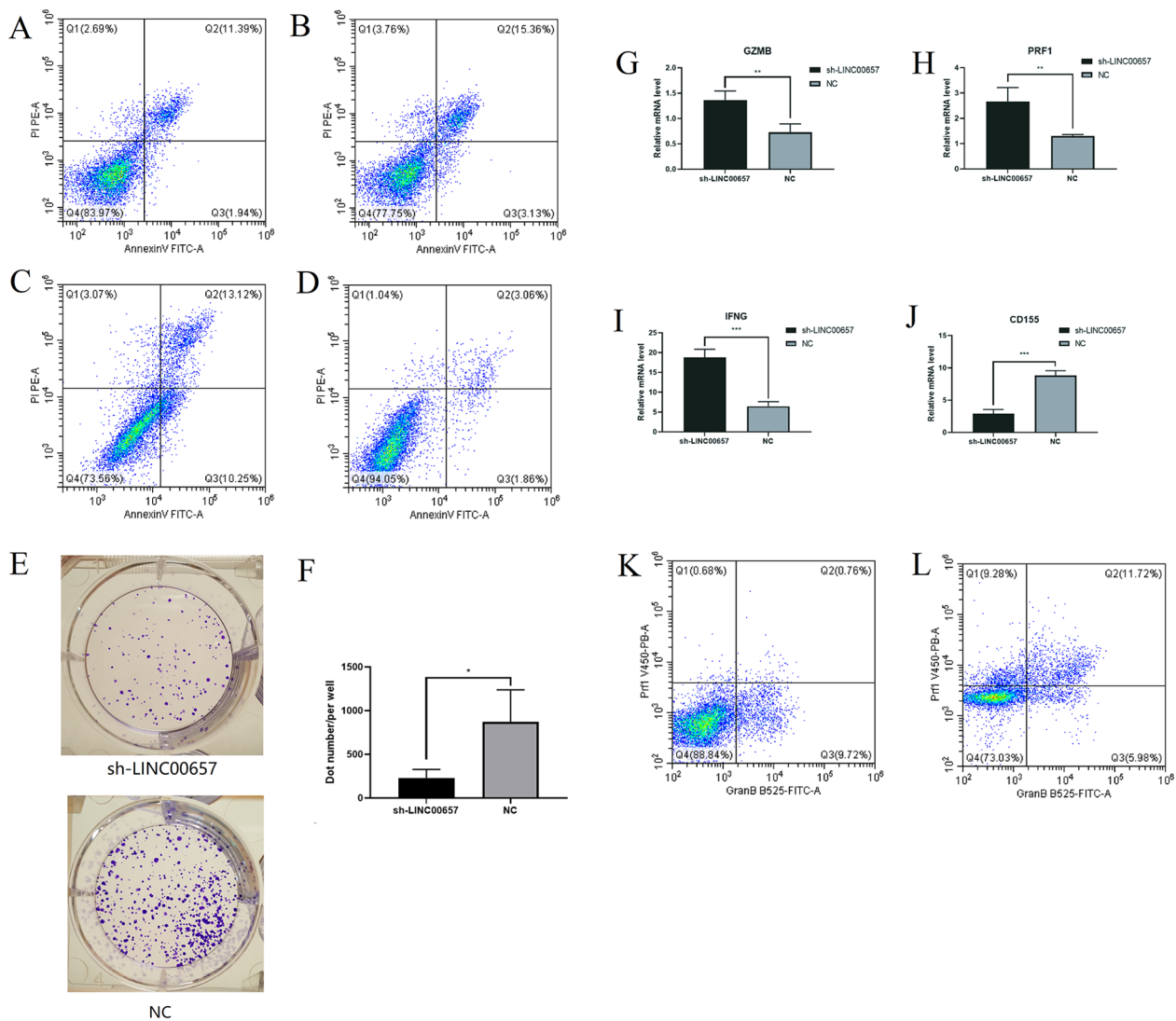
Figure 7. The experimental verification of LINC00657 in the co-culture system. (A) The apoptosis assay of the NC group (HCT116). (B) The apoptosis assay of the sh-LINC00657 group (HCT116). (C) The apoptosis assay of the NC group (CD8+T cell). (D) The apoptosis assay of the sh-LINC00657 group (CD8+ T cell). (E) The colony formation assay of the NC and sh-LINC00657 group. (F) Histogram of clone formation assay. (G-I) The mRNA expression of GZMB, PRF1, and IFNG in sh-LINC00657 and NC group (CD8+ T cells). (J) The mRNA expression of CD155 in sh-LINC00657 and NC group (HCT116). (K) The expression of PRF1 and GZMB in CD8+ T cells by flow cytometry (NC group). (L) The expression of PRF1 and GZMB in CD8+ T cells by flow cytometry (sh-LINC00657 group).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

suppressor gene in different cancers.[44,45] In CRC, it could promote stem-like invasion to promote the development of CRC.[46] However, there is little research on the immunosuppressive function of LINC00657. Interestingly, by analyzing the expression of LINC00657 between MSI-H and MSI-L/ MSS CRC patients, we found LINC00657 was higher in MSI-L/MSS CRC patients. Besides, correlation analysis also showed that LINC00657 was positively associated with MMR protein (MLH1, MSH2, MSH6, and PMS2). We performed tumor cell and CD8+ T cell co-culture experiments to verify our findings. After knockdown of LINC00657, GZMB, IFNG, and PRF1 in CD8+ T cells were upregulated, and CD8+ T cell apoptosis was significantly reduced. To sum up, LINC00657 in colorectal tumor cells could significantly suppress anti-tumor immunity, potentially interacting with MMR protein.

To explore the potential regulatory mechanism of LINC00657, we constructed a LINC00657-derived CD8+ T cell infiltration ceRNA network. hsa-miR-338-5p and hsa-miR-1224-3p were core miRNAs and have not been reported in CRC. There were 4 mRNAs in the ceRNA network (ETS2, SCD, UBE2H, YY1), mainly expressed on CRC tumor cells. Ets-2 deletion mouse could significantly increase the infiltration of CD8+ T cells and decrease T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in a pancreatic cancer mouse model.[47] The upregulation of SCD could promote CRC development,[48] and it was shown upregulated in high relapse risk stage II CRC patients.[49] UBE2H was reported associated with a poor prognosis of lung cancer[50] and pancreatic cancer,[51] while there is no such report on CRC, which deserves further research. In addition, YY1 was proven a tumor promotor in CRC by a ceRNA mechanism.[52]

CD155 (PVR) is a member of adhesion molecules upregulated on various tumor cells, including CRC.[53,54] CD155 on tumor cells could induce the degradation of CD226 (DNAM-1) on CD8+ T cells to resist immunotherapy.[54] In addition, checkpoint receptors TIGIT and CD96 could bind with CD155 to suppress T and NK cells,[55] which could be promising immunotherapy targets, and further clinical studies are warranted. Recent research showed that TIGIT/CD155 could promote immune escape and the progression of CRC, indicating targeting TIGIT/CD155 could be a novel immunotherapy strategy in treating CRC.[23,56] In our ceRNA network, we were pleased to find the expression of LINC00657 was significantly positively correlated with CD155, and the downstream mRNAs (ETS2, SCD, UBE2H, YY1) showed the same trend. To sum up, we speculated that the LINC00657-derived CD8+ T cell infiltration ceRNA network could interact with CD155 to regulate the CD8+ T infiltration and cytotoxic function, and the specific molecular mechanism needed further exploration.

Conclusions

In conclusion, we constructed an lncRNA LINC00657-derived CD8+ T cell infiltration ceRNA network in CRC to predict the potential molecular regulatory relationship using bulk-seq and scRNA-seq data. By establishing a tumor cell and CD8+ T cell co-culture system, we found that LINC00657 could impair CD8+ T cell cytotoxicity, which could be a novel immunotherapy target in CRC.

Author Contributions

DZ and YW designed this study. QW, ZZ, and MJ performed data analysis and prepared the main manuscript. TY and YJ established the co-culture system. YC, JC, and JZ collected the data. DT provided technical support for flow cytometry. All authors reviewed the final manuscript.

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Supplemental Material

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