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Simple Summary: This study explored the dysregulation of long noncoding RNA (lncRNA)-miRNA/mRNA network in circulating CD4+ T cells of patients with hepatocellular carcinoma (HCC) using next-generation sequencing. The study found different expression of 34 transcripts, including two lncRNAs (XIST and MIR222HG), 29 mRNAs, and three other types of RNA and miRNAs in the circulating CD4+ T cells of HCC patients. The expression of the lncRNA XIST-related miRNAs and their target mRNAs was confirmed using real-time quantitative polymerase chain reaction on samples from healthy controls and HCC patients. The dysregulation of the lncRNA-miRNA/mRNA network in HCC might yield novel insights into the etiology or progression of HCC.

Abstract: Hepatocellular carcinoma (HCC) is one of the most common cancers and the main cause of cancer-related death globally. Immune dysregulation of CD4+ T cells has been identified as a role in the development of HCC. Nevertheless, the underlying molecular pathways of CD4+ T cells in HCC are not completely known. Thus, a better understanding of the dysregulation of lncRNA-miRNA/mRNA network might yield novel insights into the etiology or progression of HCC. In this study, circulating CD4+ T cells were isolated from the whole blood of 10 healthy controls and 10 HCC patients for next-generation sequencing of the expression of lncRNAs, miRNAs, and mRNAs. Our data showed that there was different expression of 34 transcripts (two lncRNAs, XIST and MIR222HG, 29 mRNA, and 3 other types of RNA) and 13 miRNAs in the circulating CD4+ T cells of HCC patients. The expression of the lncRNA XIST-related miRNAs and their target mRNAs was confirmed using real-time quantitative polymerase chain reaction (qPCR) on samples from 100 healthy controls and 60 HCC patients. The lncRNA–miRNA/mRNA regulation network was created using interaction data generated from ENCORI and revealed there are positive correlations in the infiltration of total CD4+ T cells and negative correlations in the infiltration of Th1 CD4+ T cells.

Keywords: hepatocellular carcinoma (HCC); CD4+ T cells; next generation sequencing (NGS); long non-coding RNAs (lncRNAs); microRNAs (miRNAs)

1. Introduction

Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer, accounting for 90% of all occurrences of primary liver cancer [1]. HCC is a recognized inflammation-related cancer form.

The immunological microenvironment of the liver plays a crucial role in the etiology of illness [2]. Infection with the hepatitis B virus or the hepatitis C virus [3], persistent alcohol consumption, nonalcoholic steatohepatitis, and exposure to aflatoxin B are all variables that might increase the likelihood of developing hepatocellular carcinoma [4]. When certain infectious pathogens generate persistent inflammation, innate and adaptive immune system modifications influence disease development [5]. Persistent inflammation promotes the development of HCC by the infiltration of diverse immune cells, including dendritic cells, macrophages, natural killer cells, neutrophils, T cells, and B cells [6].

T cells are recognized to have a crucial role in the development of HCC. Among the subtypes of T cells, the dysregulation of the function of CD4+ T cells is emerging as a factor in HCC. By secreting cytokines and activating CD8+ T lymphocytes, CD4+ T cells play a role in antitumor immune responses [7]. Activated CD4+ T cells stimulate CD8+ cytotoxic T lymphocytes (CTLs) via dendritic cells or by directly secreting IL-2. After receiving signals from dendritic cells, CD4+ T cells are activated and polarized to the Th1 phenotype, producing effector cytokines such as IFN and TNF. CD4+ T cells also stimulate the differentiation and maturation of B cells into plasma cells [8]. After specific cytokine signaling and transcription factor expression, CD4+ T cells may differentiate into several subsets, including T helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), follicular helper T cells (Tfh), and T-regulatory cells (Tregs) [9]. The steady decline of CD4+ T lymphocytes is substantially related with increased mortality and a reduced survival time in patients with HCC [10].

Long noncoding RNAs (lncRNAs) play a significant role in a variety of physiological and pathological processes via several regulatory mechanisms [11]. Many evidence points to that abnormal expression of certain lncRNAs in HCC contributes to the progression of the illness [12-14]. Several studies demonstrated that lncRNAs are essential for T cell development, activation, and pathogen response [15-17]. Accumulating evidence indicates that lncRNA and microRNA (miRNA) interact to control gene expression [18, 19]. LncRNAs may act as miRNA sponges or bind to sites on the target genes of miRNAs to reduce the interaction of miRNA and its target mRNA. On the other hand, miRNAs can regulate the stability and half-life of lncRNA by target lncRNA. In addition, some lncRNAs containing miRNA sequences can be cleaved by Dicer and/or Drosha to produce miRNAs [20].

There has been no extensive research of the lncRNA–miRNA/mRNA network of circulating CD4+ T cells in HCC. In this work, circulating CD4+ cells from HCC clinical samples were extracted and compared to those from healthy people. The expression landscape of lncRNAs, miRNAs, and mRNAs is analyzed, and the regulatory network of miRNA–mRNA/ lncRNA is created.

2. Materials and Methods

2.1. Study Patient Population

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (IRB number: 201900911B0), and all participants provided written informed consent. Blood samples were taken from patients with or without HCC. We employed 10 healthy controls and 10 HCC patients in our NGS study. The demographics of these twenty people are presented in Supplemental Table 1. To validate the expression of discovered genes, one hundred samples of healthy controls and sixty samples of HCC were utilized. The demographics of these individuals from the validation cohort are given in Supplemental Table 2.

Supplemental Table 1. The clinical data of 20 people who participated in the NGS assessment of differential mRNA, lncRNA, and miRNA gene expression.

	Health (n = 10)	HCC (n = 10)
Age (years, mean ± SD)	36.70±7.32	66.40±10.84
Gender		
Male, n (%)	1 (10%)	8 (80%)
Female, n (%)	9 (90%)	2 (20%)

Supplemental Table2. The clinical data of 160 individuals who had their transcript expression validated using real-time qPCR.

	Health (n = 100)	HCC (n = 60)
Age (years, mean ± SD)	38.19±8.50	65.40±10.03
Gender		
Male, n (%)	24 (24%)	48 (80%)
Female, n (%)	76 (76%)	12 (20%)

2.2. Isolation of Circulating CD4+ Cell and RNA Extraction

PBMC were isolated using density gradient centrifugation and Ficoll-PaqueTM PREMIUM solution (GE Healthcare, 17544202). Anti-human CD4 Particles - DM magnetic nanoparticles (BD IMagTM, 557767) were used to isolate CD4+ cells according to the manufacturer's procedure. The collected CD4+ cells were lysed using 700 ul of QIAzol Lysis Reagent, and RNA was extracted using the miRNeasy Mini Kit (QIAGEN, 217004) per the manufacturer's instructions. Then, 50 l RNase-free water was added to the RNA-isolated samples.

2.3. RNA Quality Determination and NGS

The extracted RNA samples were quantified using a NanoDrop 2000 (Thermo Scientific) spectrophotometer and a Qubit 2.0 Fluorometer (Thermo scientific). Qubit / NanoDrop ratio (1±0.3 assign as well) and Caliper RNA Quality Score (RQS) adopting Caliper LabChip GX Electrophoresis System were utilized to evaluate RNA quality (Caliper Life Sciences). Using the TruSeq® Stranded Total RNA Sample Preparation Instructions, cDNA libraries were constructed (Illumina, 15031048). Equal samples from each library were pooled and sequenced with a NextSeq 500. (Illumina).

For RNA-Seq, the row sequences were assessed for quality using FastQC v0.11.8. and trimmed for primer-adaptor sequences using the RNA-seq alignment tool from BaseSpace (Illumina), followed by alignment to the human reference genome (hg38) with STAR v2.7.3a. The expression levels and differential expression analysis were estimated using DESeq2 v1.24.0. (selection criteria: adjusted P value of <0.05 and |fold change| > 2).

For miRNA-Seq, the sequences were assessed for quality and adapter trimming using Trim_Galore v. 0.4.4, followed by alignment to the human reference genome (hg38) from UCSC with bwa v. 0.7.15. The expression profiles were produced using BCGSC miRNA Profiling Pipeline and differential expression analysis were estimated using DEseq2 v. 1.16.1.

2.4. GO and KEGG Enrichment Analysis

Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed using the R package clusterProfiler (version 3.18.1) to detect the enrichment of signaling pathway. The GO analysis included analyses of biological processes, cellular components, and molecular functions. GO and KEGG terms with p < 0.05 were considered significantly enriched by differentially expressed genes (DEGs).

2.5. Real-Time Quantitative Polymerase Chain Reaction

For measuring the expression of mRNAs and lncRNAs, the transcripts were reverse transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). 7500 Real-Time PCR System and Power SYBR Green PCR Master Mix (ABI 4367659) were utilized for gene expression investigation (Applied Biosystems). The sequences of the primers used are shown in Supplemental Table 3.

For miRNA, the transcripts were reverse transcribed to cDNA by TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, A28007). TaqMan Advanced miRNA Assay (Applied Biosystems, A25576) and TaqMan Fast Advanced Master Mix were utilized for the miRNA expression study (Applied Biosystems, 444557).

2.6. The Interaction of miRNA-lncRNA and miRNA-mRNA

The Encyclopedia of RNA Interactomes (ENCORI; https://starbase.sysu.edu.cn/; vers. 3.0) was used to obtain information on the miRNA-lncRNA or miRNA-mRNA interaction [21]. ENCORI is an open-source platform for researching the interactions between miRNAs and their targets. At least three miRNA-target prediction databases, including PITA, miRmap, and TargetScan, were utilized to identify the targeted miRNAs of candidate genes in the present work.

Supplemental Table 3. The primers sequences used in real-time quantitative polymerase chain reaction.

Targeted genes	Order	Sequences
XIST	forward	5'-ACGCTG CATGTGTCCTTAG-3'
	reverse	5'-GAGCCT CTTATAAGCTGTTTG-3'
ID1	forward	5'-GCAGGTAAACGTGCTGCTCTACGA-3'
	reverse	5'-CGAGTTCAGCTCCAACTGAAGGTC-3'
IL-1β	forward	5'-TCCATGTCCTTTGTACAAGGAGAAGAAAGT-3'
	reverse	5'-TCGCTTTTCCATCTTCTTCTTTGGGTAAT-3'
ANXA3	forward	5'-ACTGAGATCCTGTGTTTAAGGAGCTT-3'
	reverse	5'-GGCTCGATGCAGTCTTTCGG-3'
PER1	forward	5'-GCCTCGGTACCAGCCATTCC -3'
	reverse	5'-GGTGTGTGCCGCGTAGT-3'
FN1	forward	5'-CCAGCCTACGGATGACTC-3'
	reverse	5'-AATGACCAC TGCCAAAGC-3'

2.7. CD4$^+$ T Cells Infiltration Analysis

The association between CD4$^+$ T cells infiltrates and differential express gene was evaluated using TIMER2.0 website (http://timer.cistrome.org/), which provides robust estimation of immune infiltration levels using website algorithms, including TIMER, EPIC, xCell, CIBERSORT, and quanTIseq [22].

2.8. Statistical Analysis

The Windows version of GraphPad Prism 5 was used for all statistical tests (version 5.01). The data were provided as a mean ± standard error (SEM). The Mann–Whitney U test was used to conduct the pairwise comparisons, and the resulting p values were then shown. A value of P 0.05 was used to indicate statistical significance in all two-tailed tests.

3. Results

3.1. NGS Analysis

RNA-Seq analysis of circulating CD4$^+$ cells from 10 HCC patients vs. 10 health control showed that the expression of 34 transcripts were significantly differently expressed in the circulating CD4+ T cells. Two of the 34 transcripts were lncRNAs (XIST and MIR222HG), 29 were mRNAs, and three were of other types of RNAs (Table 1). Among these two lncRNAs, MIR222HG was up-regulated and XIST was down-regulated in HCC patients. Among the 29 mRNAs, 12 were up-regulated and 17 was down-regulated in HCC patients (Table 1). The GO analysis revealed that the biological processes of 34 differentially expressed genes (DEGs) were predominantly associated with leukocyte-mediated immunity, myeloid leukocyte activation, neutrophil activation, granulocyte activation, and humoral immune response (Figure 1A). The cellular component of these DEGs was engaged in granule lumen and vesicle lumen. The molecular function of these DEGs was involved in glycosaminoglycan binding, immunoglobulin binding and MHC protein complex binding. The results of the KEGG pathway enrichment analysis showed that DEGs were mostly implicated in the primary immunodeficiency and B cell receptor signaling pathways (Figure 1B).

The miRNA-seq study revealed that the expression of 13 miRNAs, one of which was up-regulated and twelve of which were down-regulated, was significantly different between HCC patients and healthy controls (Table 2). The predicted target genes of these miRNAs with differential expression are reported in Table 4. The GO analysis revealed that these miRNA target genes are crucial to tissues development. Protein activity was associated with the biological function of these miRNA target genes. KEGG pathway enrichment result for these predicted target genes was involved in TGF−β signaling pathway, cellular senescence, protein processing in endoplasmic reticulum, spliceosome and several signaling pathway (Figure 2).

Table 1. Differential expression transcripts from NGS analysis in the patients with HCC compared with healthy controls.

Genes	Fold change (log2)	P-adj	Chromosome	Type
RPS9	-7.13	0.0090	chr19	protein_coding
TAP2	-7.09	0.0104	chr6	protein_coding
LILRB1	-5.71	0.0020	chr19	protein_coding
XIST	-3.08	0.0001	chrX	lncRNA
SNORD3A	-2.95	0.0000	chr17	snoRNA
PCDH9	-2.76	0.0163	chr13	protein_coding
FCRL5	-2.15	0.0001	chr1	protein_coding
IGHM	-2.04	0.0006	chr14	IG_C_gene
GATA2	-1.98	0.0000	chr3	protein_coding
MS4A2	-1.89	0.0194	chr11	protein_coding
FCRL2	-1.73	0.0125	chr1	protein_coding
TCL1A	-1.70	0.0000	chr14	protein_coding
MS4A1	-1.45	0.0000	chr11	protein_coding
CD79A	-1.40	0.0442	chr19	protein_coding
LRRN3	-1.28	0.0079	chr7	protein_coding
LGALS12	-1.27	0.0471	chr11	protein_coding
FCRL1	-1.20	0.0389	chr1	protein_coding
PTPRS	-1.16	0.0156	chr19	protein_coding
RETN	-1.13	0.0021	chr19	protein_coding
AEBP1	-1.12	0.0184	chr7	protein_coding
U2AF1	1.03	0.0064	chr21	protein_coding

PER1	1.03	0.0000	chr17	protein_coding
IL1B	1.32	0.0000	chr2	protein_coding
G0S2	1.36	0.0119	chr1	protein_coding
RN7SL1	1.46	0.0061	chr14	misc_RNA
MIR222HG	1.64	0.0473	chrX	lncRNA
MAFF	2.15	0.0000	chr22	protein_coding
CEACAM8	2.54	0.0021	chr19	protein_coding
CTSG	2.67	0.0079	chr14	protein_coding
ID1	2.86	0.0000	chr20	protein_coding
ANXA3	2.89	0.0104	chr4	protein_coding
MMP8	3.03	0.0021	chr11	protein_coding
CAMP	3.15	0.0015	chr3	protein_coding
FN1	3.76	0.0471	chr2	protein_coding

Table 2. Differential expression miRNAs from NGS analysis in HCC patients compared with healthy controls.

| miRNAs	Fold change (log2)	P-adj
hsa-miR-34a-5p	1.8277	2.96E-07
hsa-miR-11400	-2.3323	5.06E-03
hsa-miR-495-3p	-1.4377	8.24E-05
hsa-miR-369-3p	-1.3807	4.40E-04
hsa-miR-493-5p	-1.3633	1.75E-03
hsa-miR-889-3p	-1.2994	3.01E-02
hsa-miR-493-3p	-1.2379	6.44E-03
hsa-miR-494-3p	-1.2308	7.77E-03
hsa-miR-370-3p	-1.2273	1.10E-05
hsa-miR-411-5p	-1.1649	3.01E-02
hsa-miR-379-5p	-1.1397	2.53E-03
hsa-miR-654-3p	-1.1235	6.53E-03
hsa-miR-543	-1.0776	1.43E-03

Figure 1A.
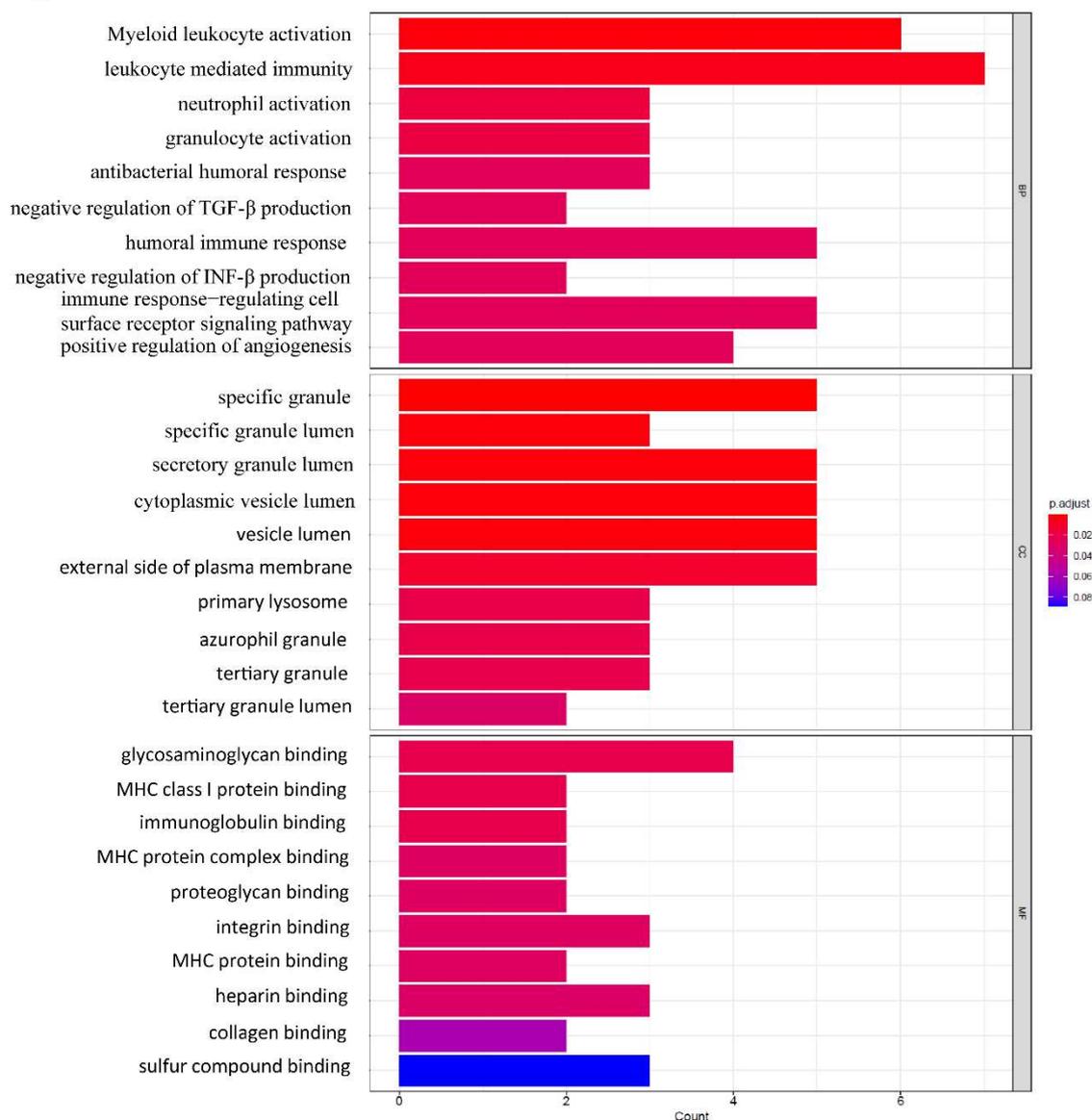
Figure 1B.
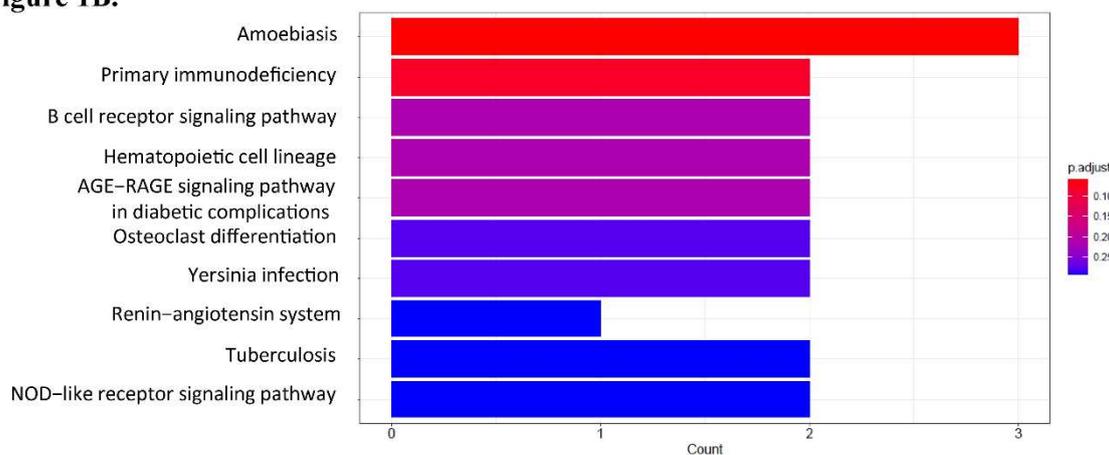
Figure 1. Functional enrichment analysis of differentially expressed genes in CD4+ T cells of HCC. (A) GO terms and (B) KEGG pathways of 34 differentially expressed genes.
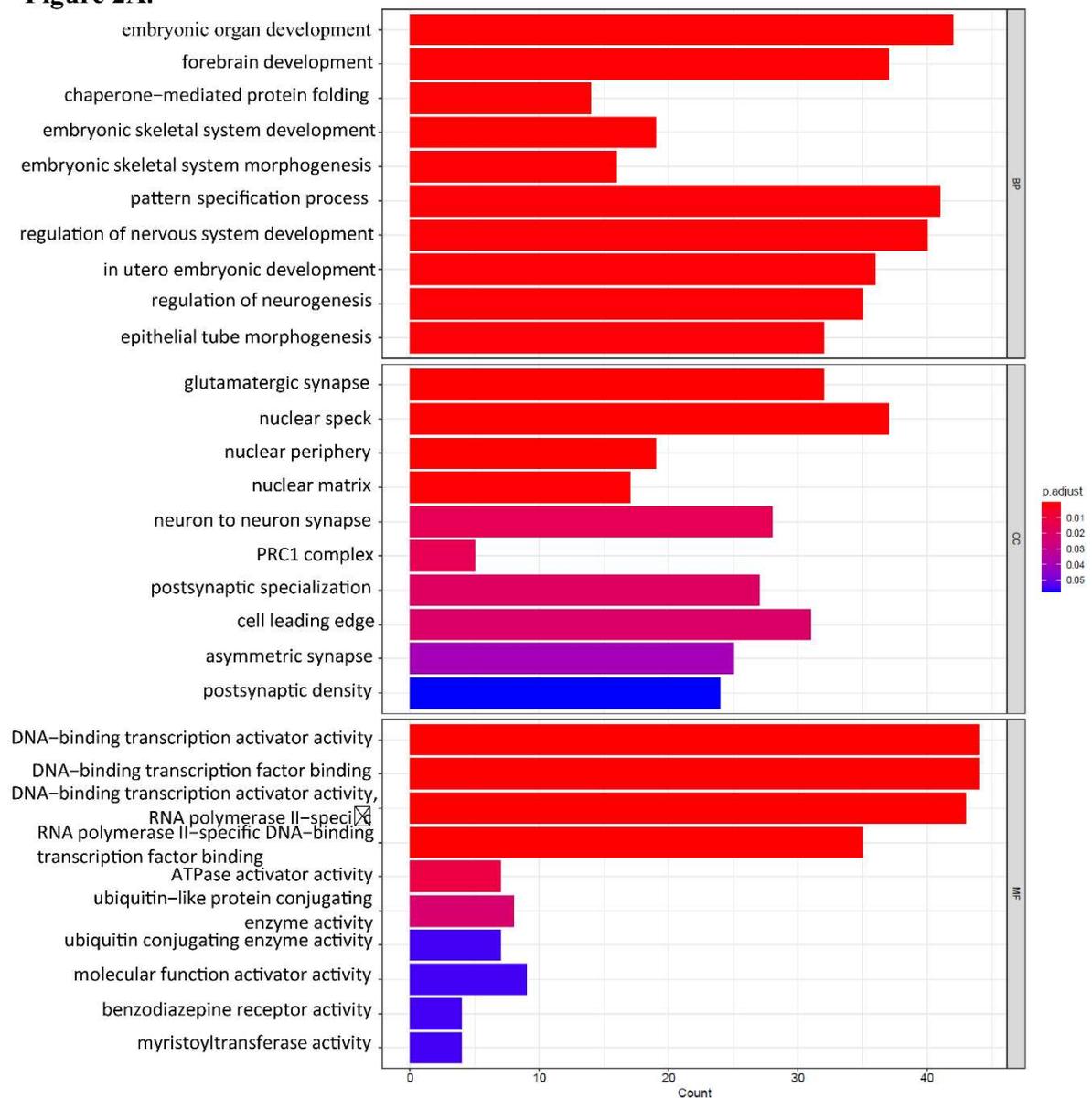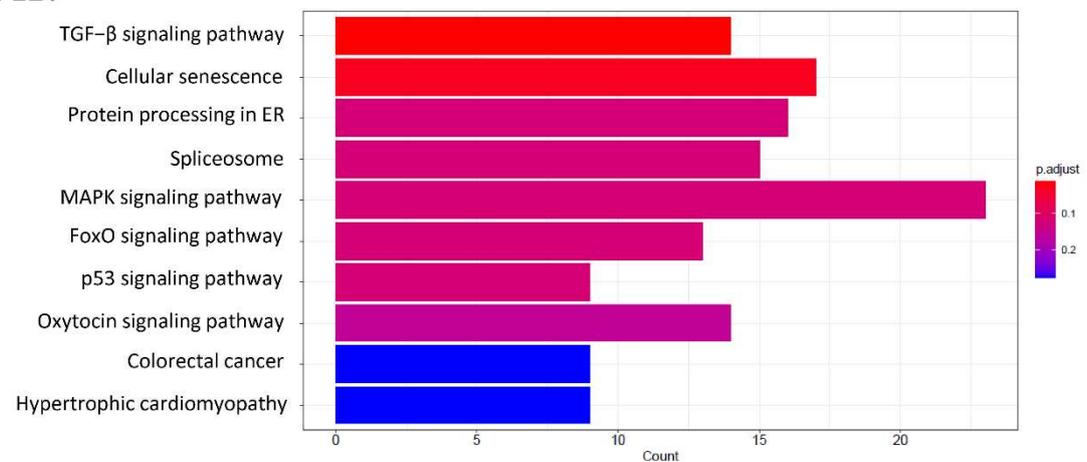
Figure 2A.

Figure 2B.

Figure 2. Functional enrichment analysis of predicted target genes of differentially expressed miRNA in CD4+ T cells of HCC. (A) GO terms and (B) KEGG pathways of predicted target genes of differentially expressed miRNA.

3.2. The miRNA-lncRNA or miRNA-mRNA Interaction by ENCORI

ENCORI was used to study the interaction between miRNA and lncRNA as well as miRNA and mRNA. Based on ENCORI analysis, there are 8 of 13 differential expression miRNAs to XIST (Table 3), including hsa-miR-34a-5p, hsa-miR-369-3p, hsa-miR-370-3p, hsa-miR-494-3p, hsa-miR-495-3p, hsa-miR-543, hsa-miR-654-3p and hsa-miR-889-3p. However, there is no differential expression miRNAs be related to MIR222HG. The target mRNAs of eight XIST-related miRNAs predicted by ENCORI were identified (Supplemental Table 4) and compared to their expression level from the DEGs in this study (Table 4). Due to the limited number of isolated CD4+ T cells, only those up-regulated mRNAs that are related with down-regulated miRNAs were chosen for further real-time qPCR validation in this work. Five mRNAs were identified: Inhibitor of DNA binding 1 (ID1), Interleukin 1 beta (IL1β), Annexin A3 (ANXA3), Period circadian regulator 1 (PER1), and Fibronectin 1 (FN1).

Table 3. XIST-related miRNAs predicted by ENCORI.

miRNAs	LncRNA	Target sequence
hsa-miR-34a-5p	XIST	gcugaCACAUACAUACACUGCCu
hsa-miR-369-3p	XIST	acauuuuugaaAAGUAUUAUu
hsa-miR-369-3p	XIST	accacccccugAUGUAUUAUu
hsa-miR-369-3p	XIST	uuAGAUUGA-CA-GUAUUAUg
hsa-miR-370-3p	XIST	ugaccacugcugggCAGCAGGa
hsa-miR-370-3p	XIST	gagagcugaguCUUCAGCAGGu
hsa-miR-370-3p	XIST	cuuucuUUUCCUCCCAGCAGGg
hsa-miR-370-3p	XIST	ccccuuUUGCAGUACAGCAGGg
hsa-miR-370-3p	XIST	cccuuuUUGCAGUACAGCAGGg
hsa-miR-370-3p	XIST	ugcccuUUGCAGUACAGCAGGg
hsa-miR-370-3p	XIST	cuuccuUUCCCUUCCAGCAGGg
hsa-miR-370-3p	XIST	ccccuuUUGCAGUACAGCAGGg
hsa-miR-370-3p	XIST	ucaccuUUCCCUUCCAGCAGGg
hsa-miR-370-3p	XIST	ccacuuUUCCCUUCCAGCAGGa
hsa-miR-370-3p	XIST	ccuccuuggCAAAGCAGCAGGa
hsa-miR-370-3p	XIST	uauuccUUUUGUCUUACAGCAGGg
hsa-miR-370-3p	XIST	cucccuUUGUAUUCCAGCAGGg
hsa-miR-370-3p	XIST	ccccauUUGCA-UUCAGCAGGg
hsa-miR-494-3p	XIST	cucugcaagguacUAUGUUUCg
hsa-miR-494-3p	XIST	ugucUUACCCAUUUCCAUGUUUCu
hsa-miR-495-3p	XIST	uaaAAGGGCUCAGGGUUUGUUc
hsa-miR-543	XIST	caaGACUGUCACUUGGAAUGUUc
hsa-miR-654-3p	XIST	--------------CAGACAUu
hsa-miR-654-3p	XIST	acuucccucaggAGCAGACAUu
hsa-miR-889-3p	XIST	cuucUGGUAGAGUGGGAUAUUAu
hsa-miR-889-3p	XIST	uuuuacagcaaggGAUAUUAa

Table 4. miRNA-mRNA interaction predicted by ENCORI.

miRNA	Predicted target genes	Expression data in the NGS analysis
hsa-miR-34a-5p	PER1	up-regulated
hsa-miR-34a-5p	IL1B	up-regulated
hsa-miR-369-3p	MS4A1	down-regulated
hsa-miR-369-3p	PCDH9	down-regulated
hsa-miR-369-3p	ANXA3	up-regulated
hsa-miR-370-3p	PER1	up-regulated
hsa-miR-494-3p	MS4A1	down-regulated
hsa-miR-494-3p	PCDH9	down-regulated
hsa-miR-494-3p	ID1	up-regulated
hsa-miR-495-3p	PCDH9	down-regulated
hsa-miR-495-3p	IL1B	up-regulated
hsa-miR-495-3p	ID1	up-regulated
hsa-miR-543	PCDH9	down-regulated
hsa-miR-543	FN1	up-regulated
hsa-miR-543	GATA2	down-regulated
hsa-miR-654-3p	MS4A1	down-regulated
hsa-miR-654-3p	ID1	up-regulated
hsa-miR-889-3p	LILRB1	down-regulated
hsa-miR-889-3p	ID1	up-regulated
hsa-miR-889-3p	GATA2	down-regulated

3.3. Validation of the Expression of Transcripts

Real-time qPCR was used to determine the expression levels of XIST, eight XIST-related miRNAs, and their target mRNAs in 160 validation samples (100 health control and 60 HCC samples). Figure 3A demonstrates that miR-34a-5p was substantially up-regulated while miR-369-3p, miR-494-3p, miR-495-3p, and miR-543 were significantly down-regulated in HCC patients using real-time qPCR. These results are consistent with those disclosed by NGS. Nevertheless, the expression levels of three (miR-370-3p, miR-654-3p, and miR-889-3p) of the eight miRNAs associated with XIST were either modest or undetectable.

We then measure the identified potential target mRNAs utilizing real-time qPCR. These mRNAs were chosen based on the intersection of the target mRNAs of the aforementioned substantially downregulated miRNAs and those presenting increased expression in the NGS experiments. Five mRNAs (ID1, IL1β, ANXA3, PER1, and FN1) were selected for the real time qPCR experiment. ID1, which is a target for miR-494-3p, miR-495-3p, miR-654-3p, and miR-889-3p, was significantly up-regulated in HCC patients. IL1β as a target for miR-34a-5p and miR-495-3p, ANXA3 as a target of miR-369-3p, PER1 as a target for miR-34a-5p and miR-370-3p, and FN1 as a target for miR-543 had significant upregulation in HCC patients than the healthy subjects (Figure 3B).

Given that XIST is a sex-linked lncRNA, the amount of XIST expression for male and female was detected separately by real-time qPCR analyses. XIST expression was considerably suppressed in male HCC patients but not in female HCC patients (Figure 3C). In this study, the expression levels of XIST-related miRNAs and their regulated mRNAs were evaluated in male and female HCC patients. There were no remarkable difference regarding the expression of miRNAs and the regulated mRNAs between male and female HCC patients (Supplemental Figure S1 and S2).
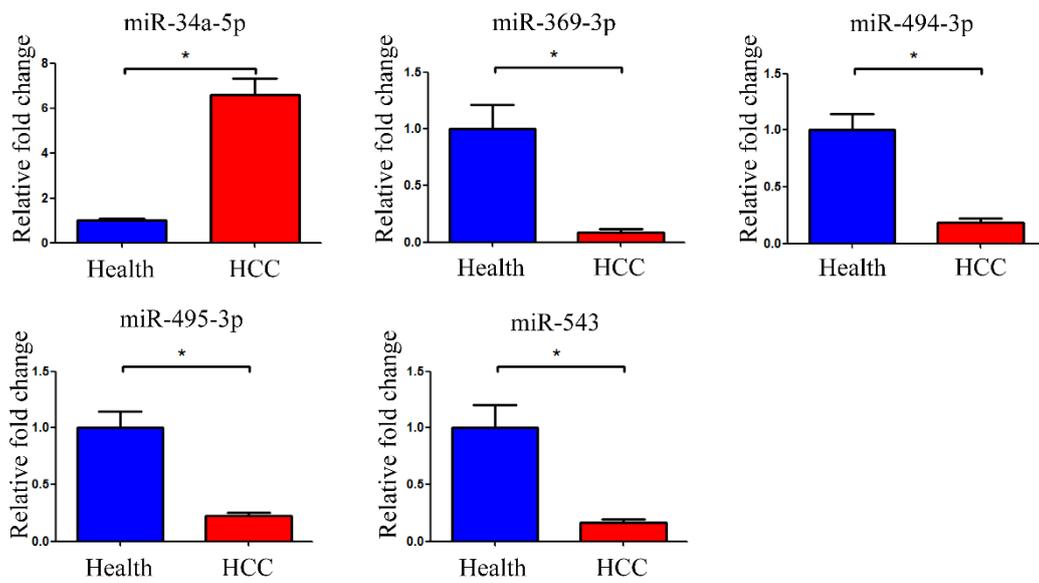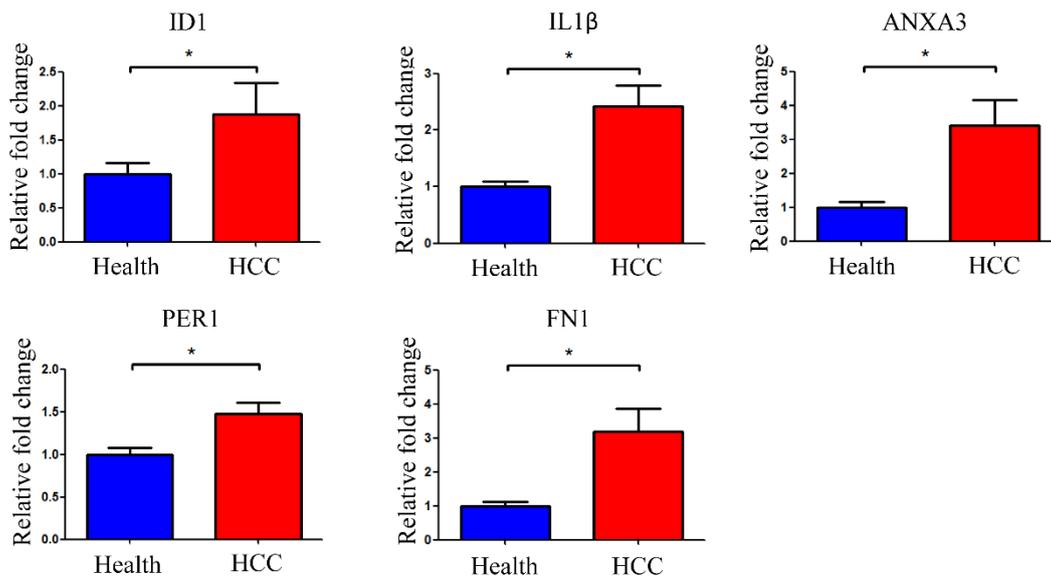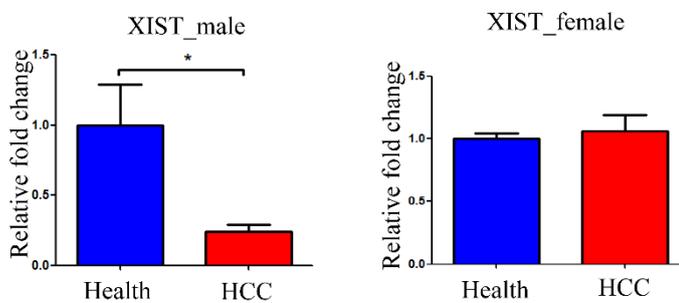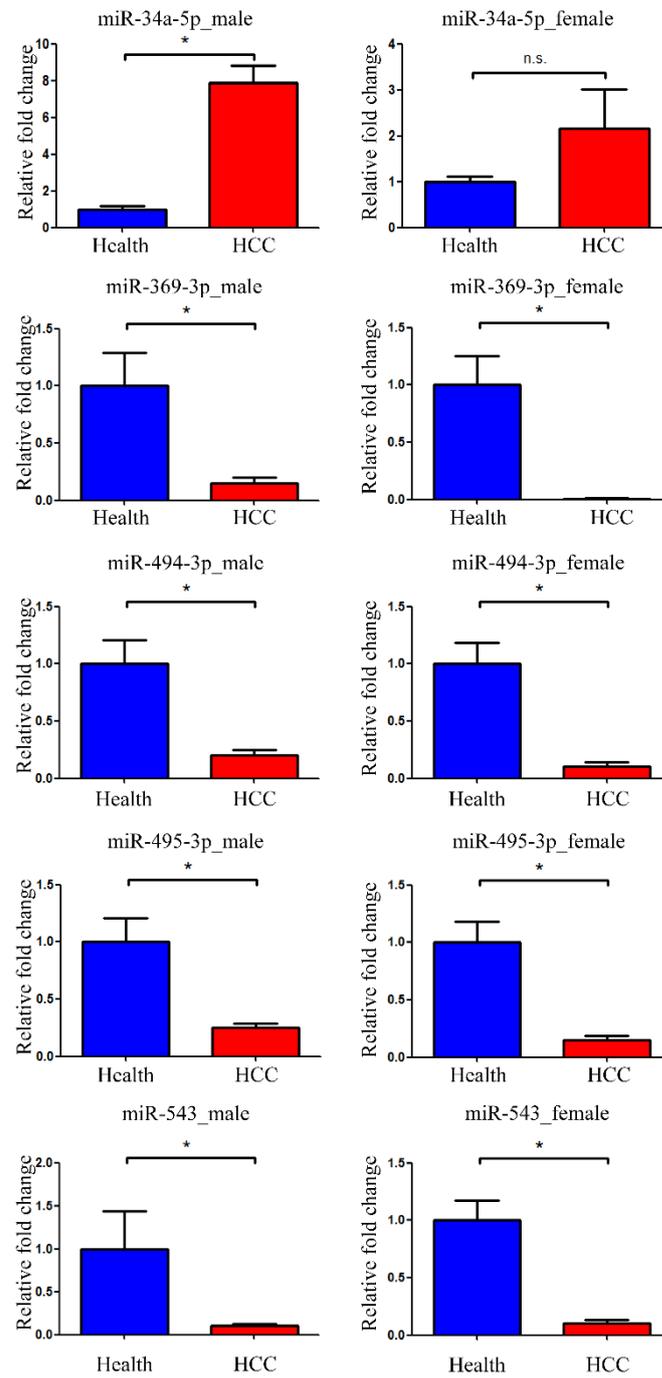
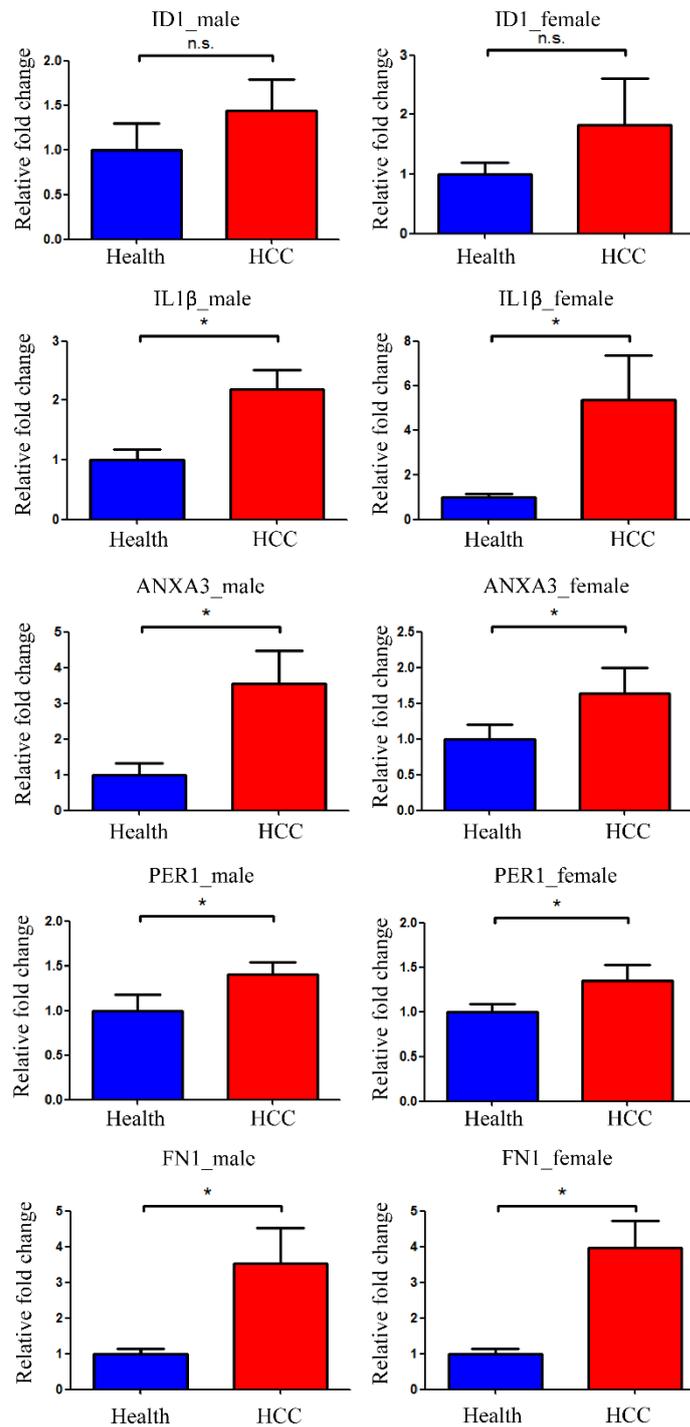**Figure 3A.**

Figure 3B.

Figure 3C.

Figure 3. miRNA / mRNA / lncRNA expression level verification. Quantification of (A) miRNAs, (B) differentially expressed miRNA-targeted mRNAs and (C) XIST expression by real-time qPCR.

Supplemental Figure S1.

Supplemental Figure 1. The expression of XIST-related miRNAs in circulating CD4^{+} T cells of male and female patients with HCC versus healthy controls.

Supplemental Figure S2.

Supplemental Figure 2. The expression of the regulated mRNA target genes of XIST-related miRNAs in circulating CD4+ T cells of male and female patients with HCC versus healthy controls.

3.4. Analysis of CD4+ T Cells Infiltrates Correlation

Using the TIMER2.0 website, we investigated the link between the expression of miRNA target genes and the amount of CD4+ T cell infiltration in HCC. The findings revealed a strong correlation between the expression of these miRNAs' target genes and the infiltration of total CD4+ T cells, particularly resting memory CD4+ T cells (Figure 4). Nevertheless, there was a negative correlation

between the infiltration of Th1 CD4+ T cells and the expression of miRNAs' target genes.3.2. Figures, Tables and Schemes

Figure 4.
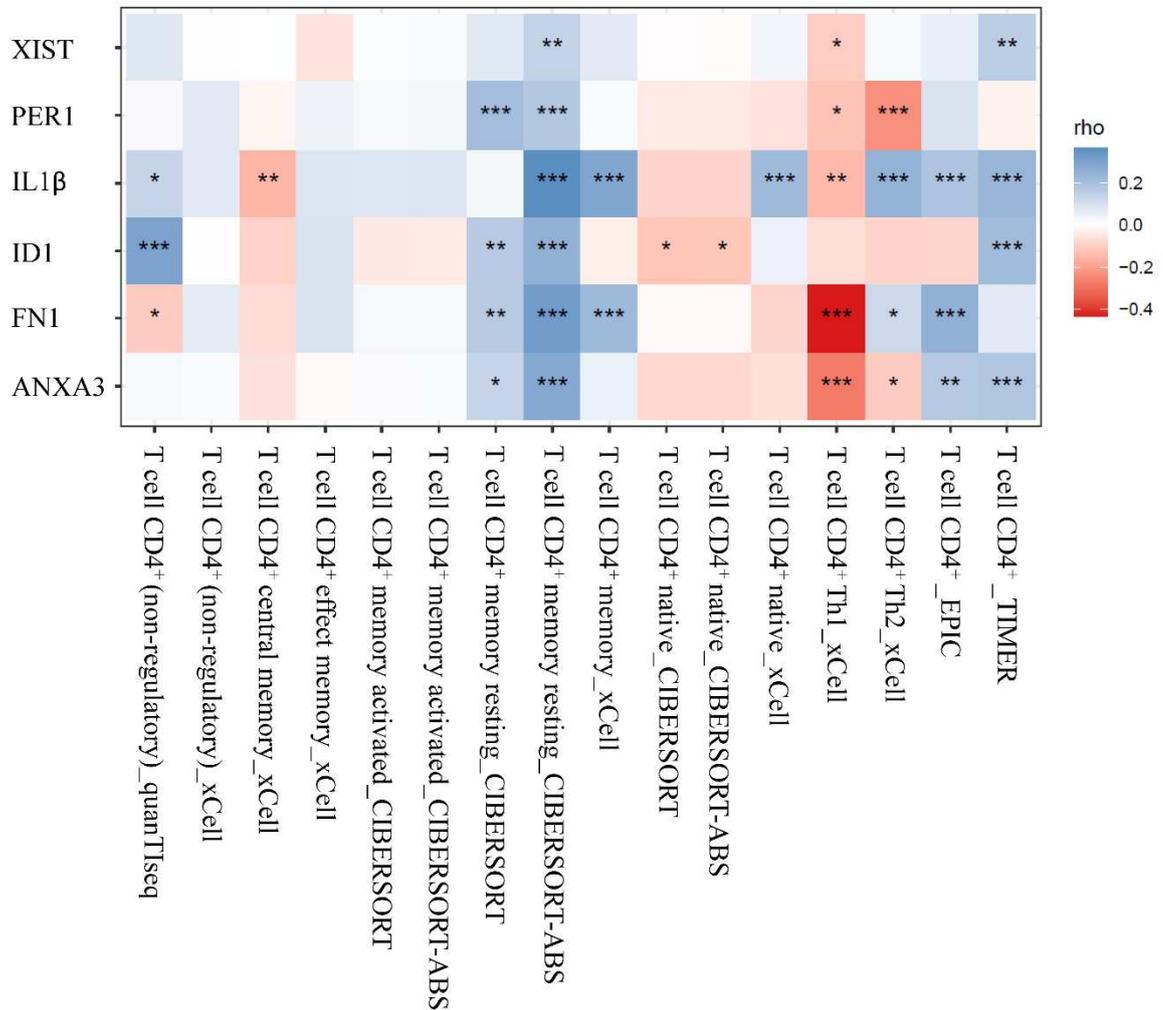
Figure 4. Analysis of CD4⁺ T cells infiltrates correlation. The correlation between the expression of miRNA-targeted genes and the level of CD4⁺ T cells infiltration in HCC. (* indicated P < 0.05; ** P < 0.01; and *** P < 0.001).

4. Discussion

In this investigation, we discovered circulating CD4+ T cells with differently expressed lncRNAs, miRNAs, and mRNAs in HCC patients. These transcripts are involved in the activation of numerous kinds of white blood cells, including myeloid leukocyte, neutrophil, and granulocyte. A XIST-related lncRNA–miRNA/mRNA network, which contains five miRNAs (hsa-miR-34a-5p, hsa-miR-369-3p, hsa-miR-494-3p, hsa-miR-495-3p, hsa-miR-543) and five miRNA target genes (ID1, IL1β, ANXA3, PER1 and FN1), was rebuilt. This regulatory network associated with XIST may be positively connected with the infiltration of CD4+ T cells, particularly resting memory CD4+ T cells, but negatively correlated with Th1 CD4+ T cells. Our finding indicated that the regulation of circulating CD4+ T cells in HCC patients differs from that of healthy individuals.

XIST plays a role in each stage of X-chromosome inactivation. XIST recruits chromatin complexes that deposit heterochromatic alterations throughout the X, including H3K27me3 and ubiquitin-H2A, leading in transcriptional silence [23]. In SLE patients, XIST was predominantly up-

regulated in activated T cell subsets and was associated by more skewed X-chromosome allelic expression [24]. Mature nave T lymphocytes exhibit distributed patterns of XIST, but the inactive X lacks epigenetic changes. Activation of mature T cells restores the dormant X gene's XIST transcripts and epigenetic changes [25]. The expression levels of XIST-related miRNAs and their regulated mRNAs did not change significantly between male and female HCC patients, despite the fact that XIST is a sex-linked lncRNA.

Regarding the XIST-related miRNAs found in this investigation, miR-34a was revealed to target 14 essential components of the NF-κB signaling pathway and to function as a major regulator of NF-κB in T cells [26]. The rise of miR-34a correlates with CD4+ and CD8+ T cell activation [27]. Targeting CXCR3, miR-34a-5p inhibits CXCR3 expression in CD4+ and CD8+ T cells. High levels of miR-34a-5p in naïve CD4+ T cells inhibit Th1 cell polarization by downregulating CXCR3, leading in diminished activation of cytotoxic T lymphocytes, natural killer cells, and natural killer T cells, as well as possible lymphopenia [28]. In addition, miR-369-3p regulates the inflammatory response as the signaling core. The upregulation of miR-369-3p inhibits the LPS-induced inflammatory response, reduces the release of TNF, IL-6, IL-12, IL-1, and IL-1, and increases the production of anti-inflammatory cytokines IL-10 and IL-1RA [29]. Nuclear translocation of NF-κB is likewise reduced by miR-369-3p [29]. In viral infections, inhibiting the expression of miR-369-3p dramatically reduces the expression of antiviral response genes, including CCL3, CCL5, CTSS, CXCL11, and IRF3 [30]. In addition, miR-494-3p has a function in macrophage polarization and activation via activating Wnt signaling to modulate M1/M2 polarization [31].

The appearance of T cell types may play a crucial role in the development of HCC. For instance, it has been observed that the ratio of Th1 to CD4+ T cells is greater in HCC patients than in healthy controls, and that patients with a higher tumor-infiltrating Th1 cell count had a considerably longer life time (26). In addition, the decrease of CD4+ T cells is linked to a poor prognosis and a high rate of HCC recurrence (10). ID1-expressing CD4+ cells greatly boosted IL-2 production and NF-B activity in response to anti-CD3 stimulation in order to improve proliferation and survival of naïve CD4+ cells (23). IL-1 promotes the proliferation and development of antigen-specific CD4+ T cells toward a proinflammatory Th phenotype via the production of IFN-, IL-17, TNF-, and IL-4 (24,25). Th1 cells have strong ANXA3 expression (26). In the stress hormone signaling pathway, the elevation of PER1 expression inherently suppresses Th1 polarization in naïve CD4+ T cells (27). Local manipulation of FN1 levels may be advantageous for boosting T cell concentration and is essential for T cell interstitial migration (28). These cytokines may contribute to communication between immune cells and affect CD4+ T cell development. Despite indications suggesting these miRNAs are involved in the regulation of T cells, further research is required to determine the effect of the XIST-miRNAs/mRNAs network on the function of circulating CD4+ T cells in HCC.

5. Conclusions

In this work, the circulating CD4+ cells of HCC patients exhibited down-regulation of XIST, dysregulation of five XIST-related miRNAs, and up-regulation of five miRNA target genes, including ID1, IL1β, ANXA3, PER1, and FN1. The explored XIST-miRNA/mRNA network might play a crucial role in controlling the function of T cells.

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