Decreased Expression of Programmed Death Ligand-L1 by Seven in Absentia Homolog 2 in Cholangiocarcinoma Enhances T-cell–mediated antitumor activity

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Supplement Materials and Methods

Patients and tissue specimens

The study protocol was approved by the clinical research ethics committee of the Eastern Hepatobiliary Surgery Hospital and Changhai Hospital. Written informed consent was obtained from all patients according to the policies of the committee. Any information that identifies patients was not included in this article. This study included 21 CCA tissues for N6-methyladenosine RNA immunoprecipitation, quantitative RT-PCR, and RT-PCR. The clinical characteristics of the HCC cohort are listed in **Table S4 (cohort-1)**. Additional 20 CCA tissues were evaluated by Western blotting and the clinical characteristics of the CCA cohort are listed in **Table S4 (cohort-2)**. Primary cells from two CCA patients' tumors were cultured as previously reported (1). All CCA specimens were obtained immediately after hepatectomy. Tissues were then fixed in 10 % buffered formalin and embedded in paraffin. Fresh specimens used in this study were snap-frozen from tissues prior to formalin fixation, transferred to liquid nitrogen, and stored at −80 °C, until use.

Cell culture and pharmacologic drug treatment

RBE is from National Laboratory Cell Resource Sharing Center (Beijing, China). HUCCT1 is from JCRB Cell Bank (Japanese Collection of Research Bioresources). Cells were cultured at 37 ºC in an atmosphere of 5% CO_2 in RPMI-1640 (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% FBS (GIBCO) and 1% penicillin–streptomycin solution (GIBCO). The genetic identity of RBE and HUCCT1 were confirmed by short tandem repeat profiling. Cell lines are routinely tested for Mycoplasma contamination every three months.

Cycloheximide (CHX, protein synthesis inhibitor; MCE, catalog no. HY-12320), MG132 (proteasome inhibitor; Selleck, Shanghai, China, catalog no. S2619) and actinomycin D (an inhibitor of DNA transcription and replication; MCE, catalog no. HY-17559) were used at a final concentration of 10 mg/mL, 50 mmol/L, and 5 mg/mL, respectively. 3-Deazaadenosine (a global methylation inhibitor; MCE, catalog no. HY-W013332) was used at a final concentration of 0 to 50 mmol/L. Pembrolizumab (anti-

PD-L1 blockade antibody; Selleck, catalog no. A2004) or control IgG (BioXcell, West Lebanon, NH, USA; catalog no. BE0297) was used at 10 mg/mL for cell treatment.

N6-methyladenosine RNA immunoprecipitation quantitative RT-PCR of fragmented mRNA assay

Enrichment of m6A-modified mRNA was performed as previously reported (2,3). Immunoprecipitated m6A modified mRNA and“input”mRNA were subjected to quantitative RT-PCR with indicated primers. Fold enrichment was determined as the ratio of m6A modified mRNA to“input”mRNA.

Experimental animal models

For in vivo tumor growth assays, indicated treated CCA cells were subcutaneously injected at 2×10^6 cells per mouse into nude mice. Xenografted tumor growth was monitored as previously reported (4).

Co-Immunoprecipitation (IP)

For transient transfection and co-immunoprecipitation experiments, CCA cells (1×10^6) were transfected for 48 h. Transfected cells were lysed in 0.5 mL of the lysis buffer. For each immunoprecipitation, a 0.4 mL aliquot of the lysate was incubated with 4 μL of the indicated antibody or control IgG overnight at 4 ℃, after which 20 μL of magnetic beads (Millipore, Billerica, MA, USA) were added for additional 2 h. Precipitates were then analyzed by standard Western blot procedures. The anti- primary antibody was listed in **Table S2**.

In vitro T-cell killing assay

In vitro T-cell killing assay was performed as previously reported (5). Pre-activated HPBMC were co-cultured with tumor cells. After incubation, the viability of tumor cells was measured by the Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) and apoptosis of tumor cells was detected by the Annexin VPE Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China; catalog no. C1065S).

Supplement reference

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Supplement Table 1. shRNA-lentivirus seed sequences.

Name	**Seed sequence（5‘-3’）**
sh-NC	GAAGGTCGGAGTCAACGGATTC
sh-METTL14#1	GAAGATATTTGTTGGATTAAAC
sh-METTL14#2	ACATTGACTTAATTATCACAGC
sh-YTHDF2#1	GCAAAACAGCAACCTAAACTGC
sh-YTHDF2#2	CTACTCTGAGGACGATATTCAC
sh-SIAH2#1	GTCATGTTTTGGCCATCACTTC
sh-SIAH2#2	AACCTTGGAATCAATGTTACTC

Supplement Table 2. Primary antibodies.

Antigens	Manufacturer	Application
FTO	Abcam	For WB 1:1000
METTL3	Abcam	For WB 1:1000
METTL14	Abcam	For WB 1:1000
ALKBH5	Abcam	For WB 1:1000
ACTIN	Abcam	For WB 1:10000
Siah2	Abcam	For WB 1:1000
YTHDF2	Abcam	For WB 1:1000
PD-L1	Abcam	For WB 1:1000
Anti-Ubiquitin (Linkage-specific K48)	Abcam	For WB 1:10000
Flag	Abcam	For WB 1:1000
Anti-Ubiquitin (Linkage-specific K63)	Abcam	For WB 1:1000
Anti-Ubiquitin (Linkage-specific K48)	Abcam	For WB 1:1000
Siah2	Abcam	For IHC 1:50
PD-L1	Abcam	For IHC 1:50

Supplement Table 3. Quantitative RT-PCR primers.

Primer name	Property	Sequence
Siah2	Forward (5′-3′)	5′-TAACCAATGCCGCCAGAAGT-3′
	Reverse (5′-3′)	5′-CCCGTGGTGGCATACTTACA-3′
PD-L1	Forward (5′-3′)	5′-TGGCATTTGCTGAACGCATTT-3′
	Reverse (5′-3′)	5′-TGCAGCCAGGTCTAATTGTTTT-3′
ACTIN	Forward (5′-3′)	5′-TGACGGGGTCACCCACACTG-3′
	Reverse (5′-3′)	5′-AAGCTGTAGCCGCGCTCGGT-3′
PRF1	Forward (5′-3′)	5′-GGCTGGACGTGACTCCTAAG-3′
	Reverse (5′-3′)	5′-CTGGGTGGAGGCGTTGAAG-3′
GZMB	Forward (5′-3′)	5′-CCCTGGGAAAACACTCACACA-3′
	Reverse (5′-3′)	5′-GCACAACTCAATGGTACTGTCG-3′
GNLY	Forward (5′-3′)	5′-CAGGCTCCCTGCCCATAAAA-3′
	Reverse (5′-3′)	5′-CTCAAGGCCTGGGTTGCC-3′
IFN	Forward (5′-3′)	5′-TCGGTAACTGACTTGAATGTCCA-3′
	Reverse (5′-3′)	5′-TCGCTTCCCTGTTTTAGCTGC-3′
YTHDF2	Forward (5′-3′)	5′-AGCCCCACTTCCTACCAGATG-3′
	Reverse (5′-3′)	5′-TGAGAACTGTTATTTCCCCATGC-3′

Supplement Table 4. Clinicopathological characteristics of CCA patients in the study cohort.

Clinicopathological Feature		**cohort-1**	**cohort-2**
		21	**20**
Gender	Male	15	13
	Female	6	7
Age, Year	≥60	17	15
	<60	4	5
Tumor size，cm	≥5	6	7
	<5	15	13
Tumor number	Multiple	4	5
	single	17	15
Lymph node metastasis	Absent	15	14
	Present	6	6
Vascular invasion	Absent	18	17
	Present	3	3
CEA, μg/L	≥ 5	10	11
	< 5	10	9
CA199, U/ml	≥ 40	16	12
	< 40	5	8
CA125, U/ml	≥ 35	9	10
	< 35	12	10
AFP，μg/L	≥ 25	15	15
	< 25	6	5