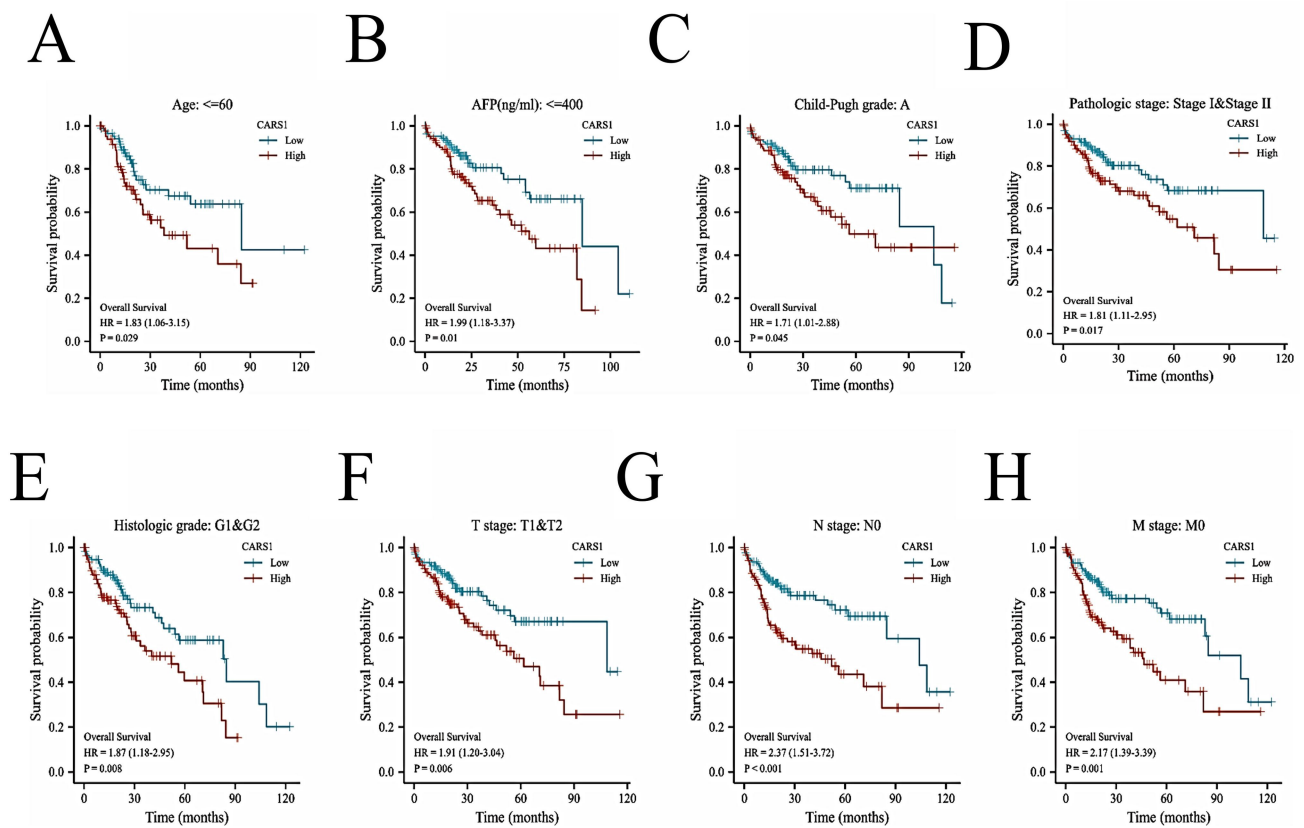


Supplementary Materials

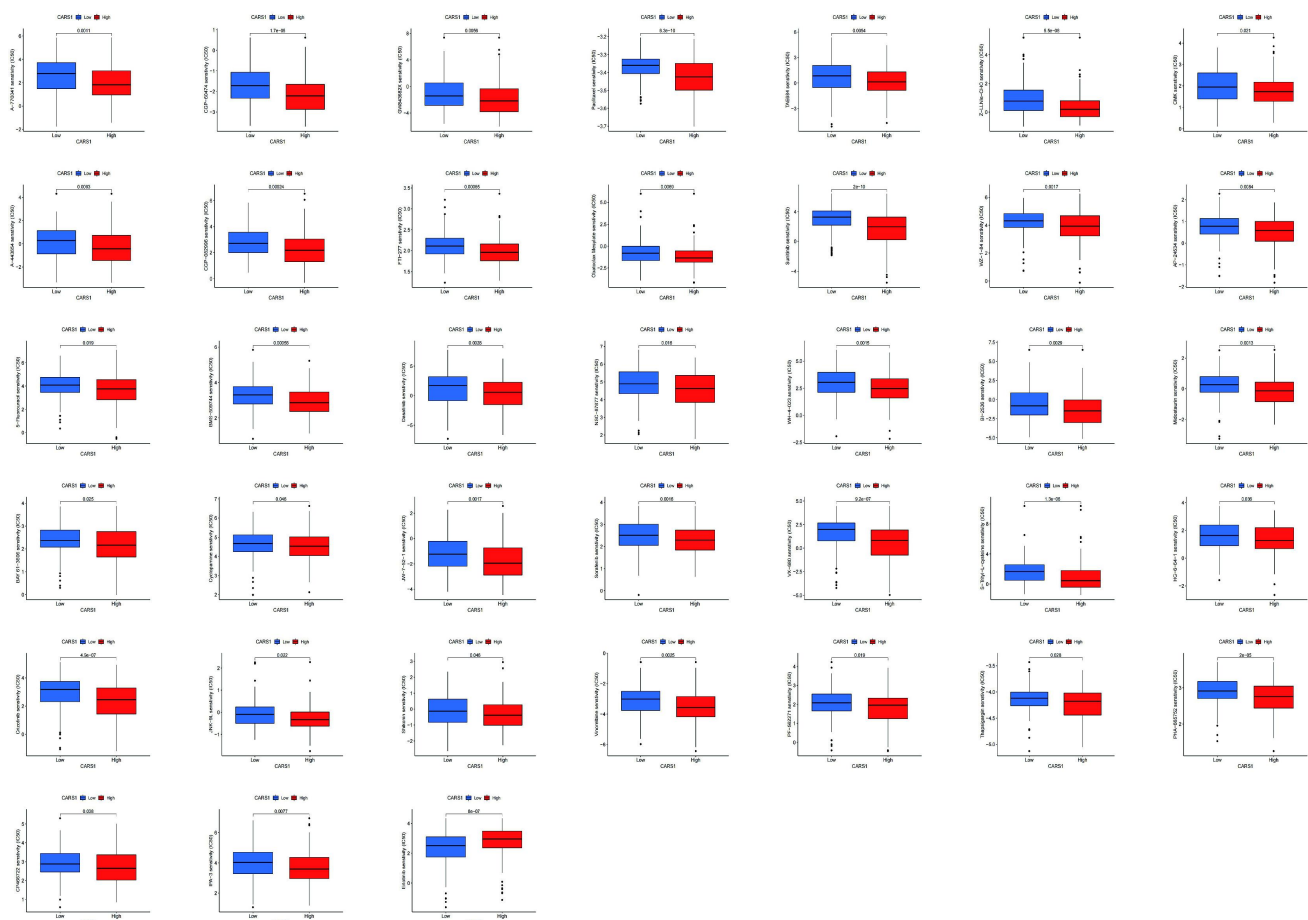
CARS1 as a Prognostic Biomarker and Candidate Therapeutic Vulnerability in Hepatocellular Carcinoma: Insights into Tumor Progression and the Immune Microenvironment

Supplementary Fig.1.



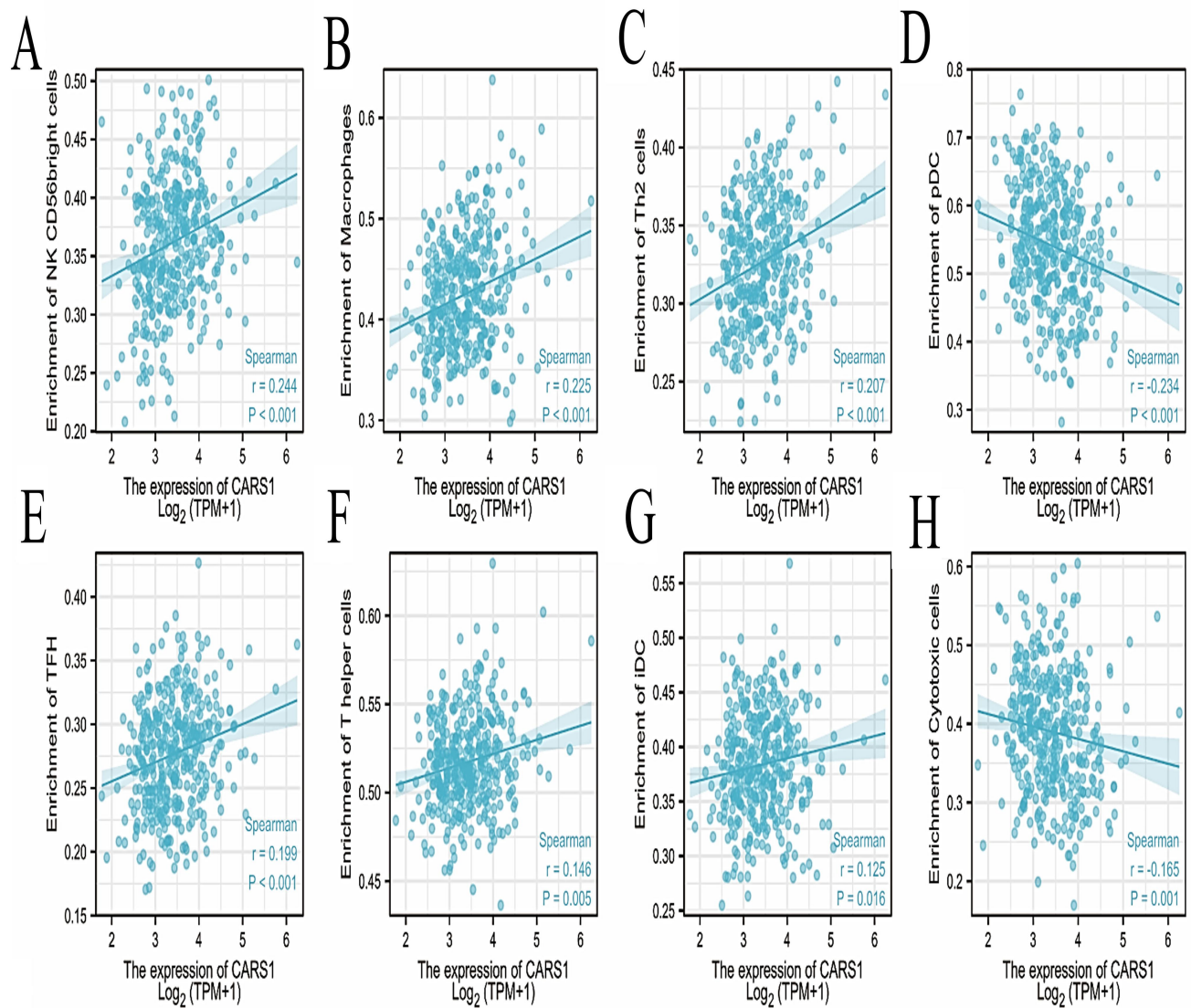
Supplementary Fig.1. Subgroup overall-survival analyses according to CARS1 expression in TCGA-LIHC. Kaplan-Meier curves compare overall survival between the CARS1-low and CARS1-high groups among patients aged ≤ 60 years (A), with alpha-fetoprotein ≤ 400 ng/mL (B), Child-Pugh class A (C), pathological stage I–II (D), histological grade G1–G2 (E), T stage T1–T2 (F), N0 disease (G), and M0 disease (H). Hazard ratios, 95% confidence intervals, log-rank P values, and censoring marks are shown in the corresponding panels.

Supplementary Fig. 2.



Supplementary Fig. 2. Predicted drug sensitivity according to CARS1 expression in TCGA-LIHC. Box plots compare pRRophetic-predicted half-maximal inhibitory concentration (IC50) values between CARS1-low (blue) and CARS1-high (red) tumors for the indicated compounds. TCGA-LIHC FPKM data were transformed as $\log_2(\text{FPKM} + 1)$, and group differences were evaluated using two-sided Wilcoxon rank-sum tests. The erlotinib comparison is shown in the final panel. Higher predicted IC50 values indicate lower predicted drug sensitivity.

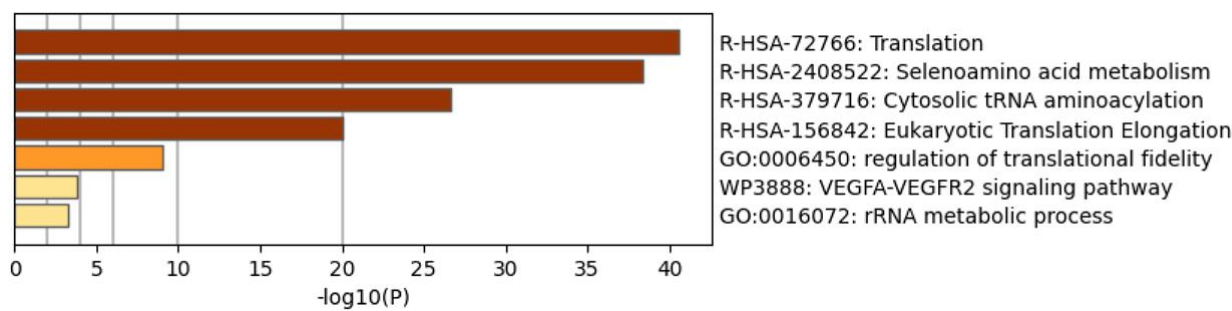
Supplementary Fig.3.



Supplementary Fig. 3. Correlations between CARS1 expression and immune-cell enrichment scores in TCGA-LIHC.

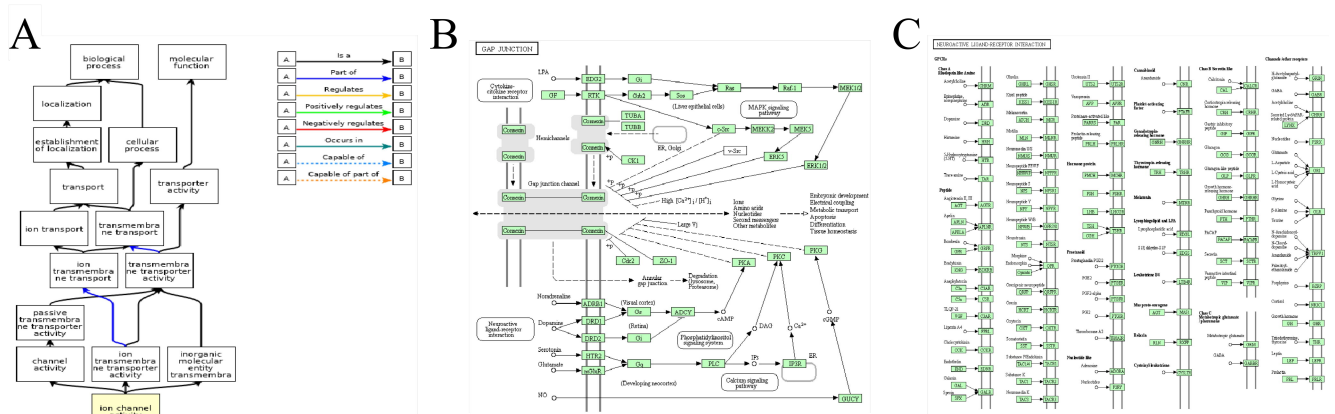
Spearman correlations are shown for NK CD56bright cells (A), macrophages (B), Th2 cells (C), plasmacytoid dendritic cells (D), follicular helper T cells (E), T-helper cells (F), immature dendritic cells (G), and cytotoxic cells (H). Shaded bands indicate the 95% confidence intervals around the fitted regression lines.

Supplementary Fig. 4.



Supplementary Fig. 4. Functional enrichment of the CARS1-centered STRING interaction network. The bar chart summarizes the most significantly enriched Reactome, Gene Ontology, and WikiPathways terms among proteins included in the CARS1-centered interaction network. These analyses identify candidate functional processes and do not demonstrate direct downstream regulation by CARS1.

Supplementary Fig. 5.



Supplementary Fig. 5. Expanded functional annotations and representative pathway maps derived from CARS1 co-expression analysis. (A) Hierarchical Gene Ontology representation of ion-transport-related terms. (B) KEGG gap-junction pathway map. (C) KEGG neuroactive ligand–receptor interaction pathway map. These database-derived diagrams illustrate enriched functional contexts and do not establish direct downstream regulation by CARS1.

Supplementary Tables

Supplementary Table 1. Clinicopathological data for the TCGA-LIHC cases included in each analysis (provided as a separate Excel file).

Supplementary Table 2. De-identified clinicopathological data, four-tier CARS1 immunohistochemical expression scores, and derived CARS1-low/high categories for the 60-patient institutional HCC cohort.

Cell Line Authentication – STR Profiling Report

Sample Type: Cell Line

Testing Type: STR

Sample code:

Table 1. Sample Code

Company Code	Cell line name
1711	MHCC97-H

Sample Number:1

Sample Type: Cell line

Testing Type: STR

Sample From: Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd.

Testing Method:

DNA was extracted by a commercial kit from CORNING (AP-EMN-BL-GDNA-250G). Twenty short tandem repeat (STR) loci plus the gender determining locus, Amelogenin, were amplified by six multiplex PCR and separated on ABI 3730XL Genetic Analyzer. The signals were then analyzed by the software GeneMapper.

Data Interpretation:

Cell lines were authenticated using Short Tandem Repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization (SDO) and in Capes-Davis et al.,

Match criteria for human cell line authentication: Where do we draw the line? Int J Cancer.

2013;132(11):2510-9.

Test Results:

1. Result

Table 2. Matching information on the cell lines

Sample Code	Multi-allele	Cell line matched	Cell Bank	EV
1711	NO	MHCC97-H	EXPASY	0.94

- Multi-allele means some STR contain more than two loci.

2. Sample Description

1711:

A. The STR results showed that no multiple alleles were found in this cell line, and no cross contamination of human cells was found in the cell line.

B. The DNA of the cell lines found to match the type of cell lines in a cell line retrieval, EXPASY database shows that cells called MHCC97-H, corresponding to the cell number CVCL 4972.

3. Genotyping Result

Table 3. STR and Amelogenin Genotyping Results of Cell line 1711

Loci	Sample information			Cell Bank information		
	Sample name: 1711			Cell line name: MHCC97-H		
	Allele1	Allele2	Allele3	Allele1	Allele2	Allele3
D5S818	12	13		12	13	
D13S317	8	8		8	11.3	
D7S820	10	10		10	10	
D16S539	12	12		12	12	

Certificate of STR Analysis

VWA	14	14	14	14
TH01	9	9	9	9
AMEL	X	Y	X	Y
TPOX	8	8	8	8
CSF1PO	11	13	11	13
D12S391	18	18		
FGA	21	24		
D2S1338	20	20		
D21S11	31.2	31.2		
D18S51	13	22		
D8S1179	12	13		
D3S1358	15	16		
D6S1043	12	20		
PENTAE	17	17		
D19S433	13	14		
PENTAD	8	9		
D1S1656	11	16		

The allele match algorithm compares the 8 core loci plus amelogenin only, even though alleles from all loci will be reported when available.

Others:

1. Genotyping Strategy and Site Distribution

Attached Table. Experimental Strategy and Sites

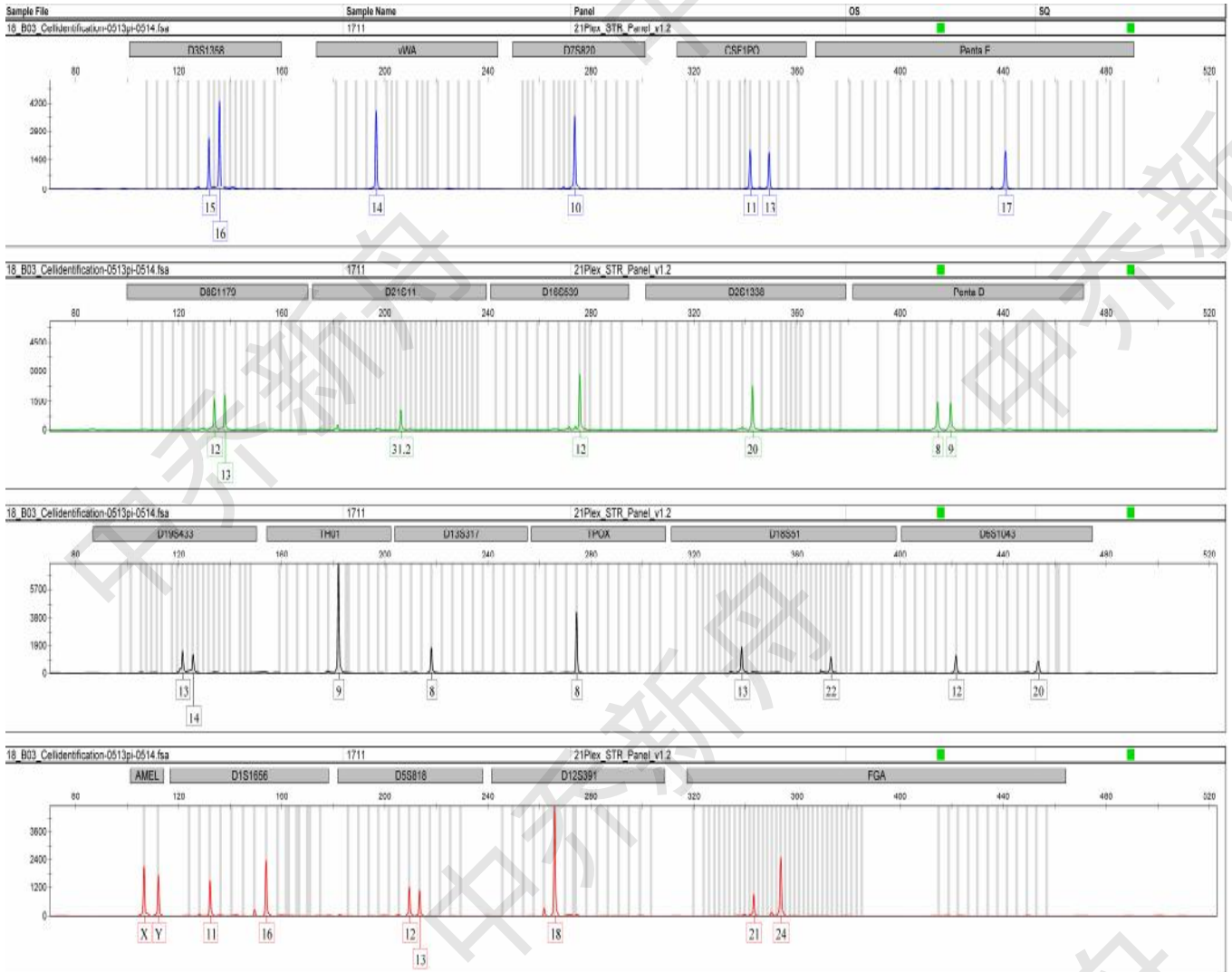
	Strategy 1	Strategy 2	Strategy 3	Strategy 4
1	D3S1358	D8S1179	D19S433	AMEL
2	VWA	D21S11	TH01	D1S1656
3	D7S820	D16S539	D13S317	D5S818
4	CSF1PO	D2S1338	TPOX	D12S391
5	PENTAE	PENTAD	D18S51	FGA
6	D6S1043			

2. DSMZ tools was used to carry on the cell line comparison, which contains 2455 cell lines STR data from ATCC, DSMZ, JCRB, ECACC, GNE and RIKEN databases. If the cell is not included in the above cell library, users need to compared with other databases.



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Certificate of STR Analysis



Cell Line Authentication – STR Profiling Report

Sample Type: Cell Line

Testing Type: STR

Sample code:

Table 1. Sample Code

Company Code	Cell line name
1861	HEP 3B2.1-7

Sample Number:1

Sample Type: Cell line

Testing Type: STR

Sample From: Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd.

Testing Method:

DNA was extracted by a commercial kit from CORNING (AP-EMN-BL-GDNA-250G). Twenty short tandem repeat (STR) loci plus the gender determining locus, Amelogenin, were amplified by six multiplex PCR and separated on ABI 3730XL Genetic Analyzer. The signals were then analyzed by the software GeneMapper.

Data Interpretation:

Cell lines were authenticated using Short Tandem Repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization (SDO) and in Capes-Davis et al., Match criteria for human cell line authentication: Where do we draw the line? Int J Cancer. 2013;132(11):2510-9.

Test Results:

1. Result

Table 2. Matching information on the cell lines

Sample Code	Multi-allele	Cell line matched	Cell Bank	EV
1861	YES	HEP 3B2.1-7	EXPASY	0.94

- Multi-allele means some STR contain more than two loci.

2. Sample Description

1861:

A. The STR results showed that multiple alleles were found in this cell line, and no cross contamination of human cells was found in the cell line.

B. The DNA of the cell lines found to match the type of cell lines in a cell line retrieval, DSMZ database shows that cells called HEP 3B2.1-7, corresponding to the cell number HB-8064.

3. Genotyping Result

Table 3. STR and Amelogenin Genotyping Results of Cell line 1861

Loci	Sample information			Cell Bank information		
	Sample name: 1861			Cell line name: HEP 3B2.1-7		
	Allele1	Allele2	Allele3	Allele1	Allele2	Allele3
D5S818	13	13		13	13	
D13S317	12	14		12	14	
D7S820	8	8		8	10	
D16S539	10	10		10	10	

VWA	17	17	17	17
TH01	6	7	6	7
AMEL	X	X	X	X
TPOX	9	9	9	9
CSF1PO	8	8	8	8
D12S391	17	17		
FGA	18	18		
D2S1338	21	25		
D21S11	30	31		
D18S51	20	20		
D8S1179	12	12		
D3S1358	15	15		
D6S1043	12	17		
PENTAE	5	16		
D19S433	12.2	14		
PENTAD	12	14		
	16.3	17	17.3	

The allele match algorithm compares the 8 core loci plus amelogenin only, even though alleles from all loci will be reported when available.

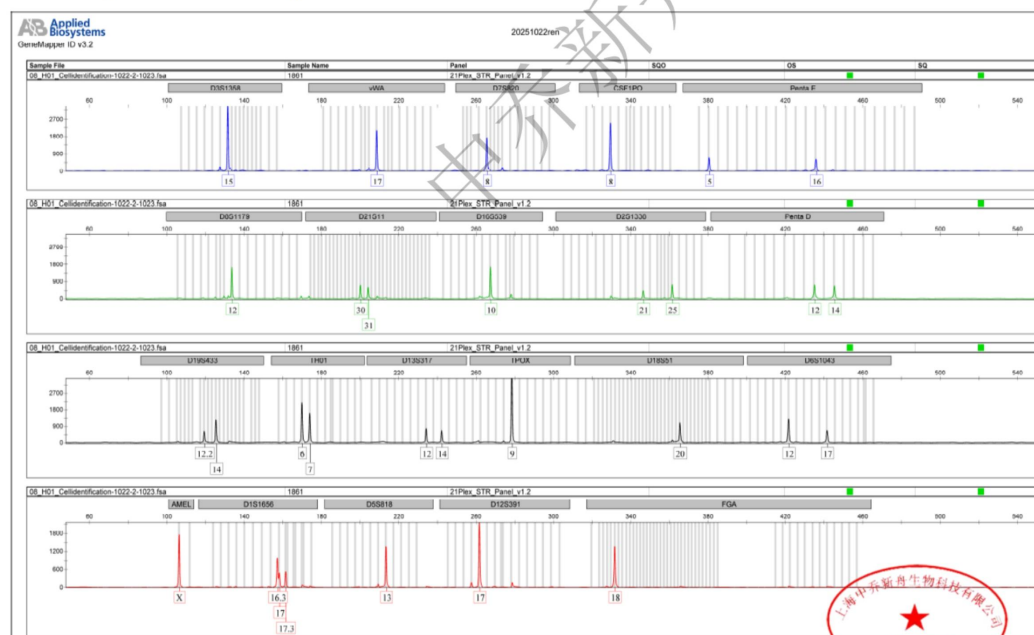
Others:

1. Genotyping Strategy and Site Distribution

Attached Table. Experimental Strategy and Sites

	方案 1	方案 2	方案 3	方案 4
1	D3S1358	D8S1179	D19S433	AMEL
2	VWA	D21S11	TH01	DIS1656
3	D7S820	D16S539	D13S317	D5S818
4	CSF1PO	D2S1338	TPOX	D12S391
5	PENTAE	PENTAD	D18S51	FGA
6			D6S1043	

2. DSMZ tools was used to carry on the cell line comparison, which contains 2455 cell lines STR data from ATCC, DSMZ, JCRB, ECACC, GNE and RIKEN databases. If the cell is not included in the above cell library, users need to compared with other databases.



Mycoplasma Detection Report

Cell lines: MHCC97H and Hep3B

Sample type: Cells cultured in T25 flasks

Sample preparation: Cells were maintained in antibiotic-free culture medium for more than 48 h until they reached approximately 90% confluence. Samples were collected and tested according to the manufacturer's instructions for the One-Step Mycoplasma Detection Kit (LAMP method; catalog no. SNCD-004).

Test results:

Colorimetric LAMP result:



Tube order (left to right): negative control, positive control, MHCC97H, and Hep3B.

Conclusion: Both MHCC97H and Hep3B cell cultures tested negative by the LAMP assay. The color of both test samples matched the negative control and differed from the positive control, indicating no detectable mycoplasma contamination.