

Integrated Bioinformatics analysis and clinical validation reveals that high expression of mucin 1 in intrahepatic cholangiocarcinoma predicts recurrence after curative resection

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Abstract. Intrahepatic cholangiocarcinoma (ICC) is a cancer type with high malignancy and a current lack of biomarkers to predict recurrence. In the present study, to identify potential biomarkers, five ICC datasets from the Gene Expression Omnibus database were analyzed to construct initial datasets by using a robust rank aggregation approach. A total of 19 upregulated genes were identified in the initial datasets. The genes identified were then further analysed using data from The Cancer Genome Atlas. Only mucin 1 (MUC1) exhibited significance regarding differential expression and survival prediction. Finally, the expression levels of MUC1 were assessed using reverse transcription-quantitative PCR in 61 pairs of ICC tumor and matched non-cancerous samples. The expression of MUC1 was significantly elevated in ICC tissues compared with that in matched non-cancerous counterparts (P=0.001). Patients with high MUC1 expression levels had

significantly shorter overall survival (OS, P=0.009) and recurrence-free survival (RFS, P=0.012). MUC1 was identified as an independent prognostic factor for OS [hazard ratio (HR)=2.364, 95%CI: 1.214-4.485; P=0.023] and RFS (HR=2.552 95%CI: 1.294-5.032; P=0.007) in the multivariate analysis. Using receiver operating characteristic analysis, a co-index including MUC1 had a high accuracy for predicting survival [MUC1 combined with serum levels of CEA and cancer antigen 19-9, and lymph node metastasis, area under curve (AUC)=0.746, 95%CI: 0.620-0.872] and recurrence (MUC1 combined with bile duct invasion and lymph node metastasis, AUC=0.729, 95%CI: 0.605-854). In conclusion, MUC1 is highly expressed in ICC tissue and is a potential prognostic biomarker and therapeutic target for ICC.

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common type of primary liver malignancy after hepatocellular carcinoma (1,2). ICC originates from the intrahepatic bile duct and affected patients present with a focal liver mass (3). The incidence of ICC is increasing worldwide and mortality rates have not declined (4-6). The poor prognosis of ICC is of particular concern. Resection is the only curative treatment for ICC, but even with resection, patients with ICC have a poor prognosis (7,8). For early ICC, the 5-year survival of patients with resection is around 30-35% (9).

Gene dysregulation contributes to the tumorigenesis of ICC (10). Identification of the dysregulated genes may be used to identify novel treatment targets and predictors for response to chemotherapy and radiotherapy (11). Numerous genes appear to be promising diagnostic biomarkers for ICC, as well as predictors of prognosis and targets for therapy (10,12,13). Gene expression studies are being used to identify these genes.

Integrated data analysis has a key role in the analysis of high-throughput data (14). The R package RobustRankAggreg may be used for data integration with the robust rank aggregation (RRA) method (15). The algorithm assigns a P-value

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Abbreviations: adjP, Bonferroni correction adjusted P-value; GEO, Gene Expression Omnibus; ICC, intrahepatic cholangiocarcinoma; logFC, log₂-transformed fold change; MUC1, mucin 1; OS, overall survival; RRA, robust rank aggregation; RFS, recurrence-free survival; TCGA, The Cancer Genome Atlas

Key words: intrahepatic cholangiocarcinoma, Bioinformatics analysis, mucin 1, gene dysregulation

to each item that indicates how much better it is positioned in the ranked list than expected by chance. This P-value is used for re-ranking the items and to determine statistical significance (16).

In the present study, an integrated Bioinformatics analysis was used to identify genes associated with ICC. Clinical samples were then analyzed to validate a gene that was identified and its clinical significance.

Patients and methods

Data selection and datasets. A search for ICC expression profiling array data was performed using Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) (17) for entries added until May 2017. The search strategy was based on a combination of the following terms: {(intrahepatic cholangiocarcinoma) OR [cholangio* AND (cancer* OR tumor* OR tumor* OR carcinoma)]}. All data series were carefully screened. Studies on more than one type of tumor, but that included ICC, were also evaluated. Studies that profiled different histologic subtypes but that did not include non-cancerous tissue, and studies using only cell lines, were excluded. Gene expression data and corresponding clinical information for the cholangiocarcinoma dataset were downloaded from The Cancer Genome Atlas (TCGA, <https://www.cancer.gov/tcga>) data portal in May 2017.

Dataset construction. The series of data from the GEO datasets (GSE89749, GSE76297, GSE57555, GSE32879 and GSE26566) were analyzed separately using the limma application (version 3.30.13) (18). The expression level of each gene was log₂-transformed for further analysis. Bonferroni correction was used to adjust P-values; a correction that adjusted each P-value (adjP) <0.01 for a gene with a lfold change (FC)>2 was applied to each data series. Identification of dysregulated genes associated with cholangiocarcinoma was performed using the edgeR application (version 3.16.5) (19). log₂-transformation and Bonferroni correction were also applied for each gene. For a more stringent limit, the applied adjusted P-value was <0.001 and the llog₂-transformed FCI was >4.

RRA was used for the integration of the GEO data series. The R package (version: 1.1) was used to detect genes that were ranked consistently better than expected under the null hypothesis of uncorrelated inputs and to assign a significance score for each gene (16). All genes identified were statistically significant (P<0.01) after the Bonferroni correction was performed.

Validation of potential dysregulated genes using reverse transcription-quantitative PCR (RT-qPCR). A total of 61 pairs of ICC and adjacent non-cancerous tissues were used to validate the potential dysregulated genes. All tissues were ground separately in liquid nitrogen. The total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Complementary DNA synthesis was performed using a PrimeScript™ reverse transcriptase reagent kit (Takara Bio, Inc.). Amplification and quantification were performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR® Premix Ex Taq™ (Tli RNaseH Plus; Takara Bio, Inc.).

GAPDH was used as an endogenous control. The expression level of the analyte gene was normalized to that of GAPDH and the 2^{-ΔΔC_q} method was applied (20). The primers used are presented in Table SI.

Patients. The study protocol was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China). ICC was defined as adenocarcinoma arising from the second-order or greater distal branches of the intrahepatic bile ducts. The inclusion criteria were as follows: i) The patient received a curative resection between January 2009 and December 2012 at Zhongshan Hospital, Fudan University (Shanghai, China); ii) the patient's ICC was diagnosed by two experienced pathologists; iii) each patient received the operation from the same surgical group; iv) no concurrent malignant tumors of other types were present; v) no anti-tumor treatments were given prior to the surgery; vi) the complete follow-up information was available. All tissues were frozen immediately after the hepatectomies.

The tumor-nodes-metastasis staging was performed according to the guidelines of the eighth edition of the American Joint Committee on Cancer/International Union against Cancer (21).

Follow-up. After resection, each patient had an appointment for a follow-up exam every 3-4 months during the first 2 years, and then every 4-6 months during the next year. Liver function indicators, serum α-fetoprotein and hematological parameters were examined at each follow-up visit. Liver ultrasonography was performed at each visit by physicians who were not involved in the patient's treatment and had no access to treatment information. Contrast computed tomography (CT) scans of the chest, abdomen and pelvis were performed once every 6 months. A bone scan or a magnetic resonance imaging (MRI) scan was performed if it was considered necessary due to any clinical indication. If tumor recurrence in the liver was suspected, a CT scan or MRI with intravenous contrast was performed. Biopsies of lesions were performed when a definite diagnosis could not be elucidated made by other methods.

Statistical analysis. All R packages used were based on R software (version 3.3.2). Comparison of expression levels between tumors and paired adjacent non-cancerous tissues was performed using paired t-tests.

Overall survival (OS) time was measured from the date of surgery to the date of death. The recurrence-free survival (RFS) time was calculated from the date of surgery to the date of the first clinically-documented tumor recurrence or metastasis, or the date of death. Inter-group comparisons were performed using the chi-squared or Fisher's exact test. The OS and RFS times were calculated using the Kaplan-Meier method; log-rank tests were used to assess differences between survival curves. The Cox linear hazards model was used logistic regression analysis for the univariate and multivariate analysis. The accuracy of predicting prognosis was evaluated using receiver operating characteristic (ROC) curves, and the predictive performance was determined by calculating the area under the ROC curve (AUC). All statistical analyses were performed using the SPSS 22.0 software

package (IBM Corp.). $P < 0.05$ was considered to indicate statistical significance.

Results

Characteristics of the data series and associated dysregulated genes. A total of five data series from GEO datasets deposited between 2012 and 2017 were included for further analysis (22–26). The characteristics of these studies/data series are presented in Table I.

A total of 345 tumor samples and 175 control counterparts were included in the integrated dataset. The control samples varied across the studies (Table I). Different microarray platforms were used in the studies; the number of mRNA probes ranged from 22,185 to 70,753. Use of the limma R package ($\log_{2}FC > 2$, $\text{adj}P < 0.01$) revealed that at least 31 dysregulated genes were contained in one data series. In total, the median number of significantly upregulated genes was 46 (range, 14–4,889), and the median number of downregulated genes was 534 (range, 17–3,801) (Table I).

The RRA analysis identified 19 upregulated genes and 130 downregulated genes (Table SII). The P -values for all dysregulated genes were statistically significant ($\text{adj}P < 0.01$) after Bonferroni correction. The corrected P -values for the dysregulated genes ranged from 1.38×10^{-7} to 9.86×10^{-3} . To identify specific biomarkers, the 19 upregulated genes were selected for further analysis (Fig. 1A and Table II).

Mucin 1 (MUC1) is upregulated in ICC and has clinical significance in TCGA data. Data from the TCGA were used to study the upregulated genes identified. There were 36 ICC tumor tissues and nine normal tissues in the TCGA dataset. The edgeR package was used to reveal the dysregulated genes. A total of 769 upregulated genes and 713 downregulated genes were identified ($\log_{2}FC > 4$, $\text{adj}P < 0.001$). The results for the upregulated genes are presented in Fig. 1B and Table SIII. Intersection of the two datasets revealed that 13 upregulated genes were identified in the GEO as well as TCGA datasets (Fig. 1C). All 13 genes were upregulated in ICC tissue compared with normal liver tissue (Fig. 1D and E), but only MUC1 had a statistically significant impact in the survival analysis (Figs. 1F and S1). The expression of MUC1 in ICC patients was selected for further study.

Validation of MUC1 expression in patients with ICC. A total of 61 patients with ICC met the inclusion criteria and were enrolled in the present study. All of them had ICC that was diagnosed and pathologically confirmed at the Liver Surgery Department of Zhongshan Hospital, Fudan University (Shanghai, China) between January 2009 and December 2012. The clinicopathological characteristics of these patients are presented in Table III.

MUC1 expression was examined using RT-qPCR analysis. Consistent with the results of the dataset analysis, the expression of MUC1 was significantly higher in ICC tumor tissue than in adjacent non-tumor tissue ($P = 0.001$; Fig. 2A). The enrolled patients were divided into two groups based on the median MUC1 expression value (i.e., low MUC1 expressed in ICC tissue, MUC1^{low}, $n = 30$; high MUC1 expressed in ICC tissue, MUC1^{high}, $n = 31$). Inter-group comparisons of the

Table I. Features of the Gene Expression Omnibus datasets included in the present analysis.

Series accession	First author (year)	Region	Array type	Tumor samples (n)	Type (number) of control samples	Upregulated genes (n)	Downregulated genes (n)	(Refs.)
GSE89749	Jusakul (2017)	Asia	Illumina HumanHT-12 V4.0 expression beadchip	118	Non-tumor bile duct from the same patients as the tumor tissue (n=2)	18	767	(22)
GSE76297	Chaisaingmongkol (2017)	North America	[HTA-2_0] Affymetrix Human Transcriptome Array 2.0 [transcript (gene) version]	91	Matched peritumoral liver tissue (n=91)	46	242	(23)
GSE57555	Murakami (2015)	Asia	Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381	16	Matched peritumoral liver tissue (n=16)	14	17	(24)
GSE32879	Oishi (2012)	North America	[HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version]	16	Paired non-tumor tissue from the same patients as the tumor tissue (n=7)	159	534	(25)
GSE26566	Andersen (2012)	Europe	Illumina human Ref-8 v2.0 expression beadchip	104	Matched peritumoral liver tissue (59)	4,889	3,801	(26)

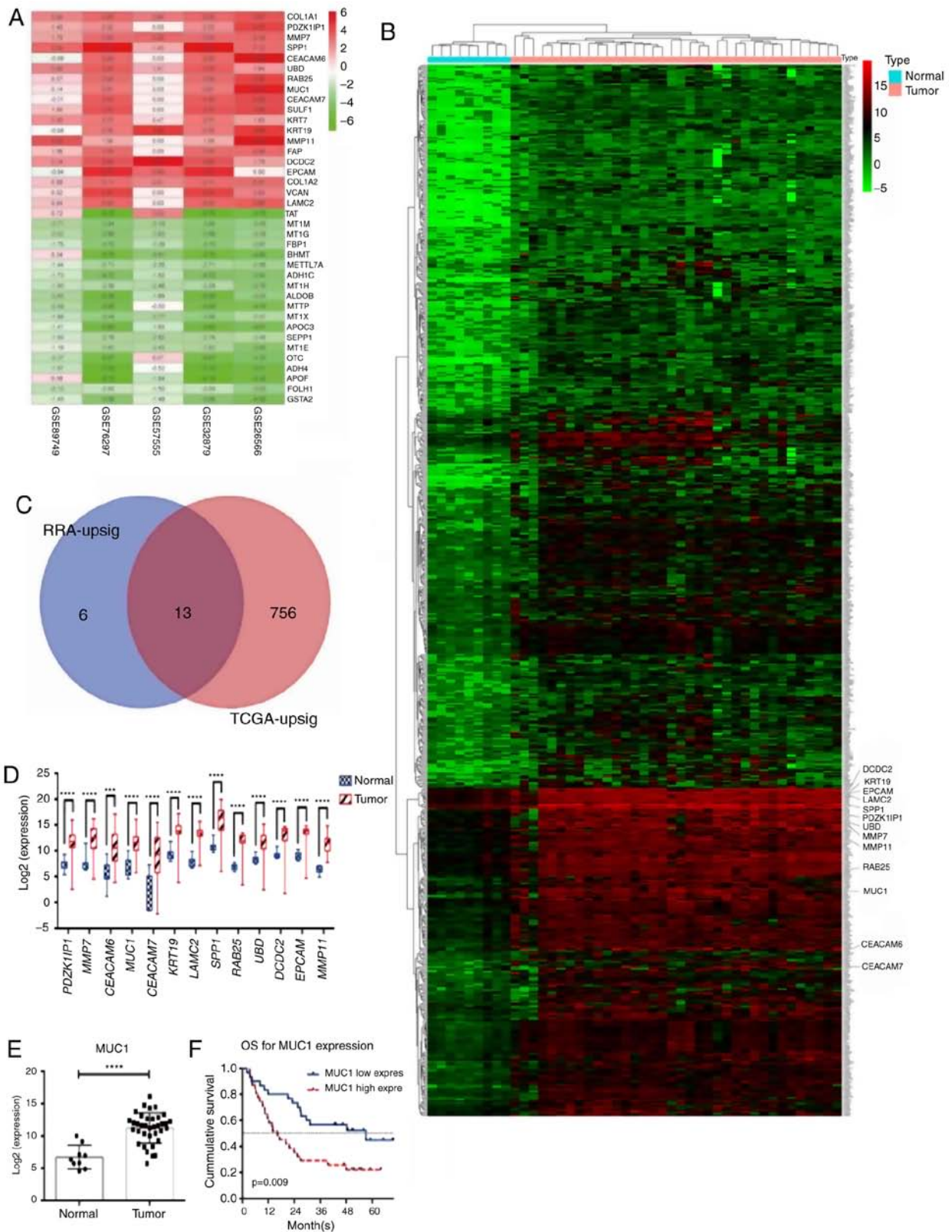


Figure 1. MUC1 is upregulated in ICC tissue, as validated using databases. (A) The 19 upregulated genes and the 19 most significant downregulated genes linked to cholangiocarcinoma identified using robust rank aggregation. Numbers in the colored fields indicate the log2-transformed fold change. 0.00 or white boxes indicates that the gene was absent from the respective dataset. (B) Upregulated genes linked to cholangiocarcinoma identified using the edgeR package (TCGA dataset). The enlarged gene symbols adjacent to the heatmap are the 13 intersected genes. (C) 13 genes were identified to be upregulated in the RRA integrated datasets and the TCGA data. (D and E) The expression levels of (D) the 13 genes and (E) MUC1 were significantly higher in ICC tissue, validated using TCGA data. (F) Among the 13 genes, only MUC1 predicted OS. RRA-upsig refers to the significantly upregulated genes identified using RRA; TCGA-upsig refers to significantly upregulated genes among the TCGA data. ***P<0.001; ****P<0.0001. MUC1, mucin 1; TCGA, The Cancer Genome Atlas; ICC, intrahepatic cholangiocarcinoma; OS, overall survival; RRA, robust rank aggregation; PDZK1IP1, PDZK1 Interacting Protein 1; MMP7, matrix metalloproteinase 7; CEACAM6, carcinoembryonic antigen related adhesion molecules 6; MUC1, mucin 1; CEACAM7, carcinoembryonic antigen related adhesion molecules 7; KRT19, keratin 19; LAMC2, laminin subunit gamma-2; SPP1, secreted phosphoprotein 1; RAB25, Ras Genes from Brain Protein 25; UBD, ubiquitin D; DCDC2, doublecortin domain containing 2; EPCAM, epithelial cell adhesion molecule; MMP11, matrix metalloproteinase 11.

Table II. The 19 upregulated genes and the 19 most significant downregulated genes associated with cholangiocarcinoma, identified using robust rank aggregation.

A, Upregulated			
Gene symbol	P-value	Adjusted P-value	logFC
COL1A1	4.14x10 ⁻¹¹	2.22x10 ⁻⁶	3.147
PDZK1IP1	1.08x10 ⁻⁹	5.82x10 ⁻⁵	2.093
MMP7	2.16x10 ⁻⁹	1.16x10 ⁻⁴	2.864
SPP1	2.99x10 ⁻⁹	1.61x10 ⁻⁴	3.904
CEACAM6	5.02x10 ⁻⁹	2.70x10 ⁻⁴	2.195
UBD	1.26x10 ⁻⁸	6.79x10 ⁻⁴	2.610
RAB25	1.50x10 ⁻⁸	8.06x10 ⁻⁴	2.208
MUC1	2.06x10 ⁻⁸	1.11x10 ⁻³	2.284
CEACAM7	2.74x10 ⁻⁸	1.47x10 ⁻³	2.174
SULF1	3.78x10 ⁻⁸	2.03x10 ⁻³	2.511
KRT7	4.01x10 ⁻⁸	2.16x10 ⁻³	2.040
KRT19	4.26x10 ⁻⁸	2.29x10 ⁻³	2.819
MMP11	4.89x10 ⁻⁸	2.63x10 ⁻³	2.526
FAP	5.34x10 ⁻⁸	2.87x10 ⁻³	2.099
DCDC2	5.95x10 ⁻⁸	3.20x10 ⁻³	3.545
EPCAM	6.92x10 ⁻⁸	3.72x10 ⁻³	2.401
COL1A2	1.36x10 ⁻⁷	7.34x10 ⁻³	2.544
VCAN	1.56x10 ⁻⁷	8.37x10 ⁻³	2.353
LAMC2	1.83x10 ⁻⁷	9.86x10 ⁻³	2.228

B, Downregulated

Gene symbol	P-value	Adjusted P-value	logFC
TAT	2.57x10 ⁻¹²	1.38x10 ⁻⁷	-3.201
MT1M	1.18x10 ⁻¹¹	6.32x10 ⁻⁷	-3.387
MT1G	2.20x10 ⁻¹¹	1.18x10 ⁻⁶	-3.279
FBP1	7.71x10 ⁻¹¹	4.15x10 ⁻⁶	-2.892
BHMT	8.03x10 ⁻¹¹	4.32x10 ⁻⁶	-3.980
METTL7A	8.36x10 ⁻¹¹	4.49x10 ⁻⁶	-2.438
ADH1C	8.58x10 ⁻¹¹	4.62x10 ⁻⁶	-3.404
MT1H	8.81x10 ⁻¹¹	4.74x10 ⁻⁶	-2.657
ALDOB	1.03x10 ⁻¹⁰	5.54x10 ⁻⁶	-3.811
MTTP	1.42x10 ⁻¹⁰	7.62x10 ⁻⁶	-3.601
MT1X	2.89x10 ⁻¹⁰	1.56x10 ⁻⁵	-2.816
APOC3	3.31x10 ⁻¹⁰	1.78x10 ⁻⁵	-3.859
SEPP1	4.31x10 ⁻¹⁰	2.32x10 ⁻⁵	-2.490
MT1E	6.40x10 ⁻¹⁰	3.44x10 ⁻⁵	-2.646
OTC	7.06x10 ⁻¹⁰	3.79x10 ⁻⁵	-3.225
ADH4	7.06x10 ⁻¹⁰	3.80x10 ⁻⁵	-4.379

Table II. Continued.

B, Downregulated			
Gene symbol	P-value	Adjusted P-value	logFC
APOF	7.06x10 ⁻¹⁰	3.80x10 ⁻⁵	-3.828
FOLH1	7.43x10 ⁻¹⁰	3.99x10 ⁻⁵	-2.194
GSTA2	7.95x10 ⁻¹⁰	4.28x10 ⁻⁵	-3.152

P-values were adjusted using Bonferroni correction. LogFC, log₂-transformed fold change. COL1A1, collagen type I alpha 1 chain; PDZK1IP1, PDZK1 Interacting Protein 1; MMP7, matrix metalloproteinase 7; SPP1, secreted phosphoprotein 1; CEACAM6, carcinoembryonic antigen related adhesion molecules 6; UBD, ubiquitin D; RAB25, Ras Genes from Brain Protein 25; MUC1, mucin 1; CEACAM7, carcinoembryonic antigen related adhesion molecules 7; SULF1, Sulfatase 1; KRT7, keratin 7; KRT19, keratin 19; MMP11, matrix metalloproteinase 11; FAP, Fibroblast Activation Protein Alpha; DCDC2, doublecortin domain containing 2; EPCAM, epithelial cell adhesion molecule; COL1A2, collagen type I alpha 2 chain; VCAN, versican; LAMC2, laminin subunit gamma-2. TAT, tyrosine aminotransferase; MT1M, metallothionein 1M; MT1G, metallothionein 1G; FBP1, fructose-bisphosphatase 1; BHMT, betaine-homocysteine S-methyltransferase; METTL7A, methyltransferase like 7A; ADH1C, alcohol dehydrogenase 1C (Class I), gamma polypeptide; MT1H, metallothionein 1H; ALDOB, aldolase, fructose-bisphosphate B; MTTP, microsomal triglyceride transfer protein; MT1X, metallothionein 1X; APOC3, apolipoprotein C3; SEPP1, selenop; MT1E, metallothionein 1E; OTC, ornithine carbamoyltransferase; ADH4, alcohol dehydrogenase 4 (Class II), Pi Polypeptide; APOF, apolipoprotein F; FOLH1, folate hydrolase 1; GSTA2, glutathione S-transferase alpha 2.

clinicopathological characteristics are presented in Table III. The MUC1^{high} group included more patients with tumors that invaded the surrounding tissue (P=0.040). Inter-group differences for the other clinicopathological characteristics were not statistically significant. The levels of MUC1 expression in tumors that invaded the surrounding tissue were higher than in those without surrounding tissue invasion (P=0.027; Fig. 2B).

Clinical validation of the prognostic significance of MUC1 expression. Survival analysis was performed to identify whether MUC1 levels may be used to predict prognosis in ICC patients. The median survival times of the MUC1^{low} and MUC1^{high} groups were 55.06 and 17.25 months, respectively (P=0.009). The 1-, 3- and 5-year OS rates for the MUC1^{low} group were 73, 57 and 45%, respectively, as opposed to 35, 26 and 20%, respectively, for the MUC1^{high} group. The differences in OS rates according to the log-rank test was statistically significant between the two groups (P=0.009; Fig. 2C). A total of 9 patients (30.0%) in the MUC1^{low} group and 17 (54.8%) in the MUC1^{high} group experienced tumor recurrence. The inter-group difference in the RFS rates was statistically significant (P=0.012; Fig. 2D).

The results of the univariate analysis indicated that MUC1 expression levels were a significant prognostic factor for OS [hazard ratio (HR)=2.233, 95%CI: 1.214-4.485, P=0.011; Table IV]. The serum levels of carcinoembryonic antigen

Table III. Clinical characteristics of enrolled patients.

Parameter	All patients (n=61)	MUC1 ^{low} (n=30)	MUC1 ^{high} (n=31)	P-value ^a
Age (years)	59 (39-77)	57 (39-72)	59 (40-77)	0.86
Gender				0.21
Male	40 (65.57)	22 (73.33)	18 (58.06)	
Female	21 (34.43)	8 (26.67)	13 (41.94)	
Number of tumors				0.85
Single	42 (68.85)	24 (80.00)	18 (58.06)	
Multiple	19 (31.15)	6 (20.00)	13 (41.94)	
Tumor size (cm)	6 (2-14)	6 (2-11.5)	6 (2.5-14)	0.79
Vascular invasion				0.28
Yes	16 (26.23)	6 (20.00)	10 (32.26)	
No	45 (73.77)	24 (80.00)	21 (67.74)	
Bile duct invasion				0.08
Yes	6 (9.84)	5 (16.67)	1 (3.23)	
No	55 (90.16)	25 (83.33)	30 (96.77)	
Nerve invasion				0.53
Yes	3 (4.92)	2 (6.67)	1 (3.23)	
No	58 (95.08)	28 (93.33)	30 (96.77)	
T stage				0.16
T1a	14 (22.95)	9 (30.00)	5 (16.13)	
T1b	17 (27.87)	8 (26.67)	9 (29.03)	
T2	26 (42.62)	13 (43.33)	13 (41.94)	
T3	0 (0.00)	0 (0.00)	0 (0.00)	
T4	4 (6.56)	0 (0.00)	4 (12.90)	
LN metastasis				0.53
Yes	3 (4.92)	2 (6.67)	1 (3.23)	
No	58 (95.08)	28 (93.33)	30 (96.77)	
Surrounding tissue invasion				0.04
Yes	4 (6.56)	0 (0.00)	4 (12.90)	
No	57 (93.44)	30 (100.00)	27 (87.10)	
Differentiation				0.11
Well	50 (81.97)	27 (90.00)	23 (74.19)	
Poor	11 (18.03)	3 (10.00)	8 (25.81)	
Type of resection				0.29
Less than hemihepatectomy	46 (75.41)	21 (70.00)	25 (80.65)	
Hemihepatectomy	13 (21.31)	7 (23.33)	6 (19.35)	
Extended hemihepatectomy	2 (3.28)	2 (6.67)	0 (0.00)	
Post-operative treatment				0.311
Yes	9 (14.75)	3 (10.00)	6 (19.35)	
No	42 (68.85)	27 (90.00)	25 (80.65)	

Values are expressed as the median (range) or n (%). Vascular invasion, bile duct invasion and nerve invasion were identified pathologically. Post-operative treatment includes transarterial chemoembolization or radiofrequency ablation. LN, lymph node; MUC1, mucin 1. ^aP-values represent comparison between MUC1^{low} group and MUC1^{high} group.

(CEA) and cancer antigen 19-9 (CA19-9), the degree of tumor differentiation, tumor diameter, tumor T stage and presence of lymph node metastasis were also significant influencing

factors of OS (Table IV). The multivariate analysis revealed that a high MUC1 expression level (HR=2.364, 95%CI: 1.127-4.960, P=0.023), high levels of CEA (HR=2.315, 95%CI:

Table IV. Univariate and multivariate analysis of prognostic factors for overall survival.

Prognostic factor	Univariate		Multivariate	
	HR (95%CI)	P-value	HR (95%CI)	P-value
MUC1 (≥ 0.056 vs. < 0.056 , ratio to GAPDH)	2.233 (1.214-4.485)	0.011	2.364 (1.127-4.960)	0.023
CEA (≥ 20 ng/ml vs. < 20 ng/ml)	2.197 (1.106-4.365)	0.025	2.315 (1.081-4.958)	0.031
CA-19-9 (≥ 37 ng/ml vs. < 37 ng/ml)	2.675 (1.368-5.231)	0.004	2.303 (1.109-4.780)	0.025
Differentiation (poor vs. well)	2.284 (1.102-4.736)	0.026	1.102 (0.485-2.502)	0.816
Tumor diameter (> 5 cm vs. ≤ 5 cm)	2.236 (1.110-4.503)	0.024	1.461 (1.127-4.960)	0.320
LN metastasis (yes vs. no)	4.440 (1.916-10.288)	0.001	2.846 (1.259-6.436)	0.012
T stage (T2-4 vs. T1)	1.435 (0.995-2.070)	0.053		
Surrounding tissue invasion (yes vs. no)	2.612 (0.797-8.557)	0.113		
Tumor number (solitary vs. multiple)	1.603 (0.838-3.064)	0.154		
Nerve invasion (yes vs. no)	1.117 (0.268-4.645)	0.879		
Vascular invasion (yes vs. no)	0.798 (0.378-1.681)	0.552		
Bile duct invasion (presence vs. absence)	1.995 (0.762-5.018)	0.163		

LN, lymph node; HR, hazard ratio; CA, cancer antigen.

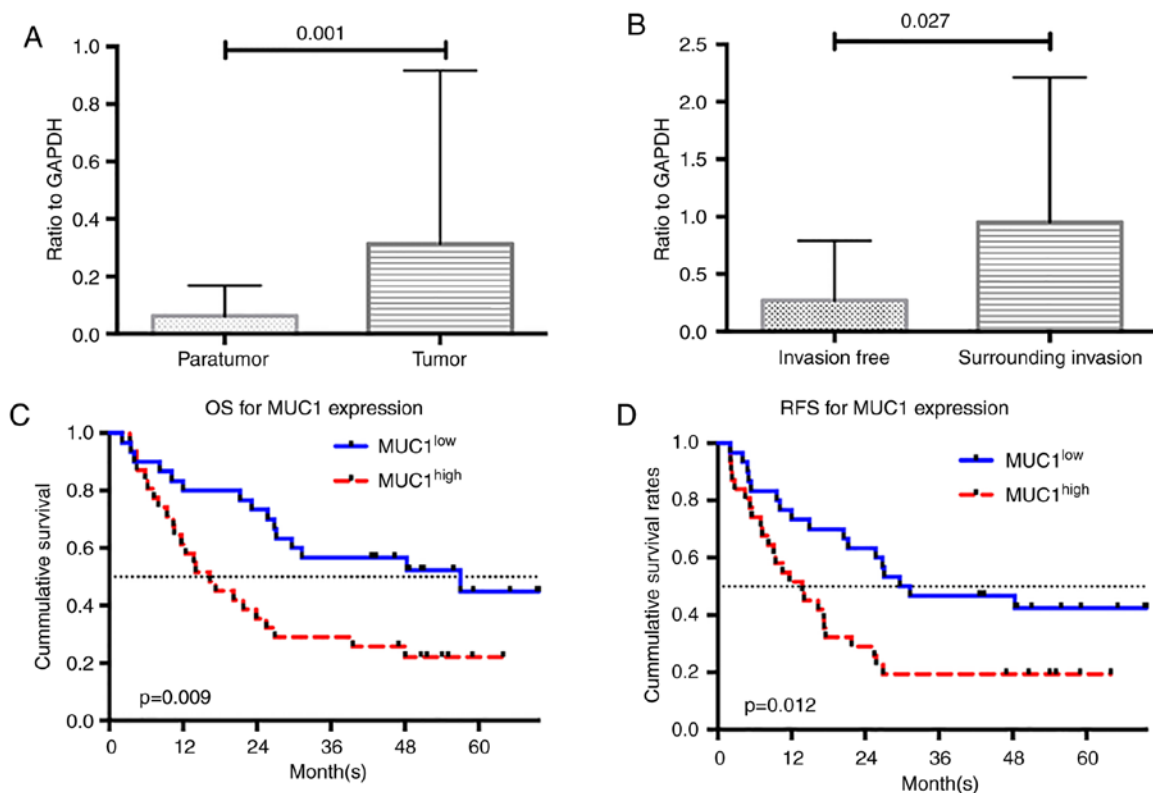


Figure 2. Clinical validation of MUC1 expression and its prognostic significance. (A) The levels of MUC1 were significantly greater in the ICC tumor tissues compared with the matched paratumor tissues. (B) MUC1 was also significantly higher in tumors with surrounding tissue invasion, compared with the tumors that did not invade. All data were presented as the mean + standard deviation. (C and D) MUC1 was a prognostic predictor for (C) OS and (D) RFS. MUC1^{low} refers to the group with low expression of MUC1 in ICC tissue and MUC1^{high} to the group with high expression of MUC1 in ICC tissue. MUC1, mucin 1; ICC, intrahepatic cholangiocarcinoma; OS, overall survival; RFS, recurrence-free survival.

1.081-4.958, $P=0.031$) and CA19-9 (HR=2.303, 95%CI: 1.109-4.780, $P=0.025$), and the presence of lymph node metastasis (HR=2.846, 95%CI: 1.259-6.436, $P=0.012$) were

independent prognostic factors for OS (Table IV). A high level of MUC1 was also a prognostic factor for RFS (univariate analysis HR=2.187, 95%CI: 1.171-4.083, $P=0.014$; multivariate

Table V. Univariate and multivariate analysis of prognostic factors for recurrence-free survival.

Prognostic factor	Univariate		Multivariate	
	HR (95%CI)	P-value	HR (95%CI)	P-value
MUC1 (≥ 0.056 vs. < 0.056 , ratio to GAPDH)	2.187 (1.171-4.083)	0.014	2.552 (1.294-5.032)	0.007
Bile duct invasion (yes vs. No)	2.445 (1.022-5.851)	0.045	3.979 (1.449-10.928)	0.007
Tumor diameter (> 5 cm vs. ≤ 5 cm)	1.977 (1.026-3.810)	0.042	1.130 (0.509-2.510)	0.764
T stage (T2-4 vs. T1)	1.468 (1.039-2.073)	0.029	1.403 (0.931-2.115)	0.106
LN metastasis (yes vs. no)	2.449 (1.256-4.776)	0.009	2.053 (1.042-4.046)	0.038
Surrounding tissue invasion (yes vs. no)	2.045 (0.628-6.656)	0.235		
CEA (≥ 20 ng/ml vs. < 20 ng/ml)	1.108 (0.567-2.167)	0.764		
CA-19-9 (≥ 37 ng/ml vs. < 37 ng/ml)	0.828 (0.452-1.516)	0.540		
Differentiation (poor vs. well)	1.930 (0.940-3.960)	0.073		
Tumor number (solitary vs. multiple)	1.504 (0.798-2.836)	0.207		
Nerve invasion (yes vs. no)	2.217 (0.679-7.239)	0.187		
Vascular invasion (yes vs. no)	0.971 (0.488-1.933)	0.933		

LN, lymph node; HR, hazard ratio; CA, cancer antigen.

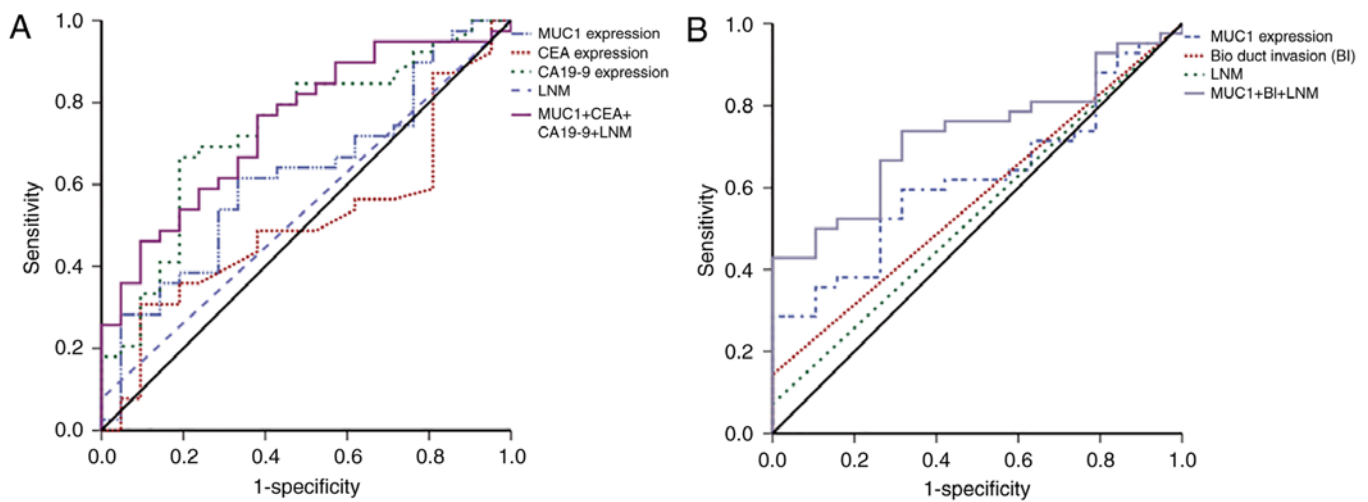


Figure 3. ROC curves for OS and RFS. (A) ROC curve for OS. (B) ROC curve for RFS. LNM, lymph node metastasis; BI, bile duct invasion; CA19-9, cancer antigen 19-9; ROC, receiver operating characteristic; MUC1, mucin 1; OS, overall survival; RFS, recurrence-free survival.

analysis HR=2.552, 95%CI: 1.294-5.032, P=0.007). Other independent risk factors for RFS time were the presence of lymph node metastasis (HR=2.053 95%CI: 1.042-4.046, 95%CI, P=0.038) and bile duct invasion (HR=3.979 95%CI: 1.449-10.928, P=0.007; Table V).

ROC curve analysis of OS and RFS was then used to examine the predictive precision of these independent prognostic factors. MUC1 expression levels alone was not a strong predictor of OS (AUC=0.623), but the combination of the serum levels of MUC1, CEA and CA19-9, and lymph node metastasis, was a better predictor of OS (AUC=0.746; Fig. 3A and Table VI). The ROC curve for RFS indicated that MUC1 combined with bile duct invasion and lymph

node metastasis was the best predictor of RFS (AUC=0.729; Fig. 3B and Table VI).

Discussion

ICC has a low incidence but is associated with high mortality. Due to the lack of effective treatments, the incidence of ICC-associated mortality is increasing worldwide (10,27,28). Gene dysregulation contributes to the genesis of ICC (10). Most of the gene expression studies that have examined how dysregulated genes contribute to the pathogenesis of ICC did not include sufficient samples to identify relevant genes or had no clinical data for validation (23-26). Therefore,

Table VI. Receiver operating characteristic analysis for OS and RFS.

A, OS	
Parameter	AUC (95%CI)
MUC1	0.623 (0.476-0.769)
CEA	0.509 (0.357-0.660)
CA19-9	0.734 (0.600-0.867)
LNM	0.538 (0.388-0.689)
Combined Co-index 1	0.746 (0.620-0.872)
B, RFS	
Parameter	AUC (95%CI)
MUC1	0.622 (0.480-0.763)
BI	0.571 (0.423-0.720)
LNM	0.536 (0.382-0.689)
Combined Co-index 2	0.729 (0.605-0.854)

The status of OS and RFS used to calculate the AUC after the very last follow-up appointment (2016.06.30). LNM refers to the presence of lymph node metastasis; BI refers to the presence of bile duct invasion. Combined co-index 1, MUC1 + CEA+ CA19-9 + LNM; Combined Co-index 2, MUC1 + BI + LNM. MUC1, mucin 1; OS, overall survival; RFS, recurrence-free survival; CA, cancer antigen.

a combined approach that included the use of integrated Bioinformatics analysis and clinical validation was used in the present study to identify genes involved in ICC. Lack of availability of raw expression datasets and difficulty in achieving rigorous normalization and integration over platforms are obstacles for the meta-analysis of gene expression data. In the present study, the RRA method was used to overcome these disadvantages (16).

MUC1 is a heterodimeric type I transmembrane glycoprotein expressed on the surface of the epithelium of most organs (e.g., mammary, gastric, respiratory, urinary, and reproductive tract) (29,30). MUC1 interacts with p53, which results in the inhibition of p53-mediated apoptosis (31). It also interacts with β -catenin to initiate the epithelial-mesenchymal transition that promotes the onset of metastasis (32). Overexpression of MUC1 is associated with the malignancy of ICC and indicates a poor outcome (33,34). However, most of the above-mentioned studies were single-center and single-ethnicity investigations. In the present study, an integrated Bioinformatics analysis of data from multiple centers (241 samples) and multiple ethnicities was performed. The results suggested that MUC1 regulates ICC tumor invasion. Certain studies have indicated that MUC1 expression is a predictor for OS in ICC patients (34,35), but few have detected a correlation between MUC1 expression and RFS. All patients included in the combined cohort of the present study had comprehensive clinical information and the longest follow-up was >5 years. Although the

cohort of patients was heterogenous with T1 or T2 tumors accounting for ~50%, there was no significant difference in T stage between the MUC1^{high} group and the MUC1^{low} group (P=0.18). Most patients with T3 or T4 tumors decided not to receive any surgery and accordingly, no samples were collected. Furthermore, patients with T3 and T4 tumors usually received pre-operative treatment and were ruled out based on the inclusion criteria. These circumstances led to heterogeneity. The present results revealed that the expression level of MUC1 was an independent prognostic factor for OS and RFS. The ROC analysis revealed that a model combining MUC1 expression, bile duct invasion and lymph node metastasis may be used to predict RFS (AUC=0.729). Using MUC1 as a predictor for RFS and OS may help identify patients with a high risk of recurrence, which may aid in the selection and development of post-operative treatment and monitoring protocols.

In the present study, a novel approach that may be used to study ICC and improve clinical practice was developed. To the best of our knowledge, the present study was the first to combine integrated Bioinformatics analysis with clinical validation to identify biomarkers for ICC. Use of the GEO and TCGA datasets provided sufficient evidence to prove the association of MUC1 with ICC. The large number of clinical samples included increases the validity of the present results. ICC is a cancer type with high malignancy, which lacks effective treatments (10,27,28). MUC1 has been previously reported to be a potential target for anti-cancer therapies (12,36,37). A glycosylated tripartite vaccine that targets MUC1 is under development (38). Another MUC1 vaccine (ONT-10) has been tested in a phase I clinical trial (39). The results of the present study provided further insight that will be useful for the development of comprehensive treatments for ICC. However, studies that use larger sample sizes should be performed to validate the present results. *In vitro* and *in vivo* experiments are also required to determine the potential mechanisms by which MUC1 drives tumorigenesis.

In the present study, it was identified that MUC1 was upregulated in ICC tissues and that it was associated with prognosis. Clinical validation revealed that a co-index including MUC1 and other clinical parameters predicts recurrence of ICC with high accuracy, which may support the selection and development of post-operative treatment and monitoring protocols.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available in the GEO: GSE89749 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89749>), GSE76297 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76297>), GSE57555 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57555>), GSE32879 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32879>); and GSE26566 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26566>) and TCGA (<https://cancergenome.nih.gov/>). All other data are included in this published article and its supplementary data.

Authors' contributions

FYC designed the study, analysed the data and drafted the manuscript. CZ, XYZ and KQZ collected and verified the data. YFP and LY analyzed and interpreted the data. JF, JZ, JH and ZW designed the study, interpreted the data, revised the manuscript and made the decision to submit it for publication. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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