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Expression of long non-coding RNA ANRIL predicts a poor prognosis in intrahepatic cholangiocarcinoma

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Abstract

Background: Intrahepatic cholangiocarcinoma (iCCA) is a deadly cancer worldwide associated with an increased incidence, limited therapeutic options and absence of reliable prognostic biomarkers. Long non-coding RNAs (lncRNA) emerge as relevant biomarkers in cancer being associated with tumor progression. However, lncRNA have been poorly investigated in iCCA.

Aim: To identify lncRNA significantly associated with the survival of patients with iCCA after tumor resection for curative intent.

Methods: Gene expression profiling and Q-RT-PCR were performed from a cohort of 39 clinically well-annotated iCCA. Univariate Cox proportional hazards model with Wald Statistic was used to identify lncRNA significantly associated with overall (OS) and/or disease-free (DFS) survival.

Results: A signature made of 9 lncRNA was identified to be significantly ($P < 0.05$) associated with OS and DFS, including 4 lncRNA (lnc-CDK9-1, XLOC_I2_009441, CDKN2B-AS1, HOXC13-AS) highly expressed in poor prognosis iCCA and 5 lncRNA (lnc-CCHCR1-1, lnc-AF131215.3.1, lnc-CBLB-5, COL18A1-AS2, lnc-RELL2-1) highly expressed in better prognosis iCCA. We further validated CDKN2B-AS1 (ANRIL) as a poor prognosis biomarker, not only in iCCA, but also in hepatocellular carcinoma, kidney renal clear cell carcinoma and uterine corpus endometrial carcinoma.

Conclusions: We report a prognosis lncRNA signature in iCCA and the clinical relevance of CDKN2B-AS1 (ANRIL) overexpression in several cancers.

Keywords

cholangiocarcinoma; prognosis; long non-coding RNA; ANRIL

Introduction

Cholangiocarcinomas (CCA) comprise several types of tumors arising from the malignant transformation of cholangiocytes lining different part of the biliary tree. Based on the anatomical location, intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) CCA are recognized ^{1,2}. Each of these CCA subtypes exhibits specific biological and pathological features, e.g. in term of clinical presentation, risk factors, prognosis and therapeutic strategies ². In the liver, iCCA represents the second most common type of malignant primary tumor after hepatocellular carcinoma (HCC), encompassing 10% to 15% of cases. However, an increase in iCCA incidence and mortality has been observed worldwide over the last decades. Today, surgical resection still remains the best curative treatment strategy of CCA, although it is associated with a high risk of tumor recurrence ¹. For patients with unresectable or metastatic CCA, mostly as a result of a late diagnosis, systemic chemotherapy (usually a gemcitabine and cisplatin combination) is proposed as a palliative treatment ¹. Recently, adjuvant chemotherapy was demonstrated effective in resected CCA ³. However, defining the optimal population that could benefit from adjuvant chemotherapy is particularly important. From a clinical point of view, the identification of innovative biomarkers for early diagnosis, prognosis and response to therapies, may improve the management and the survival of patients with iCCA. Although carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) levels may be elevated in some patients, there is no specific serum biomarkers for the diagnosis of iCCA. Several prognostic tissue biomarkers have been proposed based on genomic analyses. Notably, by combining laser capture microdissection and unsupervised gene expression profiling, we previously established clinically relevant signatures specific of the tumor microenvironment in iCCA ⁴. Thus, an increased expression of osteopontin (OPN) and lysyl oxidase like 2 (LOXL2) in the stroma was identified as a significant prognostic factor associated with a reduced survival of patients after tumor resection ^{4,5}. More recently, we identified a novel long non-coding RNA (lncRNA) up-regulated

by the transforming growth factor beta (TGF β) in human iCCA and associated with an inflammatory microenvironment, indicating that lncRNAs are also actively involved in iCCA carcinogenesis⁶.

LncRNAs belong to an emerging class of regulatory RNAs that play critical roles in modulating a large variety of biological processes and cell signaling pathways. Accordingly, altered lncRNA expression has been reported in several cancers, including CCA⁷. Recent meta-analyses of The Cancer Genome Atlas (TCGA) database identified differentially expressed lncRNA in CCA (e.g. LINC00313, NEXN-AS1, COL18A1-AS1, HULC), the expression of some of them being significantly associated with overall survival (OS) of patients^{8,9}. Mechanistically, co-expression network analysis of coding and non-coding RNA in resected CCA tumors identified lncRNA (e.g. APOC1P1, PVT1) possibly regulating inflammation and oxidative stress in malignant cholangiocytes^{10,11}. Notably, pvt1 oncogene (PVT1) was shown to promote cell proliferation and migration by epigenetically silencing angiopoietin like 4 (ANGPTL4), a tumor suppressor gene candidate¹². Cyclin dependent kinase inhibitor 1A (CDKN1A), another well-described tumor suppressor gene, was shown to be epigenetically silenced in CCA through the interaction of small nucleolar RNA host gene 1 (SNHG1) lncRNA with enhancer of zeste 2 (EZH2), the catalytic subunit of the polycomb repressive complex 2 (PRC2)¹³. Nuclear paraspeckle assembly transcript 1 (NEAT1) lncRNA may interact with EZH2 as well to repress the expression of E-cadherin (*CDH1*), thus promoting epithelial-to-mesenchymal transition (EMT) and metastasis¹⁴. Accordingly, an increased expression of SNHG1 and NEAT1 has been associated with aggressive tumorigenesis features in CCA^{13,14}. NEAT1 has been also reported to contribute to gemcitabine sensitivity¹⁵. Conversely, lncRNA may positively affect the expression of oncogenic regulators, either directly or indirectly. Thus, epigenetically-induced lncRNA-1 (EPIC1) was reported to promote CCA cell growth and cancer progression by physically interacting with MYC oncogene and possibly acting as a guide to direct MYC onto its target

genes involved in cell cycle progression¹⁶. Interestingly, LINC01296 lncRNA was demonstrated to stabilize MYCN oncogene by sponging microRNA-5095, which naturally targets MYCN mRNA for degradation¹⁷. Urothelial cancer associated 1 (UCA1) lncRNA was reported to promote CCA formation and progression by activating a proliferative AKT/GSK-3 β /CCND1 axis and by inducing EMT, migration and invasion¹⁸. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1), a lncRNA induced in several cancers, promotes cell proliferation and invasion of CCA cells by activating the PI3K/AKT pathway as well¹⁹. While interesting mechanisms of lncRNA action have been elegantly described in CCA carcinogenesis, reports on their clinical relevance are scarce. The expression of few individual lncRNA (e.g. CRNDE, UCA1, TUG1) was reported to correlate with clinical progression (e.g. tumor grade, lymph node metastasis, TNM stage) and was identified as risk factors for an unfavorable overall survival (OS) in iCCA^{16,18,20-23}. Circulating lncRNAs, including prostate cancer associated transcript 1 (PCAT1) and MALAT1, have been also reported in the plasma of patients with pCCA²⁴. However, besides individual lncRNA, there is no exhaustive report on the clinical relevance of lncRNA expression as prognostic factors in iCCA so far^{7,23}. Thus, this study was specifically designed to establish an unsupervised robust signature of lncRNA predictive of overall (OS) and/or disease-free (DFS) survival, by using pan-genomic microarrays and a cohort of clinically well-annotated iCCA.

Methods

Human iCCA samples

A cohort of 39 patients with iCCA was studied. Freshly frozen tumor samples were obtained through the French liver cancer biobanks network – INCa (BB-0033-00085). These patients underwent liver tumor resection for curative intent from January 2007 to July 2014. None of the patient underwent liver transplantation. Six patients underwent chemo- and/or radio-therapy prior surgery. For all cases, iCCA diagnosis was confirmed by a pathological examination. Tissue sections were evaluated after hematoxylin-eosin and Sirius Red staining (SI Figure 1). Written

informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008), as reflected in a priori approval by the local institution's human research committee and the institutional review board of Inserm (IRB00003888, IORG0003254). Clinical and pathological features of patients are reported in Table 1.

Gene expression profiling

Total RNA was extracted from tumor tissue sections and purified with a miRNAeasy kit (Qiagen, Courtaboeuf, France). Genome-wide expression profiling was performed using the low-input QuickAmp labeling kit and human SurePrint G3 8x60K pangenomic microarrays (Agilent Technologies, Santa Clara, USA). Gene expression data were processed using Feature Extraction and GeneSpring softwares (Agilent Technologies), as previously described⁶. Clustering analysis was performed using Gene Cluster and TreeView softwares²⁵.

Q-RT-PCR

Quantitative-RT-PCR was performed by using a SYBR Green master mix (Applied Biosystems, Carlsbad, CA) as previously described⁶. A mixture of oligo-dT (250ng) and random hexamers (100ng) was used to prime the reverse transcription of 1µg total RNA (Superscript III RT, Invitrogen, Carlsbad, CA). Quantitative analysis of PCR data was conducted with the $2^{-\Delta\Delta Ct}$ method using actin beta (ACTB) Ct values for normalization. Melting analysis was conducted to validate the specificity of PCR products. PCR analysis was performed using the following primers: CDKN2B-AS1-Forward, 5'-TCCAGTGCAAGTATGGTCTGT-3'; CDKN2B-AS1-Reverse, 5'-TAGCAGAAAGCTGCAAAGGC-3'; ACTB-Forward, 5'-GGACTTCGAGCAAGAGATGG-3'; ACTB-Reverse, 5'-AGCACTGTGTTGGCGTACAG-3'.

Statistical analysis

Statistical analysis of microarray data was as previously described²⁶. Notably, lncRNAs significantly associated with OS and/or DFS were identified using an univariate Cox proportional

hazards model with Wald Statistic^{26,27}. Up- and down-regulated lncRNA were determined by the ration of their expression in poor versus better prognosis iCCA. A meta-analysis of RNA-sequencing data generated by The Cancer Genome Atlas (TCGA) was also performed to determine the clinical relevance of CDKN2B-AS1/ANRIL expression in independent datasets. The Kaplan-Meier method was used to estimate OS and DFS, and group differences were analyzed with the log-rank test^{4,5,28}.

Results

Patient characteristics

Thirty nine patients (27 men) who underwent liver resection with curative intent for iCCA were included in the profiling study (Table 1). The median age was 61 ± 14 years. Similar to our previous cohort⁵, 69% patients had a tumor with a size > 5cm. The cohort included 15 (38%) well-differentiated, 17 (44%) moderately-differentiated, and 7 (18%) poorly-differentiated tumors. Satellite nodules were detected in 49% patients and lymph nodes invasion in 28% patients.

A lncRNA signature predictive of OS and DFS

Gene expression profiling was performed on RNA extracted from the 39 iCCA cases. In order to identify innovative prognostic biomarkers, statistical analysis was restricted to the differential expression of lncRNAs. By using an univariate Cox proportional hazards model with Wald Statistic, 16 and 17 lncRNAs were identified to be significantly ($P < 0.05$) associated with OS and DFS, respectively (Figure 1A and Table 2). Among those, 9 were predictive of both OS and DFS, including 4 up- and 5 down-regulated lncRNA in poor prognosis iCCA (Figure 1A and Table 2). CDKN2B-AS1 and COL18A1-AS2 are two examples of such prognostic lncRNAs. Indeed, CDKN2B-AS1 median expression was able to segregate iCCA tumors into two groups, where a high expression of CDKN2B-AS1 was significantly associated with a reduced OS ($P = 0.008$) and DFS ($P = 0.002$). Conversely, highly COL18A1-AS2 expressing iCCA tumors were significantly associated with a better prognosis (Figure 1B). In addition, clustering analysis of 39

iCCA tumors based on the expression of the 9 lncRNA expression signature identified two clusters: cluster A and cluster B (Figure 1C). Validating this prognostic lncRNA signature, patients included in clusters A and B exhibited a significant difference in OS and DFS, as demonstrated by Kaplan Meier plot analysis and log-rank testing (Figure 1D). Cluster A was also associated with a significantly higher number of poorly-differentiated iCCA (SI Table 1).

CDKN2B-AS1 is a poor prognosis biomarker in cancer

Quantitative RT-PCR was performed to validate the microarray data. We focused on *CDKN2B-AS1* that exhibited a high variability among the samples and the more robust statistical significance (Figure 1C and Table 2). Thus, from our initial cohort of 39 iCCA, two groups were defined based on the expression of *CDKN2B-AS1*, including 15 highly expressing tumors and 15 tumors with low or no *CDKN2B-AS1* expression (Figure 2A). Validating our previous observation, a marked difference was observed in the OS of these two groups of patients (Figure 2A). No significant association with other clinical and pathological variables was identified in our cohort of iCCA (SI Table 2). More interestingly, a high expression of *CDKN2B-AS1* was shown to be significantly ($P < 0.05$) associated with a reduced survival of patients with hepatocellular carcinoma, kidney renal clear cell carcinoma and uterine corpus endometrial carcinoma (Figure 2B), identifying *CDKN2B-AS1* as a poor prognosis biomarkers in several cancers.

Discussion

CCA is a deadly cancer worldwide waiting for innovative biomarkers and therapeutic strategies to improve the survival of patients^{1,2}. In iCCA, several clinical variables have been identified as important prognostic factors after surgical resection for curative intent, including tumor size, pathological lymph node involvement and vascular invasion^{1,29,30}. Omics approaches (e.g. genomics, proteomics, metabolomics) have been extensively used to screen for novel candidate serum, bile and tissue molecular biomarkers in iCCA³⁰. As an example, by proteomic

analysis, a high expression of SSP411 in the serum and/or the bile has been significantly associated with iCCA diagnosis³¹. Serum and bile CA 19-9 level has been also associated with lymph node metastasis and OS although the specificity and sensitivity was highly heterogeneous³⁰. By focusing more specifically on the alterations of the gene expression in the stroma of iCCA^{4,32}, we previously identified and validated specific mRNA and associated proteins, including OPN, LOXL2 and epithelial cell adhesion molecule (EPCAM), as independent prognostic tissue biomarkers associated with a reduced OS of patients with iCCA after tumor resection^{4,5,28}. Here, we specifically focused on the expression of lncRNA and we identified a set of lncRNA predictive of OS (n=16) and/or DFS (n=17), among those 9 were predictive of both OS and DFS. Except for HAND2-AS1, HOXC13-AS, and CDKN2B-AS1, the identified lncRNA were poorly characterized. Our data indicated that HAND2-AS1 down-regulation in poor prognosis iCCA is predictive of DFS. Accordingly, HAND2-AS1 has been recently shown to inhibit cancer cell proliferation, migration, and invasion in esophagus squamous cell carcinoma and colorectal cancer by sponging oncogenic microRNA, including miR-21^{33,34}. HOXC13-AS (up-regulated in poor prognosis iCCA from our lncRNA signature) has been shown to positively affects cell proliferation and invasion in nasopharyngeal carcinoma by sponging miR-383-3p³⁵. CDKN2B-AS1, also known as antisense non-coding RNA in the INK4 locus (ANRIL), was identified as a prognostic biomarker, not only in iCCA, but also in hepatocellular, kidney and uterine corpus carcinomas. Very promisingly, the low expressing group in iCCA showed a very high long term survival. In clinical context, one can question the need for adjuvant chemotherapy in a population with such high survival, potentially sparing toxicity for patients who do not require such therapy.

ANRIL is located at the CDKN2A/B genomic locus on chromosome 9p21.3 in human³⁶. Interestingly, this locus is associated with an increased risk of cancer and metabolic disease, including type 2 diabetes, obesity and cardiovascular diseases³⁷. ANRIL expression and function have been extensively investigated in several cancers but not in iCCA³⁸. Here, we

demonstrated that ANRIL expression is associated with a dismal outcome. Although clinically relevant differences in the expression of ANRIL expression have been highlighted in iCCA tumor tissues in our study, it remains to be determined whether ANRIL is up-regulated in iCCA as compared to normal biliary epithelium, as it was previously reported in other cancers, including HCC³⁹. Such an induction of ANRIL may suggest a key role in iCCA carcinogenesis. Indeed, gain and loss of function experiments in cancer cells demonstrated that ANRIL lncRNA favors cell proliferation and invasion. *In vivo*, ANRIL expression is associated with an increase of tumor size and growth, as well as invasive and metastatic phenotypes. Mechanistically, ANRIL was initially shown to promote cell proliferation and to inhibit apoptosis by epigenetically silencing the CDKN2A/B genomic locus⁴⁰. In non-small cell lung cancer ANRIL was reported to silence kruppel like factor 2 (KLF2) and CDKN1A/P21 expression as well⁴¹. In gastric cancer, the impact of ANRIL on proliferation and apoptosis was mediated by the epigenetic silencing of miR-99a and miR-449a, two miRNAs known to target mTOR and CDK6/E2F1 pathways⁴². In ovarian⁴³ and bladder⁴⁴ cancers, ANRIL may induce BCL2 apoptosis regulator (BCL2) and repress CDKN2B/P15, BCL2 associated X, apoptosis regulator (BAX) and cleaved caspase-9, to promote cell cycle progression and inhibit apoptosis and senescence. In nasopharyngeal carcinoma, pro-tumorigenic features of ANRIL were associated with a glucose metabolism reprogramming and an induction of side-population stem-like cancer cells⁴⁵. In thyroid cancer, ANRIL promotes tumor cell invasion and metastasis through TGF- β /Smad signaling and EMT⁴⁶. In HCC, sponging of liver enriched miR-122-5p by ANRIL induces cell proliferation, metastasis and invasion⁴⁷. Interestingly, ANRIL was reported to contribute to paclitaxel resistance in lung cancer, partly through poly(ADP-ribose) polymerase (PARP) and BCL2 modulating mitochondrial pathway⁴⁸.

The clinical relevance of ANRIL expression has been evaluated in several types of cancers. Thus, a high ANRIL expression has been associated with TNM stage, advanced lymph node metastasis, tumor size, and was highlighted as an independent predictor of OS and/or DFS,

particularly in nasopharyngeal ⁴⁵, ovarian ⁴³ or lung ^{41,49} cancers. Interestingly, it was recently reported that ANRIL was significantly up-regulated in lung cancer tissues and serum samples compared with normal controls, suggesting that ANRIL may serve as a relevant circulating lncRNA biomarker ⁵⁰. Given the significant association of ANRIL and prognosis factors, it would be interesting to evaluate its expression in body fluids, including serum, urine and/or bile. Another point of interest is to understand the mechanisms by which the expression of ANRIL is increased in poor prognosis tumors. External risk factors, including lifestyle, nutrition or environment, have been shown to influence the expression of lncRNA ⁵¹. Thus, HOTAIR and MALAT1 have been the most studied lncRNA in the context of tobacco use in lung cancer ⁵¹. However, in our analysis, no statistically significant association was identified between the expression of ANRIL and lifestyle-associated factors (e.g. obesity, diabetes, tobacco, alcohol). Interestingly, ANRIL was shown to be transcriptionally induced by the cell cycle associated E2F1 transcription factor ⁴², notably in an ATM-dependent manner following DNA damage ⁵², suggesting a positive regulation in highly proliferative tumors. In addition, MYC oncogene was shown to positively regulate ANRIL expression in lung cancer ⁵³. Epigenetic regulation of ANRIL through promoter methylation has been also reported ⁵⁴.

Altogether, these data suggest that ANRIL may represent not only a clinically relevant biomarker for cancer diagnosis, prognosis and response to treatment, but also an innovative therapeutic target due to the well described pro-tumorigenic features of this lncRNA.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the institutional review board of Inserm (IRB00003888).

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Figure legends

Figure 1. Identification of a prognostic lncRNA signature in iCCA.

Gene expression profiling of lncRNA was performed from 39 resected iCCA tumors. (A) Venn diagram analysis of lncRNA significantly ($P < 0.05$) associated with overall (OS) and/or disease-free (DFS) survival. (B) Example of Kaplan-Meier plots for CDKN2B-AS1 and COL18A1-AS2 expression significantly associated with OS and DFS. (C) Clustering analysis of 39 iCCA samples based on the expression of the 9-lncRNA signature significantly associated with both OS and DFS. Two clusters, A and B, were identified. (D) Kaplan-Meier plots for iCCA samples from cluster A and cluster B.

Figure 2. CDKN2B-AS1 expression predicts a poor prognosis in several cancers.

(A) Q-RT-PCR of CDKN2B-AS1 was performed in iCCA cases that were further ranked into low ($n=15$) and high ($n=15$) expressing tumors. Kaplan-Meier plots for iCCA classified into low vs. high CDKN2B-AS1 expressing tumors. (B) Kaplan-Meier plot analysis (OS) based on the expression of CDKN2B-AS1 in independent gene expression datasets from the TCGA consortium.

Supplementary Figure 1. Histological features of iCCA.

All iCCA tumors ($n=39$) were analyzed at the histological level on tissue sections ($10\mu\text{m}$) before RNA extraction and gene expression profiling. Shown are 3 examples of iCCA with low (upper panels), moderate (middle panels) and high (lower panels) stroma abundance, as evidenced by hematoxylin and eosin staining (left panels) and Sirius red staining (right panels). Scale bar: $250\mu\text{m}$.

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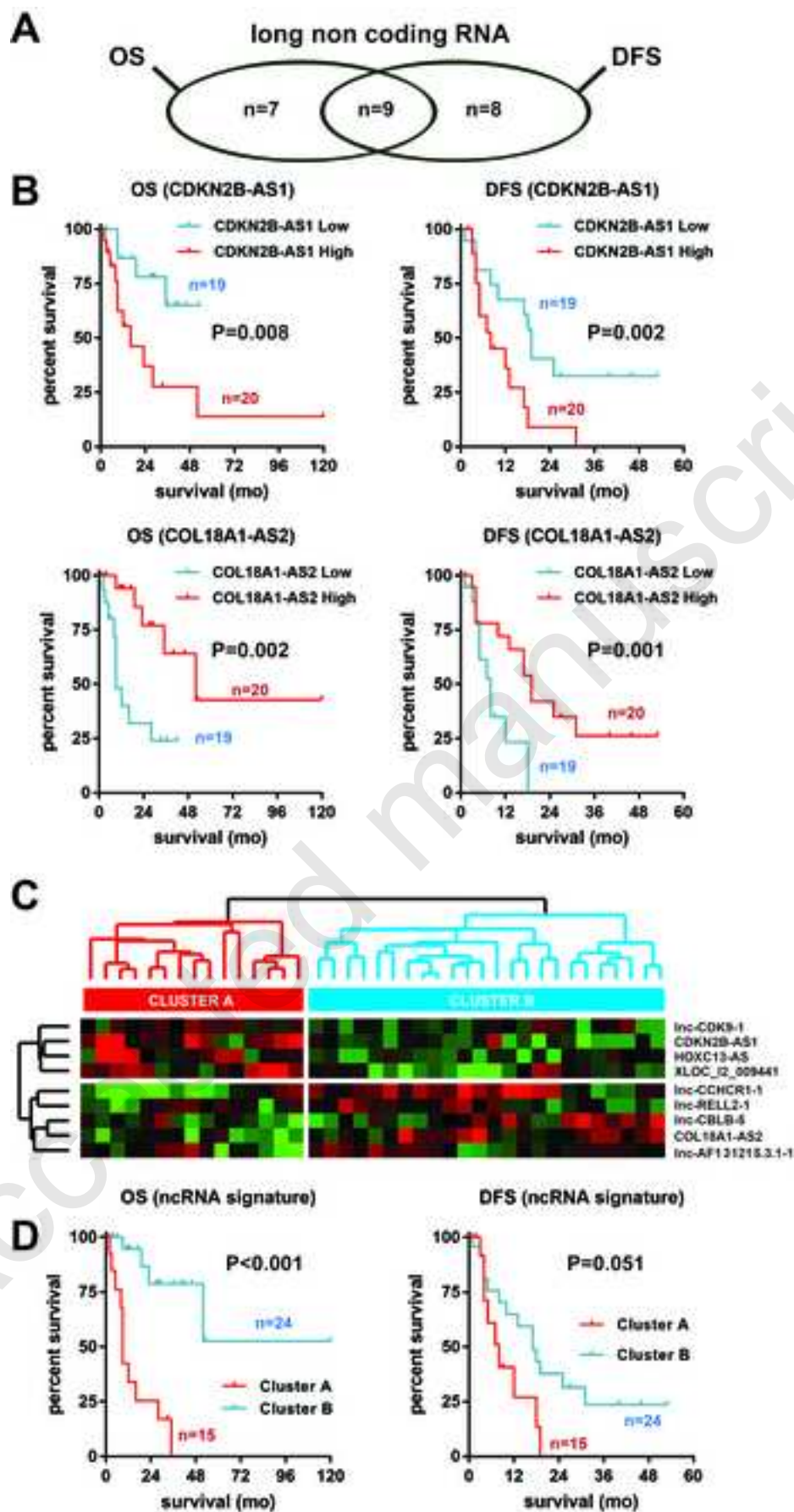


FIGURE 1

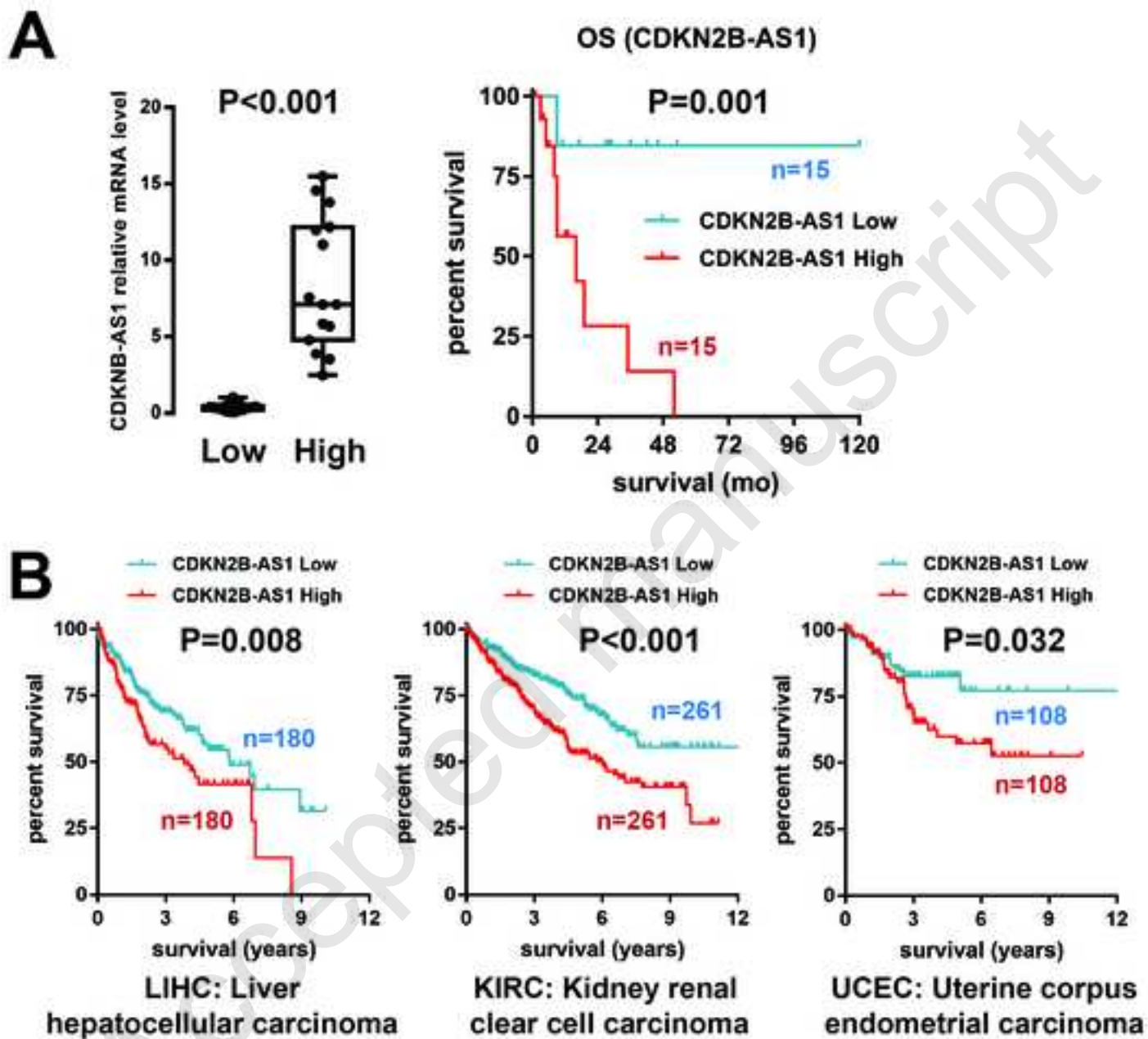


FIGURE 2

Table 1. Clinical and pathological features of iCCA patients.

Clinical and pathological features	n=39
Age (years, mean±SD)	61±14
Gender (male/female)	27/12
BMI	26±3
Tobacco use	18 (46%)
Diabetes	7 (18%)
Etiology	
HBV or HCV infection	5 (13%)
Alcohol	3 (8%)
Hemochromatosis	1 (3%)
Primary biliary cirrhosis	1 (3%)
Histologically normal liver	3 (8%)
Association of factors	6 (15%)
Undetermined	20 (51%)
Metavir fibrosis score (F0/F1/F2/F3/F4/na)	7/6/6/4/6/10
Cirrhosis	6 (15%)
Steatosis	21 (54%)
Tumor size (mm, mean±SD)	63±27
Tumor ≥50 mm	27 (69%)
OMS differentiation grade	
Well-differentiated	15 (38%)
Moderately-differentiated	17 (44%)
Poorly-differentiated	7 (18%)

Satellite nodules > 1	19 (49%)
Tumor necrosis	16 (41%)
Microvascular tumor invasion	20 (51%)
Portal invasion	6 (15%)
Biliary tract invasion	10 (26%)
Perineural invasion	15 (38%)
Capsular invasion	8 (21%)
Lymph nodes invasion	11 (28%)
Extrahepatic tissue invasion	10 (26%)

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Table 2. lncRNA signature predictive of overall (OS) and disease-free (DFS) survival.

Probe ID	Gene	Cytoband	OS		DFS	
			HR [†]	p-value [‡]	HR [†]	p-value [‡]
<u>lncRNA up-regulated in poor prognosis iCCA</u>						
A_21_P0006229	lnc-CDK9-1	hs 9q34.11	3.83	0.007	3.65	0.030
A_21_P0012328	XLOC_I2_009441	hs 22q11.1	1.99	0.018	2.01	0.010
A_19_P00322815	CDKN2B-AS1	hs 9p21.3	1.86	0.008	2.06	0.002
A_22_P00019478	HOXC13-AS	hs 12q13.13	1.83	0.047	1.75	0.048
A_21_P0014397	RALY-AS1	hs 20q11.22	24.36	0.002		ns
A_22_P00018189	lnc-ZNF609-1	hs 15q22.31	3.39	0.036		ns
A_22_P00001518	LOC101929064	hs 4q21.3	3.23	0.051		ns
A_23_P431569	LOC100049716	hs 12p13.33	2.12	0.004		ns
A_21_P0010648	XLOC_I2_001086	hs 1q21.1	2.03	0.044		ns
A_32_P850562	ZNF337-AS1	hs 20p11.1	1.88	0.022		ns
A_21_P0004202	lnc-C5orf38-1	hs 5p15.33		ns	3.13	0.009
A_21_P0009376	lnc-NLGN2-1	hs 17p13.1		ns	2.82	0.005
<u>lncRNA down-regulated in poor prognosis iCCA</u>						
A_21_P0005160	lnc-CCHCR1-1	hs 6p21.33	0.72	0.001	0.71	0.005
A_24_P312325	lnc-AF131215.3.1	hs 8p23.1	0.65	0.005	0.56	0.000

A_21_P0003146	Inc-CBLB-5	hs 3q13.12	0.60	0.020	0.56	0.024
A_22_P00025222	COL18A1-AS2	hs 21q22.3	0.55	0.002	0.55	0.001
A_22_P00012968	Inc-RELL2-1	hs 5q31.3	0.39	0.003	0.38	0.001
A_21_P0005161	Inc-CCHCR1-1	hs 6p21.33	0.59	0.001	ns	
A_22_P00004717	Inc-CTDSPL2-2	hs 15q21.1		ns	0.37	0.034
A_22_P00014082	HAND2-AS1	hs 4q34.1		ns	0.46	0.049
A_22_P00015084	Inc-SNURF-3	hs 15q11.2		ns	0.49	0.014
A_21_P0008621	Inc-EIF2AK4-2	hs 15q15.1		ns	0.64	0.032
A_22_P00010275	Inc-MTMR9-1	hs 8p23.1		ns	0.68	0.040
A_22_P00017999	Inc-ZNF132-1	hs 19q13.43		ns	0.71	0.046

Footnotes: † Hazard Ratio; ‡ p-value, as determined by a log-rank test; ns: not significant

Footnotes: † number of patients in each tested group (n=15 patients and n=24 patients for clusters A and B, respectively; although clinical data were missing for some patients); ‡ p-value, as determined by a Fisher's exact test

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Footnotes: † number of patients in each tested group (n=15 patients per group although clinical data were missing for some patients); ‡ p-value, as determined by a Fisher's exact test

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