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Research Paper

Lnc-PCDH9-13:1 Is a Hypersensitive and Specific Biomarker for Early Hepatocellular Carcinoma

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ABSTRACT

Background: Long non-coding RNAs (lncRNAs) show great potential as diagnostic tools in many diseases. We aimed to develop sensitive and noninvasive biomarkers in saliva for detecting early hepatocellular carcinoma (HCC).

Methods: Candidate lncRNA biomarkers identified by Agilent microarray were subjected to validation using qPCR for the quantification of their expression levels in independent tissue, plasma and saliva sample sets, including healthy controls, HBsAg carriers, patients with chronic Hepatitis B, liver cirrhosis, early HCC, and advanced HCC. Levels of candidate biomarkers were also measured in totally 108 saliva samples from patients with any one of other nine leading causes of cancer death in men and women.

Findings: Lnc-PCDH9-13:1 was significantly elevated in HCC tissues, plasma and saliva of HCC patients compared with healthy controls and groups of several benign liver diseases and other leading cancers. Its level was significantly reduced after curative hepatectomy but significantly elevated again if HCC recurrence occurred. Salivary lnc-PCDH9-13:1 showed reasonable specificities and sensitivities for detecting HCC compared with several control groups. Furthermore, the overexpression of lnc-PCDH9-13:1 promotes cell proliferation and migration in vitro.

Interpretation: Salivary lnc-PCDH9-13:1 is a desirable biomarker for early HCC. It may help warrant prospective validation with larger sample sizes in multi-centers.

1. Introduction

Liver cancer, over 90% of which is hepatocellular carcinoma (HCC) categorized by pathology, is the second leading causes of cancer death worldwide in men and the sixth in women, with an overall 5-year survival rate of 5%–9%. An estimated 782,500 new liver cancer cases and 745,500 deaths occurred worldwide during 2012, with China alone accounting for about 50% of the total new cases and deaths [1]. In the USA, incidence rates continue to increase rapidly for liver cancer with totally 42,220 new cases and 30,200 deaths expected to occur in 2018 [2]. In England, the incidence of liver cancer was higher in all ethnic groups compared to Whites, with the highest rates seen in Chinese (four times higher) [3]. Thus, liver cancer is one of the most fatal cancers in China, the USA, and the world to date. Curative hepatectomy can improve the 5-year survival rate to 69%, but unfortunately, approximately 80% of patients with HCC are untreatable because of advanced tumor stages at presentation [4, 6, 30]. Hepatitis B virus (HBV) which infects approximately 350 million people worldwide, is the major risk factor of HCC [7] and it accounts for up to 70% of all HCC patients [8]. Patients with chronic hepatitis B develop cirrhosis at a rate of approximately 2%–10% per year, leading to HCC in 80% to 90% of individuals finally [9]. Over 5% of the populations in Asia and sub-Saharan Africa chronically infected with the virus. Accordingly, early diagnosis and surgery are vital for treating HCC.

Studies suggest that long non-coding RNAs (lncRNAs) (≥200 bp) in HCC tissues and blood of patients with HCC show good diagnostic accuracy in detecting HCC [10]. Tissues may secrete lncRNAs into the blood by necrosis and apoptosis. Owing to the extensive blood supply in salivary glands, saliva is considered a terminal product of blood circulation. Saliva may include many components that are derived from blood, because salivary acinar cells produce saliva using blood materials. Hence, saliva can play diagnostic roles in various diseases [11].

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We aimed to identify novel and noninvasive lncRNA biomarkers in saliva to help diagnose HCC.

2. Materials and Methods

2.1. Patients and Samples

The study was conducted mainly according to the updated Standards for Reporting of Diagnostic Accuracy (STARD) 2015 reporting guideline for diagnostic accuracy studies [12]. The Healthy controls were defined as individuals with negative results by health examinations, including chest X-rays, oral examinations, abdominal ultrasounds, faecal occult-blood testing, blood cancer biomarker assays (AFP, CEA, CA19–9), HBV antigen, HCV, HIV, and syphilis antibodies. Inactive HBsAg carriers (IHC) had persistent HBV infection of the liver (positive HBsAg over 6 months with serum HBV DNA $<10^5$ copies/ml) and no laboratory indications of ongoing necroinflammatory hepatic functions: persistently normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations. Chronic hepatitis B (CHB) was defined as chronic necroinflammatory liver function caused by persistent HBV infection (positive HBsAg over 6 months with serum HBV DNA $>10^5$ copies/ml and persistent or intermittent elevation in AST or ALT concentrations). Patients with liver cirrhosis (LC) were confirmed by biopsy or two imaging modalities (hepatic ultrasound with CT or MRI) [13]. Terminal HCC (Stage IV) was diagnosed based on biopsy of the tumor or CT/MRI [14]. The histopathology results of the patients with the HCC (Stages I, II, III), lung, stomach, colon & rectum, prostate, esophagus, pancreas, leukemia, urinary bladder, non-Hodgkin lymphoma, breast, cervix uteri, ovary cancers, which were 10 leading causes of cancer death in men and women worldwide nowadays [1], were confirmed by pathology after surgical tumor resection or biopsy. Patients with other cancers and benign liver diseases were evaluated with regard to marker specificity. Normal human liver tissues were obtained from distal normal liver tissues of liver hemangioma. The normal liver tissues from liver hemangioma were also determined by pathology. No concurrent oral, infectious, autoimmune diseases and diabetes mellitus were diagnosed in all participants. All HCC patients were positive with HBsAg. Cancer patients with a diagnosis of concurrent two cancers and those undergoing chemotherapy and radiotherapy prior to sample collection were also excluded. Cancers were staged according to The American Joint Committee on Cancer: 8th Edition on Cancer Staging [15]. 100 saliva samples was obtained from 50 patients with early HCC over 1 week before and after curative surgery, and saliva from 6 HCC patients among the 50 patients with early HCC was available at the time of documented HCC recurrence with the use of radiographic imaging and, usually, pathological confirmation of recurrence (Fig. 1). Our previous study show adjacent HCC tissues were infiltrated with inflammatory cells, cytokines and cirrhosis [16]. Hence, the majority of adjacent HCC tissues were not "normal" liver tissues. So the levels of lncRNAs in adjacent HCC tissues were not measured and compared since plasma and saliva levels of the candidate lncRNA biomarkers were compared with those of normal healthy controls. The tissue, plasma and saliva samples from all participants were collected consecutively and retrospectively if they met the inclusion criterion between April 2011 and August 2016 at The Third Affiliated Hospital of Sun Yat-sen University.

Institutional review boards or ethics committees from our hospital approved the study protocol. All participants provided written informed consent for their information to be stored in the hospital database and used for research.

2.2. Procedures

Saliva samples were collected as previously described [17]. After the tissue, plasma and saliva samples were collected and stored in the −80 °C lab freezers, the following procedures were finished with seven days. Total RNAs were extracted from frozen liver tissues using TRIzol (Thermo Fisher Scientific, USA), and total RNAs in 1.2 ml of plasma or saliva were isolated by the mirVana PARIS Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocols. The lncRNA profiles of the three HCC liver tissues and three normal liver tissues assayed using Agilent Technologies, USA. The measurements of lncRNAs by quantitative polymerase chain reaction (qPCR) were performed as previously described [18]. Each qPCR reaction contained negative controls included no template control, no reverse transcriptase control, and no amplification control. All reactions including controls were carried out in triplicate. Ct values >35 were excluded from the analyses. The expression levels of each lncRNA were normalized to that of *β-actin*. All expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The indeterminate and missing data were excluded, and qualified samples would be made up the original sample sizes. The expression of candidate lncRNAs in paraffin sections was detected in situ hybridization (ISH) according to the manufacturer's instructions of the probes (Exiqon, Denmark). The expression of APF in paraffin sections was assayed by immunohistochemistry (IHC) with primary antibody purchased from Gene Company, Hong Kong. Cells with clear brown staining were regarded as positive cells. The APF levels were calculated by enzyme-linked immunosorbent assay (ELISA) with antibody obtained from Cloud-Clone Corp, USA. All experiments were performed at least three times on different days and in triplicate. The sequences of each primer and probe, and detailed

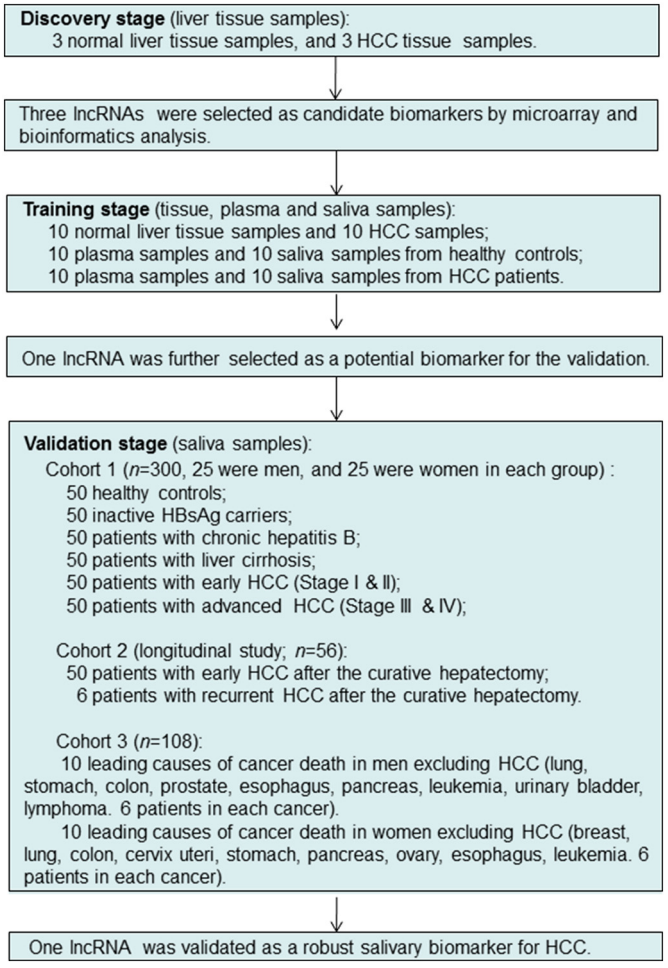

Fig. 1. Study design. Our study consisted of three stages. Candidate lncRNA biomarkers were selected by microarray and bioinformatics analysis. The levels of the selected candidate biomarkers in tissue, plasma and saliva samples were measured by qPCR and analyzed by biostatistics. The diagnostic performances of the selected lncRNAs were validated in three cohorts.

methods of ISH, IHC and ELISA are presented in the Supplementary material.

HCC cell lines, HepG2 and HepG2.2.15 were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Cells were seeded into 96-well plates at a density of 3×10^4 cells per well after transfection. Cell proliferation was examined by the Cell Counting Kit-8 (CCK8) (Dojindo Laboratories, Japan) according to the manufacturer's protocol. The CCK8 optical density (OD) was read at 450 nm for 4 consecutive days. Cell migration was examined using transwell chambers (Corning, USA). The transfected cells at a density of 1×10^5 in 200 μl serum-free medium were seeded to the upper chamber, and DMEM containing 20% fetal bovine serum were added to the bottom chamber. After 24 h, the microscope was used to count the migration cells. The transfected cells were seeded into the 6-well plates at a density of 1×10^6 and grown to reach 90% confluences overnight. Used the 100 μl pipette tip to produce the artificial wound, and washed gently with PBS three times, then cultured in DMEM without FBS. Images were photographed with the inverted microscope at 0 and 24 h. ImageJ software (National Institutes of Health, Bethesda, USA) was used to analyze the wound areas.

2.3. Statistical Analysis

LncRNA expression levels were compared using the Mann Whitney *U* test or Kruskal-Wallis H test. The differences of the lncRNA before and after surgery were studied by Wilcoxon signed-rank test. Receiver-operating characteristic (ROC) curves were used to evaluate the discriminatory power of each lncRNA for differentiating between two groups. Optimal cut-off values of the salivary biomarker were determined by the Youden index (Youden index = sensitivity + specificity − 1). When the Youden index reaches the maximum value, the corresponding cut-off value will yield the highest sum of sensitivity and specificity. The correlation between two groups was analyzed using the Spearman's correlation test. Statistical analyses were performed using the SPSS software (ver. 13.0). A two-tailed *P* value <0.05 was considered statistically significant.

The experiments and analysis were performed by three investigators working independently. All samples were procured in blinded fashion with regard to which groups they were obtained from. Investigators had no knowledge of the patient's groups. The experiments were performed in blinded ways, with subsequent data unblinded and analyzed by other co-investigators. All authors ensure the accuracy and completeness of the data and analysis and the fidelity to the technological and biostatistical protocols of this study. No adverse events occurred during performing all the tests.

3. Results

3.1. Patient Characteristics

The characteristics of the study participants are presented in Table 1. There was no significant difference in the distribution of age and sex for the four groups: healthy, inactive HBsAg carrier (IHC), chronic hepatitis B (CHB), liver cirrhosis (LC), early HCC and late HCC. All healthy, IHC, and LC participants showed normal level of serum AFP. But 31 out of 50 (62%) patients with CHB exceeded the normal level. About half of patients with early HCC and terminal HCC showed exceedingly elevated AFP levels, while another half of patients showed normal levels. The tumor sizes of early HCC were <5 cm in greatest dimension with no >3 tumors in the livers. Although 12 out of 50 terminal HCC showed smaller 5 cm in tumor size, unfortunately, a major branch of the portal or hepatic vein with direct invasion of adjacent or distal organs were detected by imaging modalities or biopsy.

Table 1
Characteristics of study participants.

Group	Variable	No.	Group	Variable	No.
Healthy	Age, years		**HCC (I, II)**	Age, years	
(*n* = 50)	Mean	55	**(*n* = 50)**	Mean	56
	SD	10		SD	11
	Sex			Sex	
	Male	25		Male	25
	Female	25		Female	25
	Serum AFP			Stage	
	<20 ng/ml	50		I	16
	>20 ng/ml	0		II	34
IHC (*n* = 50)	Age, years			Serum AFP	
				<20 ng/ml	24
	Mean	49		=20–400 ng/ml	10
	SD	13		>400 ng/ml	16
	Sex			Tumor size	
	Male	25		<5 cm	50
	Female	25		>5 cm	0
	Serum AFP			HBV DNA	
	<20 ng/ml	50		Detected	22
	>20 ng/ml	0		Not detected	28
CHB (*n* = 50)	Age, years		**HCC (III,IV)**	Age, years	
	Mean	46	**(*n* = 50)**	Mean	56
	SD	10		SD	12
	Sex			Sex	
	Male	25		Male	25
	Female	25		Female	25
	Serum AFP			Serum AFP	
	<20 ng/ml	19		<20 ng/ml	25
	=20–400 ng/ml	29		=20–400 ng/ml	12
	>400 ng/ml	2		>400 ng/ml	13
				Stage	
LC (*n* = 50)	Age, years			III	16
	Mean	49		IV	34
	SD	11		Tumor size	
	Sex			<5 cm	12
	Male	25		>5 cm	38
	Female	25		HBV DNA	
	Serum AFP			Detected	32
	<20 ng/ml	50		Not detected	18
	>20 ng/ml	0			

Abbreviations: AFP, alpha fetoprotein; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; IHC, inactive HBsAg carrier; LC, liver cirrhosis; SD, standard deviation.

3.2. The Selection of Candidate lncRNA Biomarkers for HCC

Microarray results showed that numerous lncRNAs were dysregulated in HCC tissues (Fig. 2a). The raw data of the microarray results and the detailed methods of the microarray can be downloaded in Gene Expression Omnibus (Access number GSE98269).According to the bioinformatic analysis of lncRNA-gene network, lnc-PCDH9-13:1 (Current NCBI gene ID: LINC00355), lnc-GGT1-6:1 (NCBI ID: EST 00000470591), lnc-ARRDC3-1:16 (NCBI ID: LUCAT1) played a pivotal role in HCC development with dozens of cancer genes (Fig. 2b). And no studies have reported they were also dysregulated in other cancers. Accordingly, those lncRNAs were selected as candidate biomarkers for HCC (Fig. 2c). Next, the expression levels of these three lncRNAs were measured by qPCR. 10 HCC tissue samples, plasma samples and saliva sample were from the same 10 HCC patients. Compared with normal liver tissues, lnc-PCDH9-13:1, lnc-GGT1-6:1, lnc-ARRD3-1:16 were significantly upregulated in HCC tissues according to the qPCR results (Fig. 3a). The deregulation patterns of these candidate biomarkers were consistent with those from microarray results. Compared with healthy controls, lnc-PCDH9-13:1 was significantly elevated in plasma (Fig. 3b) and saliva of HCC patients (Fig. 3c), but lnc-GGT1-6:1, lnc-ARRD3-1:16 showed no significant differences between the two groups. Hence, lnc-PCDH9-13:1 was chosen as potential salivary biomarkers and measured further in other cohorts with larger sample sizes.

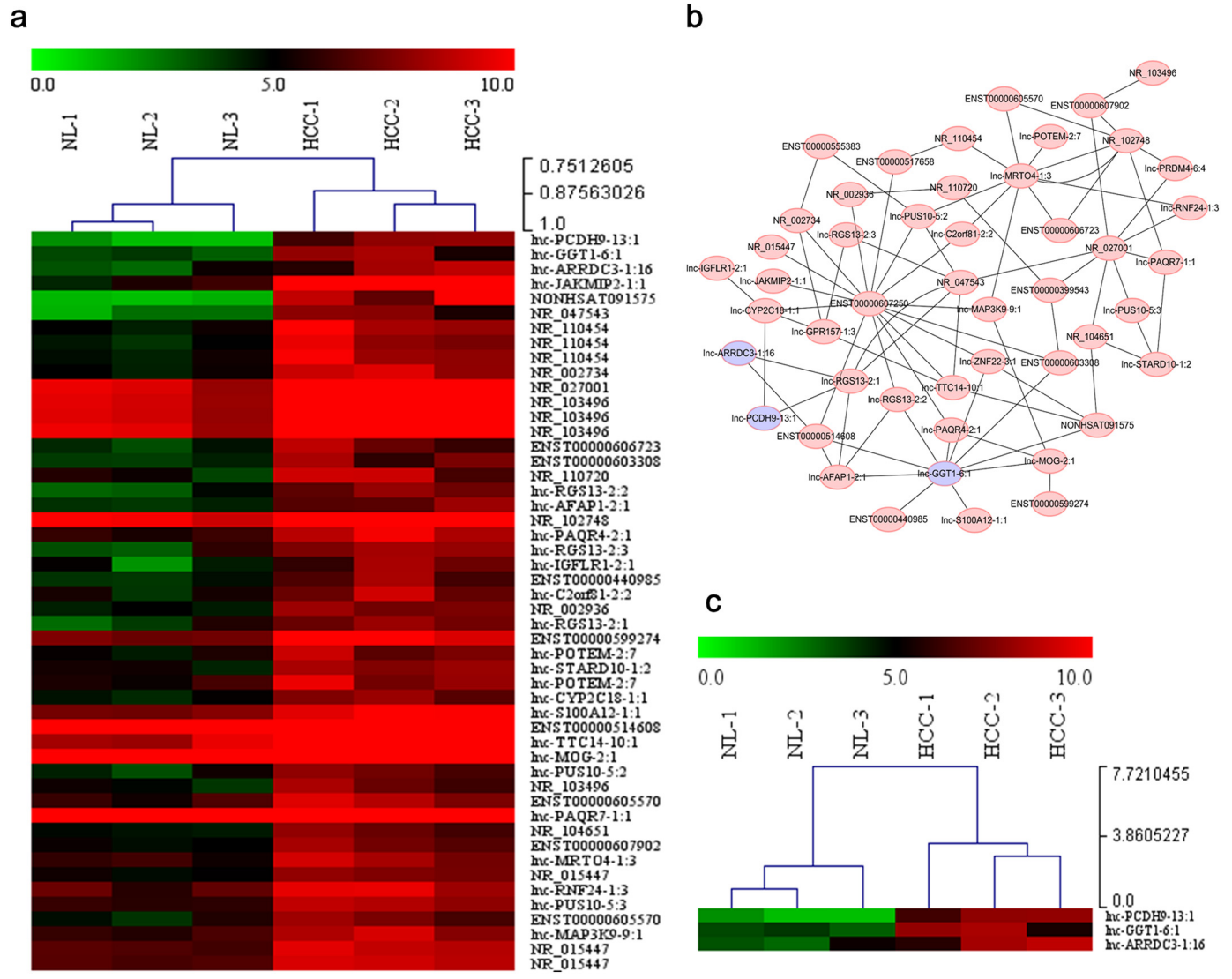

Fig. 2. Candidate lncRNA biomarkers selection by microarray results and bioinformatic analysis. (a) Microarray results showed that a number of lncRNAs were dysregulated in HCC tissues compared with normal liver tissues. According to the microarray results, the levels of lnc-PCDH9-13:1, lnc-GGT1-6:1 and lnc-ARRDC3-1:16 in HCC tissues were over 10-fold higher with $P < 0.001$ than those in normal liver tissues, and were not reported to be dysregulated in other cancers. (b) Through lncRNA-Gene network, lnc-PCDH9-13:1, lnc-GGT1-6:1 and lnc-ARRDC3-1:16 correlated with numbers of pivotal genes in HCC. (c) Taken together, these three lncRNAs were selected as candidate biomarkers for HCC and there expression levels would be measured and analyzed in further experiments with larger sample sizes. NL denotes normal liver.

3.3. The Validation of Candidate lncRNA Biomarkers

Immunohistochemical results showed that α-fetoprotein (AFP) expressed in very low levels in normal liver tissues, liver tissue with chronic hepatitis B, liver cirrhosis, and HCC tissues with serum AFP <20 ng/ml. AFP was in mild positive levels in HCC tissues of patients with serum AFP = 20–400 ng/ml and was in strong positive levels in HCC tissues with AFP >400 ng/ml (Fig. 4a). Additionally, by the ELISA results, significant correlation was observed in early HCC, late HCC, and total HCC cases between saliva AFP and serum AFP (Fig. 4b). These results demonstrated that AFP expression in HCC tissue is consistent with serum and saliva AFP levels of in the same patients. The results of in situ hybridization results showed that lnc-PCDH9-13:1(Fig. 5a) was expressed in low levels in normal liver tissues, liver tissue with chronic hepatitis B and liver cirrhosis, but significantly higher in 3 types of HCC tissues, categorized by three groups of serum AFP levels (<20 ng/ml, =20–400 ng/ml, >400 ng/ml). Additionally, the mRNA expression of the reference gene *β-actin* was also detected by ISH as comparison. The results were presented in Fig. S1. As shown in the figure, the positive signals both RNAs looked similar, so it indicated that the signals were convincing. The results of agarose gel electrophoresis (Fig. 5b) and sequencing (Fig. 5c) demonstrated that the expression levels of lnc-PCDH9-13:1 in tissue, plasma and saliva could be readily and specifically detected by qPCR. Compared with healthy controls, inactive HBsAg carriers (IHC), chronic hepatitis B (CHB), and liver cirrhosis (LC), salivary lnc-PCDH9-13:1 was significantly elevated in early (Stages I & II), terminal (Stages III & IV) and total cases of HCC patients. Salivary lnc-PCDH9-13:1 was also significantly overexpressed in HCC when healthy individuals, inactive HBsAg carriers, patients with chronic hepatitis B (CHB), and liver cirrhosis (LC) were chosen as control groups, respectively (Fig. 6a). Salivary lnc-PCDH9-13:1 was significantly reduced after curative operation, but significantly increased again after HCC recurrence (Fig. 6b). 10 HCC tissue, 10 plasma and 10 saliva samples were procured from the same 10 HCC patients. Spearman's correlation tests suggested that levels of lnc-PCDH9-13:1 in tissue correlated significantly with those in plasma and saliva (Fig. 6c).

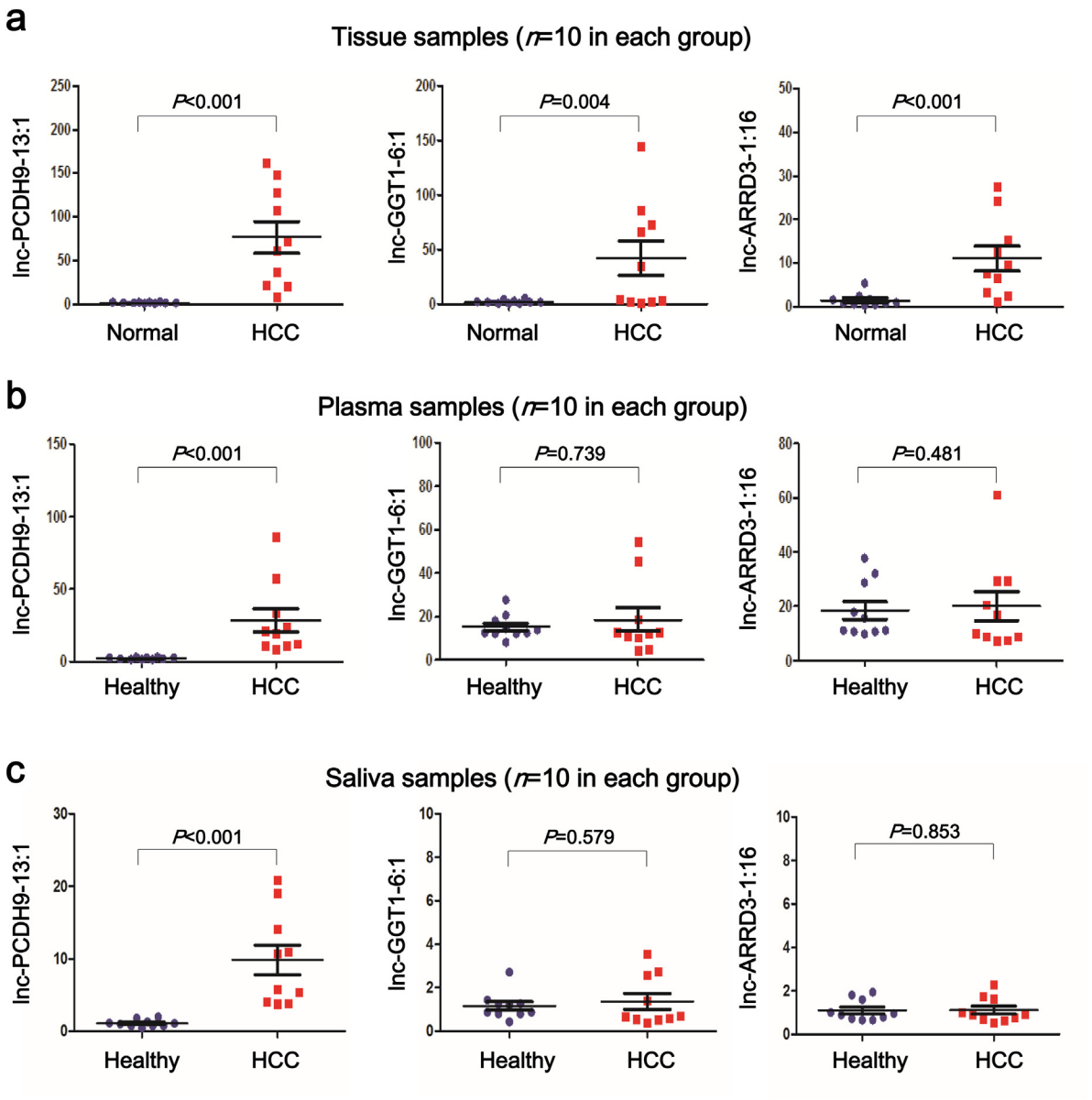

Fig. 3. The differential expression levels of candidate lncRNA biomarkers in liver tissue, plasma and saliva. (a)Compared with normal liver tissues, lnc-PCDH9-13:1, lnc-GGT1-6:1, lnc-ARRD3-1:16 were significantly upregulated in HCC tissues. (b) Compared with healthy controls, lnc-PCDH9-13:1 were significantly elevated in plasma of HCC patients, but lnc-GGT1-6:1, lnc-ARRD3-1:16 showed no significant differences between the two groups. (c) Salivary lnc-PCDH9-13:1 was significantly overexpressed in HCC patients, while the other two lncRNAs did not differ significantly between the two groups.

3.4. The Diagnostic Performances of the Salivary lncRNA Biomarkers for HCC

In order to evaluate the marker specificities, salivary levels of lnc-PCDH9-13:1in patients of 10 leading causes of cancer death excluding HCC in men and women worldwide nowadays were also measured. According to global cancer statistics,[1] 10 leading causes of cancer death in men were lung, liver, stomach, colon, prostate, esophagus, pancreas, leukemia, urinary bladder, non-Hodgkin lymphoma. And 10 leading causes of cancer death in women were breast, lung, colon, cervix uteri, stomach, liver, pancreas, ovary, esophagus, leukemia. The results suggested that salivary lnc-PCDH9-13:1 in healthy controls was similar to those in patients of 10 leading causes of cancer death in men and women excluding HCC (Fig. 7a). Through the analysis of ROC curves, salivary lnc-PCDH9-13:1 could detect HCC with sensitivities and specificities of over 85% compared with different control groups (Fig. 7b). ROC curves also indicated that salivary lnc-PCDH9-13:1 showed over 80% of sensitivities and specificities for detecting HCC with different serum AFP levels (Fig. 7c). Taken together, salivary lnc-PCDH9-13:1 showed a better diagnostic value than that of serum AFP.

3.5. Overexpressed lnc-PCDH9-13:1 Promoted Cell Proliferation and Migration In Vitro

The HepG2 and HepG2.2.15 cells were transfected with lnc-PCDH9-13:1 lentiviruses to develop stably over-expressed cell lines of lnc-PCDH9-13:1 (Fig. S2A). To primarily investigate the functions of lnc-PCDH9-13:1 in hepatocarcinogenesis, we observed its role in cell proliferation and migration. The CCK8 assay showed that lnc-PCDH9-13:1 overexpression remarkably promoted cell proliferation ability compared with the vector group in HCC cells (Fig. S2B). The transwell assay showed the number of migratory HepG2 and HepG2.2.15 cells increased after over expression of lnc-PCDH9-13:1, and indicated that cell migration ability was enhanced (Fig. S2C, D). In the wound healing

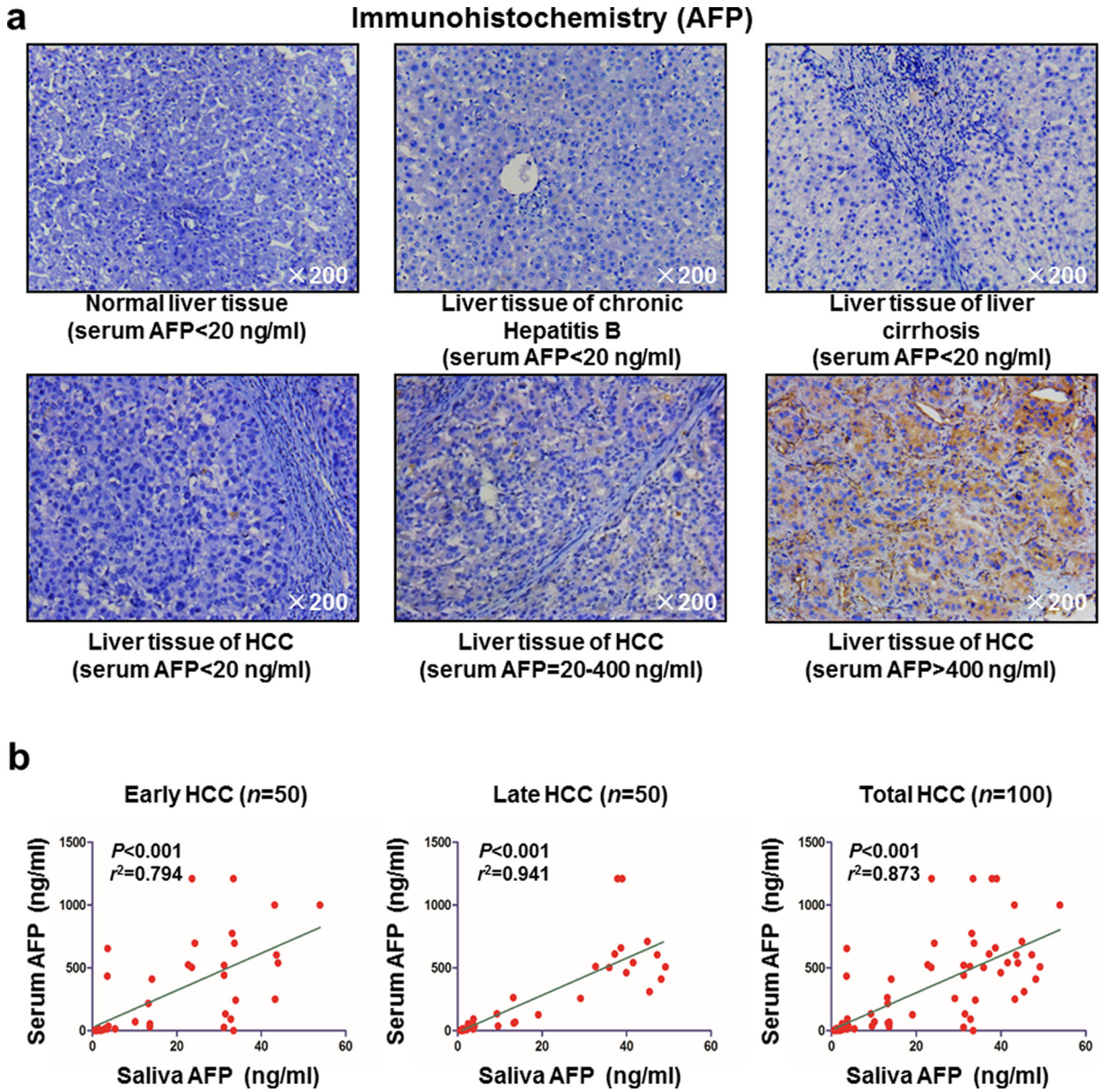

Fig. 4. The expression of AFP in different liver tissues measured by immunohistochemistry. (a) Immunohistochemical results showed that AFP expressed in very low levels in normal liver tissues, liver tissue of chronic hepatitis B, liver cirrhosis, and HCC tissues with serum AFP < 20 ng/ml. AFP was in mild positive levels in HCC tissues of patients with serum AFP = 20–400 ng/ml, and was in strong positive levels in HCC tissues of patients with serum AFP > 400 ng/ml. (b)ELISA results indicated that significant correlation was observed in early HCC, late HCC, and total HCC cases between saliva AFP and serum AFP. Clear brown staining was regarded as positive cells. r^2 represents correlation coefficient.

assay, similar results were observed, and higher potential in wound healing was possessed in the HepG2 and HepG2.2.15 cells with overexpressed lnc-PCDH9-13:1 (Fig. S2E, F).

4. Discussion

To the best of our knowledge, this study primarily reports that salivary lncRNAs can serve as biomarkers in the diagnosis of early and AFP-negative HCC, and outperform serum AFP. This study suggested that the aberrant expression of salivary lnc-PCDH9-13:1 might aid in determining the diagnosis and prognosis of HCC. Bioinformatic analysis showed that lnc-PCDH9-13:1 played significant roles with dozens of cancer genes in HCC development. Lnc-PCDH9-13:1 were overexpressed in HCC tissue and plasma and saliva of HCC patients, and its levels in tissue correlated significantly with those in plasma and saliva. Sequencing results demonstrated that qPCR could readily and specifically detect the salivary lnc-PCDH9-13:1. It is not aberrantly expressed in some benign liver diseases and other nine leading causes of cancer death. So the specificities and sensitivities of salivary lnc-PCDH9-13:1 in detecting HCC may superior to the current well-established biomarker of HCC, AFP. Elevated lnc-PCDH9-13:1 in saliva could be found in patients with early HCC, and the similarity of lnc-PCDH9-13:1 levels in early- and late-stage HCC indicated that their aberrant expression might be associated with early events in HCC development. Additionally, upregulated salivary lnc-PCDH9-13:1 appeared to reflect the HCC recurrence. Last but not least, functional experiments of lnc-PCDH9-13:1 in vitro suggested that overexpressed lnc-PCDH9-13:1 promoted proliferation and migration. Collectively, salivary lnc-PCDH9-13:1 may be predominantly derived from HCC and selectively released to the bloodstream and saliva, and it may play a role in hepatocarcinogenesis.

Although Alpha-fetoprotein-L3 (AFP-L3) and Des-γ-decarboxyprothrombin (DCP) were reported to be promising biomarkers for HCC, heir diagnostic accuracy remains controversial, and has yet translated into clinical utility. AFP is still the only well-

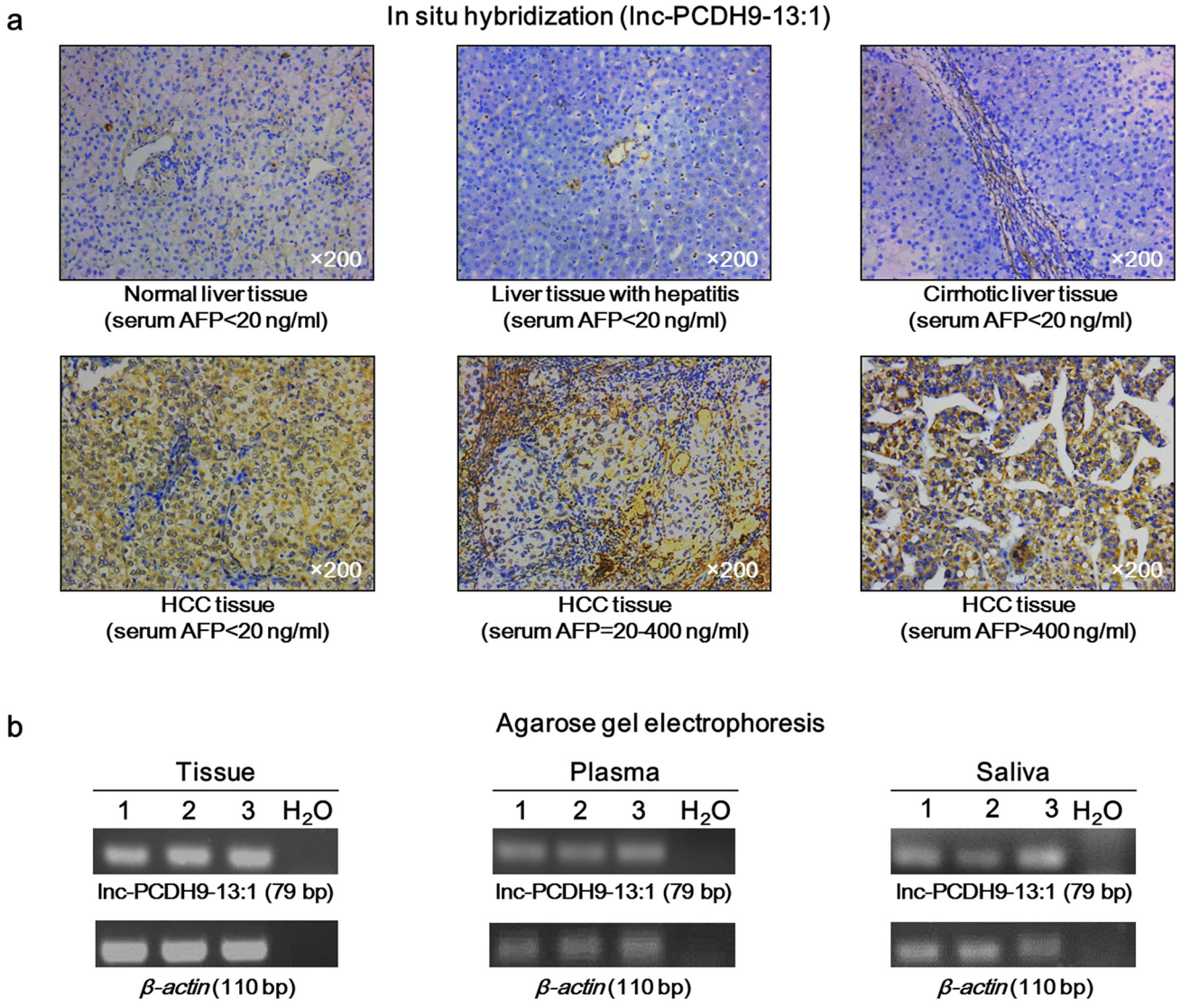

Fig. 5. Validation of candidate the lncRNA biomarker. (a) In situ hybridization results showed that lnc-PCDH9-13:1 was expressed in low levels in normal, hepatitis and cirrhotic liver tissues, but significantly higher in 3 types of HCC tissues, categorized by serum APF levels. Agarose gel electrophoresis (b) and sequencing results (c) demonstrated that in tissue, plasma, and saliva were readily and specifically detected by qPCR. Clear brown staining was regarded as positive cells.

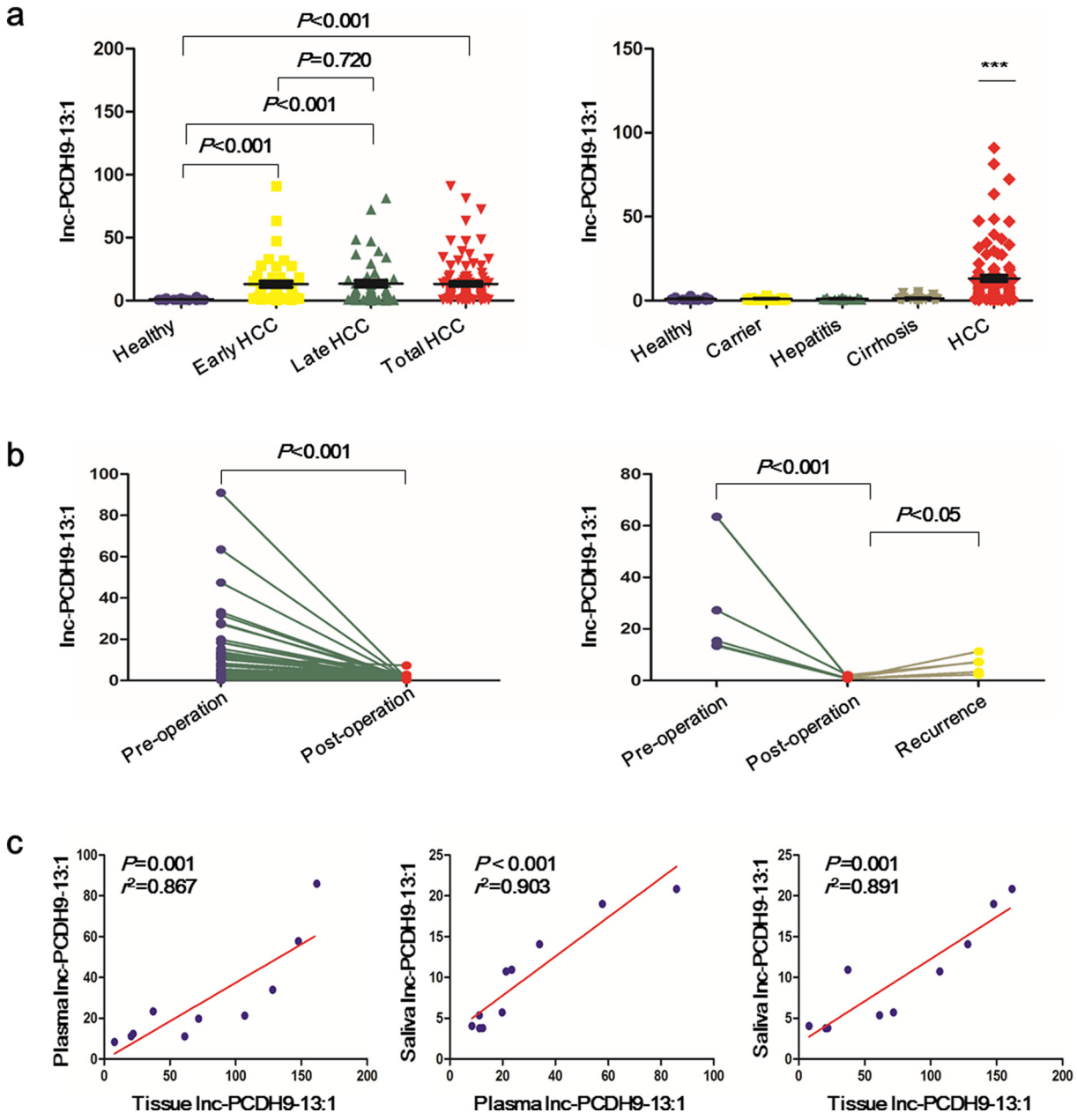

Fig. 6. The differential expression of salivary lnc-PCDH9-13:1. (a) Compared with healthy controls, inactive HBsAg carriers, chronic hepatitis B, and HBV-induced liver cirrhosis, salivary lnc-PCDH9-13:1 was significantly elevated in early, late, and total cases of HCC. (b) Salivary lnc-PCDH9-13:1 was significantly reduced after curative operation, but significantly increased after HCC recurrence. (c) According to the Spearman's correlation tests, the expression of lnc-PCDH9-13:1 in HCC tissue, plasma, and saliva of HCC patients correlated significantly with each other.

established biomarker for HCC in clinical practice [19]. However, serum AFP levels are elevated in benign liver diseases, such as hepatitis and cirrhosis. Hence, investigation of lnc-PCDH9-13:1 expression could outperform AFP in terms of helping diagnosing early-stage HCC, AFP-negative HCC and prognostic indicators. Salivary lnc-PCDH9-13:1 is a promising non-invasive and desirable biomarker for HCC. Interestingly, this study also indicated that significant correlation was observed among tissue, serum and saliva AFP. A previous study also reported strong correlation between saliva and serum AFP [5]. Hence, HCC may secret AFP into saliva by blood circulation. But the normal range of salivary AFP for healthy persons has yet determined due to lack large-scale studies.

Saliva as a multi-constituent oral fluid consists of secretions from the major and minor salivary glands, extensively supplied by blood. Molecules such as DNAs, RNAs, proteins, found in blood, could be also found in saliva. Thus, saliva is considered to be a terminal product of bloodstream. LncRNA is emerging as new regulators of diverse physiological functions. Importantly, the differential expression of lncRNAs, together with other molecular defects, plays a significant role in oncogenesis and tumor development. A study [20] reported that lncRNAs could remain stable through they were treated with freeze-thaw cycles, low/high pH, strong acid, strong base, high temperature and RNase A. Thus, lncRNAs are very attractive for the development of new target therapies and appear to be significant for the discovery of new disease-specific diagnostic makers in bodily fluids, including saliva. The term “salivaomics” was created to highlight the omics constituents in saliva that can be used for biomarker development and personalized medicine. Numerous molecules isolated from saliva have been proposed as disease biomarkers for diagnosis, prognosis, drug monitoring and pharmacogenetic studies [21]. Molecules in tumor can be shed

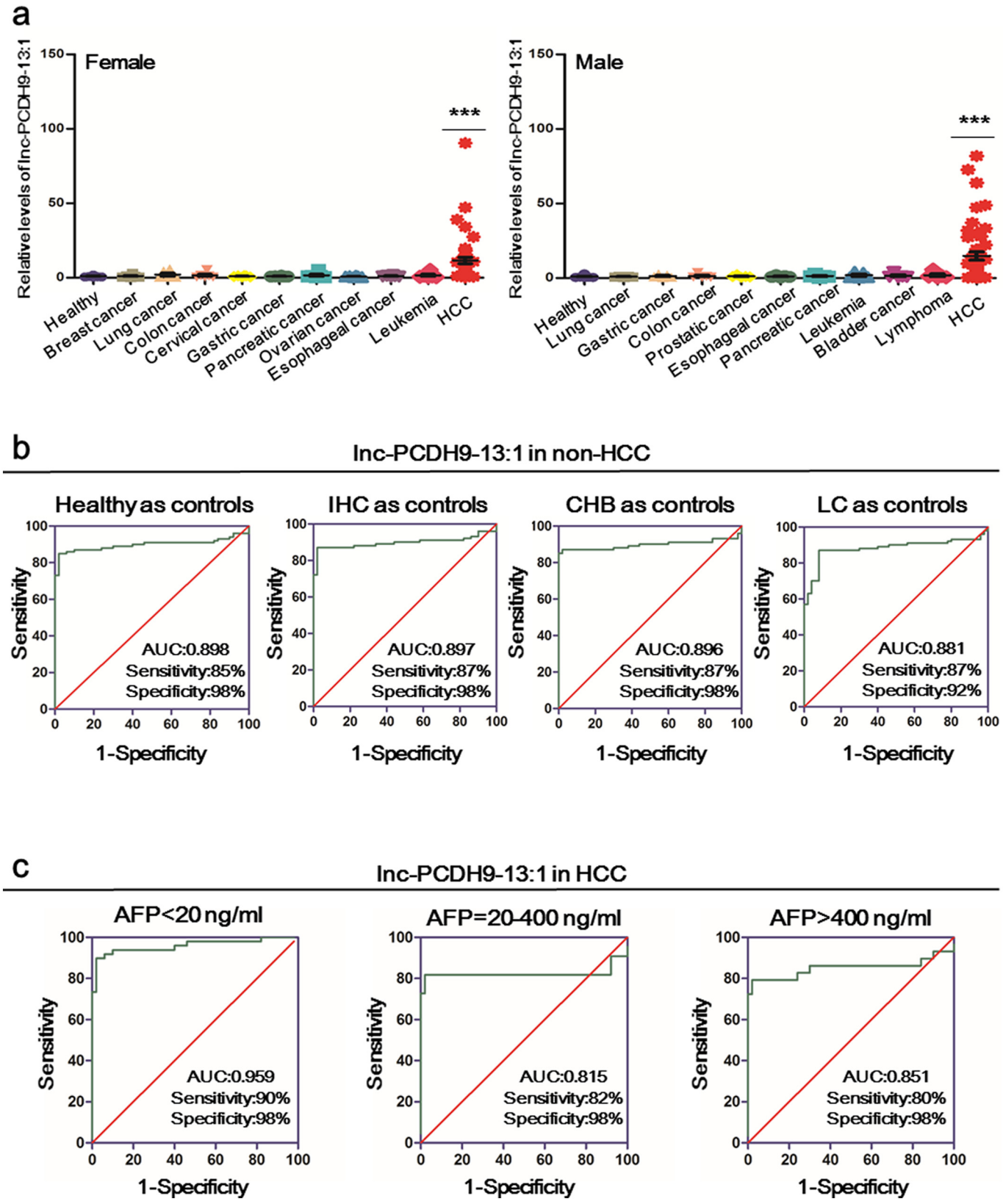

Fig. 7. The diagnostic performance of salivary lnc-PCDH9-13:1. (a) Compared with healthy controls, salivary lnc-PCDH9-13:1 did not showed significant differences with 10 leading causes of cancer death in men and women excluding HCC, but significantly overexpressed in HCC. (b) Through the analysis of ROC curves, salivary lnc-PCDH9-13:1 could detect HCC with sensitivities and specificities of over 85% compared with different control groups. (c) ROC curves indicated that salivary lnc-PCDH9-13:1 showed over 80% of sensitivities and specificities for detecting HCC with different serum AFP levels.

into the blood circulation by tumor cells undergoing apoptosis or necrosis. Serum, plasma and saliva biomarkers were showed good agreement in several studies, such as C-reactive protein (CRP), interleukin (IL)-6 [22], HIV antibody [23], microRNAs [24, 25], prostate specific antigen (PSA) [26], etc. One development and application of salivary diagnostics is the marketing of FDA-approved testing kits for AIDS, which has been widely used worldwide to screen patients with HIV infection. Oncogenic mutation of some genes could also be detected in plasma and saliva samples [27, 28]. Thus salivaomics is one of liquid biopsies, which examines physiological biofluids and performs analysis on them for improving cancer management. Notably, not 100% of the released RNAs in vitro and in vivo reflect the cellular or tissular profile. Pigati et al. reported that secreted RNAs did not necessarily reflect the abundance of RNAs in the cell of origin. Some diagnostic RNAs are selectively retained and some were selectively released by cells [29]. Lnc-GGT1-6:1 and lnc-ARRD3-1:16 were significantly overexpressed in HCC tissue, but not significantly elevated in plasma and saliva of HCC patients. According to this hypothesis, those two lncRNAs might be selectively retained in HCC, while lnc-PCDH9-13:1 was selectively released by HCC.

The most productive bodily fluids, diagnostically, are blood, saliva, and urine. Presently, blood is the most widely used for monitoring diseases. However, blood sampling is invasive and can cause pain and infection. Moreover, those collecting blood samples require professional training and examinations. It is also complex and expensive to store blood samples. For example, vacuum or anticoagulant tubes are required. Saliva has many advantages as a diagnostic sample, given its non-invasiveness, easy collection, and low cost. Those taking samples require no professional training or examinations. Saliva sampling is also safe and does not induce blood-borne diseases, anxiety, or discomfort. Even urine collection is associated with privacy issues and is not applicable to patients with anuria. Thus, saliva collection increases the willingness of people to undergo health examinations and will greatly enhance the opportunity to monitor their general health over time and to diagnose diseases at an early stage.

This study was a phase one cancer biomarker study conducted at a single institution, and the number of participants studied was small. For these reasons, replication of our findings in a larger, multi-center, prospectively studied cohort is needed before those two potential salivary biomarkers can be used in clinical practice. Future studies should also investigate why lnc-PCDH9-13:1 was selectively overexpressed in HCC tissue, plasma and saliva of HCC patients, and should address the functions in HCC development.

In conclusion, salivary lnc-PCDH9-13:1 is a novel hypersensitive and specific biomarker in diagnosis of early HCC. It may help screen for HCC in the population at risk and predict the HCC recurrence after curative hepatectomy in the future.

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Declaration of Interests

We declare no competing interests.

Author Contributions

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