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Combination of IL-34 and AFP improves the diagnostic value during the development of HBV related hepatocellular carcinoma

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Research Article

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Abstract

Background

IL34 involves in host immunity regulated carcinogenesis. Alpha-fetoprotein (AFP) is related to the development of HCC. We explored if combination of IL-34 and APF could improve the diagnostic value in HBV related hepatocellular carcinoma (HBVHCC).

Methods

Serum was obtained from HBV patients or healthy control. Liver tissue was obtained from liver biopsy in CHB, HBV related cirrhosis patients or curative resection in HBVHCC patients. Serum IL34 and MCSF were measured. Hepatic IL34, MCSF and $CD68^{+}$ tumor associate macrophages (TAMs) were determined.

Results

Serum IL34 was 1.7, 1.3 or 2.3-fold higher in HBVHCC than that of CHB, HBV related cirrhosis, or healthy control, which was inhibited following transhepatic arterial chemoembolization (TACE) in HBVHCC patients. Intrahepatic IL34 was higher in HBVHCC than that of the other three groups. Intra-hepatic IL34 was associated with high HBVDNA, $HBeAg^{-}$, poor differentiation and small tumor size of HBVHCC patients. Intra-hepatic TAMs in HBVHCC were increased 1.7 or 1.3-fold, compared to that from CHB or HBV-cirrhosis patients. Intra-hepatic TAMs were associated with high HBVDNA, high tumor differentiation, small tumor size, abnormal AFP and more tumor number. AFP plus serum IL-34, showed the highest AUC (0.837) with sensitivity (0.632) and highest specificity (0.931), suggesting that AFP plus IL-34 enhances the reliability for prediction of the development of HBVHCC among CHB patients.

Conclusions

Circulating and intra-hepatic IL34 was upregulated gradually in HBV disease progression from CHB, cirrhosis and HCC. IL-34 may be used as a diagnostic biomarker and potential therapeutic target for the management of HBVHCC.

Introduction

Hepatitis B virus (HBV) infection, a major health problem worldwide [1], is one of the major causes of hepatocellular carcinoma (HCC) [2]. HBV related HCC (HBVHCC) is a common primary liver cancer with high mortality, high recurrence, but low post-operative survival rate, mainly is due to later diagnosis. It is well known that host immunity plays a critical role in the carcinogenesis, which is elegantly demonstrated by the Nobel laureates for Medicine in 2018 [3].

Tumor microenvironment, including tumor cells, macrophages, cytokines and activated endothelial cells, plays an important role on the occurrence and development of tumor [4]. Tumor associated macrophages (TAMs) regulate the microenvironment [5], but the role of TAMs is controversial. TAMs promotes tumor invasion, formation of blood vessels and lymphatic vessel, migration of tumor cells [6, 7], perhaps *via* enhancing immunosuppression environment [8]. In contrast, TAMs may be involved in inhibiting cancer growing and metastasis [9]. The explanation of such discrepancy might be related to the differential polarization of macrophages during their maturation, i.e. namely classical activated M1 macrophages and alternatively activated M2 macrophages based on the surface biomarkers and the functionalities [5]. The differential polarization of macrophages perhaps is due to different microenvironments in different regions and/or in different individuals, mediated by different regulators [10].

IL34, a member of interleukin 1 family, is produced by a wide range of cells, including macrophages, fibroblasts and hepatocytes [11, 12]. IL34 promotes differentiation, proliferation and survival of mononuclear cells *via* binding to CSF1R [13]. Dysregulation of IL34 is involved in many diseases [14], including inflammatory bowel disease [15], rheumatoid arthritis [16], chronic heart failure [17] and ischemia/reperfusion injuryincited acute kidney injury [18]. Macrophage colony stimulating factor (MCSF) [known as (colony stimulating factor1, CSF1)] is responsible for the survival, proliferation and differentiation of mononuclear phagocytes through biding to MCSFR [19]. Although IL34 shares no apparent sequence homology with MCSF, the biological activity of IL34 is mediated *via* interacting with MCSFR, which is mainly expressed on the surface of macrophages [13].

We have demonstrated previously that IL-34 is substantially suppressed in gastric cancer, and IL-34 is an independent biomarker for predicting the development of gastric cancer [14]. Furthermore, it has been suggested IL-34 may involve in the development of HBV-HCC, using bioinformatic analysis in nude mice inoculated with human HCC, probably *via* manipulation of miR28-5p [20]. The role of miR-28-5p has been illustrated that depletion of miR-28-5p enhances the progression of HCC, in combination with IL-34 and TAM in the HCC inoculated nude mice [20]. However, it remains to be explored that the role of IL-34 during the development of HBV HCC in human *in vivo*. More recently, the correlation between IL-34 and MCSF in liver injury has been reported in HCV patients with high fibrosis scores [12]. In addition, it has been demonstrated that circulating IL34 is associated with inflammatory activity and liver fibrosis in chronic hepatitis B (CHB) patients [21]. We have illustrated that combination of inflammatory score/liver function and AFP improves significantly the diagnostic accuracy of HBV-related HCC [22].

Considering a close correlation between the severity of hepatic fibrosis and the incidence of HCC in HBV and HCV patients [23], as well as, the information from HCC inoculated nude mice [20], it is logical to hypothesis that IL-34 plays a critical role during the development of HBV-HCC, perhaps in conjunction with MCSF and TAM. Thus, it was explored that the correlation among IL34, MCSF and TAMs during the development of HBV-HCC at the different stages, particularly, the kinetics of IL34 during the progression/development of HBV related liver diseases. In addition, the accuracy of diagnostic value of the combination of AFP and IL-34 with its related TAMs in HCC was investigated. Our current finding may

be useful in the development of novel diagnostic and potential therapeutic target for the management of such devastating disease.

Materials And Methods

Study population

All of the patients were identified between April 2015 and July 2017 in Department of Infectious Diseases, Shanghai Ruijin Hospital. Serum and liver tissues were obtained from the patients with informed consents. It was selected as healthy controls (HCs) that age and sex matched healthy people for routine health check in our hospital without liver disease/HBsAg negative/negative image in CT or MRI. The selection of treatment for HBV-HCC patients were based on the guideline for treatment of primary liver cancer in China, and was conducted with a multidisciplinary diagnosis and treatment team of Ruijin Hospital, as described previously [24]. HBV-HCC patients selected for the current study were received either transhepatic arterial chemoembolization (TACE) or curative resection treatment. TACE, an interventional treatment, is one of the most common nonsurgical treatments for liver cancer [24]. Curative resection is a surgical procedure that hepatocellular cancerous tissue and a certain amount of normal tissue to be removed to obtain adequate margins. The purpose is to minimize the risk of any cancer cells being left behind.

First, the inclusion criteria of CHB patients were: adult with consecutive $HBsAg^+$ for at least six months, nucleos(t)ide (NA)naïve without cirrhosis or carcinoma. Second, the inclusion criteria of HBVHCC were: 1) Adult with consecutive $HBsAg^+$ for at least six months; 2) Diagnosed as primary HCC confirmed with pathology; 3) alpha-fetoprotein (AFP) > 400 μg/L, no other active liver disease, pregnancy, embryonic source sex reproductive system tumor and metastatic liver cancer, and could touch a swelling or hard of the liver with tumor, or imaging examination, such as computerized tomography (CT), magnetic resonance imaging (MRI) scans, and ultrasound examinations, revealed liver occupying lesions characteristic; 4) AFP ≤ 400 μg/L, more than two imaging examinations revealed liver occupying lesions which has characteristic of HCC. Third, the inclusion criteria of HBV related cirrhosis (HBVcirrhosis) were: 1) adult with consecutive $HBsAg^+$ for at least six months; 2) diagnosed cirrhosis by biopsy of the liver; 3) or imaging examination, such as CT, MRI, FibroScan or ultrasound examinations, detected enlarged livers, abnormally nodular livers, enlarged spleens, and fluid in the abdomen, suggesting cirrhosis; 4) the hospitalized patients under any event can also be diagnosed as decompensation liver cirrhosis: abdominal cavity effusion, esophageal gastric varices burst out of the blood, hepatic encephalopathy, infection.

The exclusion criteria included: 1) Coinfected with human immunodeficiency virus (HIV), hepatitis A virus (HAV), hepatitis C virus (HCV), hepatitis D virus (HDV) or hepatitis E virus (HEV); 2) Undergone liver transplantation before the study; 3) autoimmune liver disease, nonalcoholic fatty liver disease, alcoholic fatty liver disease, Wilson's disease, or hemochromatosis; 4) pregnant women or breast-feeding; 5) liver metastatic tumors; 6) CHB related acuteon-chronic liver failure (ACLF). The exclusion criteria of healthy

people were: 1) Undergone liver disease before the study; 2) Had abnormal liver function recently; 3) alcoholism (amount of alcohol: female ≥ 20g/d, male ≥ 30g/d).

This study complies with the declaration of Helsinki, and the study protocol was approved by the *Human Ethics Committee, Ruijin Hospital*. Written informed consent was obtained from all of the patients according to standards of the local ethics committees.

Routine biochemistry and cytokine quantification

It was performed that routine biochemistry [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), gamma-glutamyl transpeptidase (rGT), total bilirubin (TBil), albumin (Alb) and prothrombin time (PT)] and virologic tests [HBVDNA level, hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (antiHBs), hepatitis B envelop antigen (HBeAg), hepatitis B envelop antibody (antiHBe)]. $HBeAg^+$ is defined as positive hepatitis B envelop antigen, and $HBeAg^-$ is defined as negative hepatitis B envelop antigen. The Scheuer's scoring system was applied for pathology diagnosis of inflammation and fibrosis grading of liver tissue. Liver cirrhosis is defined as ≥ S4 of Scheuer's scoring system. Serum ALT, AST, AKP, rGT, TBil, and Alb (reflecting liver function) were quantified using Beckman coulter AU5800 automatic biochemical analyzer. HBsAg, anti-HBs, HBeAg and anti-HBe were determined using commercial ELISA kits (Abbott Diagnostics, IL). Serum HBV DNA levels were measured, using qPCR, Roche Amplicor (Roche Diagnostic Systems, Branchburg, NJ, USA). Serum IL34 and MCSF were quantified using ELISA (R&D Systems, Lille, France). Results were expressed as a concentration of cytokine production.

Immunohistochemistry (IHC)

The liver tissue blocks were obtained from surgery for HBVHCC (n = 30), liver biopsy for CHB liver (n = 5) or HBV-cirrhosis liver (n = 5) and the off cuts of liver transport donors for HCs (n = 5). HCs did not present liver disease/HBsAg negative/negative image in CT or MRI. Hepatic IL34, MCSF and CD68 were determined using immunohistochemistry, using 3,3'diaminobenzidine (DAB) color development. The primary antibodies were polyclonal rabbit antihuman IL34 (bs-18170R, Beijing Biosynthesis Biotechnology, China), polyclonal rabbit anti-human MCSF (Abcam, Cambridge, UK) and monoclonal mouse antihuman CD68 (Dako, Copenhagen, Denmark). The secondary antibody (Beijing Sequoia Jinqiao Biological Technology) was used. The specific target(s) was visualized with DAB detection kit and counterstained with hematoxylin. The IHC was repeated for twice. Negative control was applied in each labeling for every primary rabbit negative control. Intra-hepatic IL-34 or MCSF is localized in the cytoplasm of hepatocytes, which has been well documented in our previous publications [25]. IHC was quantified using a computer-assisted genuine color image analysis system (ImageProplus 9.0) for hepatic IL34, MCSF or CD68, as described previously [25, 26].

Statistical analysis

Continuous variables were expressed as means ± standard deviation or median (inter-quartile range) where appropriate. Differences between two groups were determined by unpaired t-test or the

MannWhitney U test. Among three groups were used by analysis of variance (ANOVA) or the KruskalWallis H nonparametric test. Chisquare or Fisher's exact test was employed to compare nominal variables. ROC curve and binary logistic regression analysis were used for detecting the diagnostic accuracy of serum IL-34 or MCSF for HBV-HCC. All statistical tests are twoside, and p-value < 0.05 was considered to be statistically significant. SPSS version 22.0 was used for all statistical analysis (SPSS Inc., Chicago, IL, USA).

Results

The baseline characteristics of the patients were summarized (Table 1). There were HBVHCC (n = 88), CHB (n = 64), HBVcirrhosis (n = 64) and HCs (n = 20), according to the inclusion criteria.

Table 1
Demographic, clinical characteristic, biochemical characteristic

		HC(n = 20)	CHB(n = 64)	LC(n = 64)	HCC(n = 88)	*p* value
Age (mean ± SD)		66 ± 7	38 ± 12	48 ± 12	54 ± 8	< 0.0001
Sex	Male	3/20(15%)	44/64 (68.75%)	51/64 (79.69%)	82/88 (93.18%)	< 0.05
	Female	17/20(85%)	20/64 (31.25%)	13/64 (20.31%)	6/88 (6.82%)	< 0.05
ALT (IU/L)		19.7 ± 5.68	138.23 ± 191.06	162.54 ± 352.27	72.10 ± 80.05	< 0.05
AST (IU/L)		22.45 ± 4.5	78.03 ± 121.53	129.17 ± 277.76	102.20 ± 113.52	ns
AKP (IU/L)		66.55 ± 13.38	74.53 ± 24.31	97.33 ± 52.99	146.12 ± 98.07	< 0.001
rGT(IU/L)		23.8 ± 13.35	54.33 ± 58.55	83.32 ± 103.52	129.68 ± 117.82	< 0.001
TBil(μmol/L)		12.61 ± 4.00	21.50 ± 15.67	54.58 ± 86.55	65.69 ± 87.12	< 0.001
Alb (g/L)		-	42.44 ± 9.06	35.97 ± 8.86	32.54 ± 7.00	< 0.001
PT (s)		-	12.48 ± 1.61	14.06 ± 2.44	13.95 ± 2.13	< 0.001
AFP (μg/L)		-	36.45 ± 137.73	49.14 ± 125.28	2319.21 ± 5558.19	< 0.0001
HBeAg						< 0.05
$HBeAg^-$		-	28/64 (43.75%)	34/63 (53.97%)	39/57 (68.42%)	
$HBeAg^+$		-	36/64 (56.25%)	29/63 (46.03%)	18/57 (31.58%)	
HBV-DNA (IU/ml)						< 0.001
$< 5*10^2$		-	11/63 (17.46%)	20/60 (33.33%)	35/52 (67.31%)	
$\geq 5*10^2, \leq 10^4$		-	17/63 (26.98%)	14/60 (23.33%)	8/52 (15.38%)	
$> 10^4$		-	35/63 (55.56%)	26/60 (43.33%)	9/52 (17.31%)	
IL-34 (pg/ml)		15.71 ± 4.74	21.22 ± 7.17	26.58 ± 15.83	35.74 ± 27.85	< 0.05

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AKP: alklinephosphatase; rGT: gamma-glutamyl transpeptidase; TBil: total bilirubin; Alb: albumin; PT: prothrombin time; BCLC: Barcelona Clinic Liver Cancer; LC: liver cirrhosis.

	HC(n = 20)	CHB(n = 64)	LC(n = 64)	HCC(n = 88)	*p* value
MCSF (pg/ml)	161.14 ± 146.32	134.66 ± 138.68	119.66 ± 78.98	238.31 ± 516.30	ns

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AKP: alklinephosphatase; rGT: gamma-glutamyl transpeptidase; TBil: total bilirubin; Alb: albumin; PT: prothrombin time; BCLC: Barcelona Clinic Liver Cancer; LC: liver cirrhosis.

Serum IL34 and MCSF was elevated in HBV-HCC

There was significantly difference of biochemical indices (ALT, AKP, GGT, TBil, PT and AFP) among CHB, HBVcirrhosis, and HBVHCC patients, except AST. The levels of AFP were significantly higher in HBVHCC than CHB, HBVcirrhosis ($p < 0.01$). AFP was 36.45, 49.14, or 2319.21 μg/l from CHB, HBVcirrhosis, or HBVHCC patients, respectively. Serum IL34 and MCSF were elevated in HBVHCC.

Serum IL34 was 1.7, 1.3 or 2.3-fold higher from HBVHCC groups than that from CHB (35.74 vs 21.22, $p < 0.01$), HBVcirrhosis (35.74 vs 26.58, $p < 0.05$) or HCs (35.74 vs 15.71, $p < 0.01$) (Table 1, Fig. 1A). Serum MCSF was 1.8 or 2.0-fold higher from HBVHCC groups than that from CHB (238.3 vs 134.7, $p < 0.01$) or HBVcirrhosis (238.3 vs 119.7, $p < 0.05$). However, there was no significant difference of serum IL34 between HBVcirrhosis patients and HCs (26.58 vs 21.22, $p > 0.05$), nor between CHB patients and HCs (21.22 vs 15.71, $p > 0.05$). In addition, there was no significant difference of serum MCSF between HCs and CHB, HBVcirrhosis or HBVHCC groups (Table 1, Fig. 1B).

Serum IL34 or MCSF during anti-tumor treatment in HBVHCC

We further determined the serum IL34 or MCSF in HBV-HCC patients received TACE or surgery. Serum IL34 was decreased significantly by > 20% post TACE in HBVHCC patients, compared to that prior to the treatment (28.82 vs 21.23, $p < 0.01$) (Fig. 1C); but no significant difference of MCSF was observed in the HBVHCC patients between prior to and post TACE treatment (175.6 vs 171.7, $p > 0.05$) (Fig. 1D). In addition, in HBVHCC patients undergoing surgery, there was no significant change of circulating IL34 (15.01 vs 14.01, $p > 0.05$) (Fig. 1E) and MCSF (88.32 vs 73.23; $p > 0.05$) (Fig. 1F) between prior to and post-surgery.

The factors that are associated with HBV-HCC incidence

Whether there is any correlation between IL-34 or MCSF and incidence of HBVHCC, as well as other HBV related influence factor, including ALT, AST, HBVDNA, HBsAg, HBeAg, AFP, we used Spearman rank correlation to analysis (Table 2). Serum IL34 was positively correlated with the incidence of HBVHCC ($r_s = 0.257$, $p < 0.01$), as well as, MCSF ($r_s = 0.223$, $p < 0.01$) and AFP ($r_s = 0.525$, $p < 0.01$). HBsAg, HBeAg or HBVDNA were inversely correlated with HBVHCC ($r_s = -0.441$, $p < 0.01$; $r_s = -0.557$, $p < 0.01$; or $r_s = -0.428$, $p < 0.01$, respectively).

Table 2
Factors associated with the incidence of HBVHCC

Marker	HBV-HCC	
	r_s	*p* value
IL-34(pg/ml)	0.257	< 0.01
MCSF (pg/ml)	0.223	< 0.01
ALT (IU/L)	-0.109	ns
AST (IU/L)	0.190	< 0.01
AKP (IU/L)	0.428	< 0.01
GGT (IU/L)	0.346	< 0.01
TB (μmol/L)	0.324	< 0.01
Alb (g/L)	-0.425	< 0.01
PT (s)	0.182	< 0.05
AFP (μg/L)	0.525	< 0.01
HBsAg	-0.441	< 0.01
HBeAg	-0.557	< 0.01
HBV-DNA (IU/ml)	-0.428	< 0.01

We further determined the diagnostic accuracy of serum IL-34 or MCSF for detecting HBV-HCC using ROC curve. AUC, sensitivity, specificity, LR+, and LR- for serum IL-34, MCSF or AFP for diagnosing HBV-HCC are shown in Fig. 2B. AUC values for serum IL-34, MCSF or AFP were 0.683 (0.605–0.761), 0.635 (0.556–0.714) or 0.810 (0.746–0.874), respectively (Fig. 2). Considering the limited sensitivity or specificity of the three markers, we tried to utilize AFP combined with serum IL-34 and MCSF for detecting HBV-HCC. Finally, AFP plus serum IL-34, showed the highest AUC (0.837) with sensitivity (0.632) and highest specificity (0.931). MCSF could not be combined with other two factors. Based on these parameters and binary logistic regression analysis, the final equation was established: Y = 0.031 × IL-34 + 0.025 × AFP, which could be used for prediction of HBV-HCC among CHB patients.

Intrahepatic IL34 from CHB, HBVcirrhosis, HBVHCC patients

According to the inclusion criteria, we obtained specimens of liver tissue from HBVHCC (n = 30), CHB patients (n = 5), HBVcirrhosis (n = 5), HCs (n = 5). There was no significantly difference of biochemical indices (ALT, AST, AKP, GGT, TBil, PT and AFP) among CHB, HBVcirrhosis, and HBVHCC patients, except Alb (**Supplement** Table 1). Intrahepatic IL34 and MCSF from HBVHCC patients was significantly higher

than that of CHB, HBVcirrhosis and HCs (Fig. 3, 4) (all of the $p < 0.05$). Intrahepatic $CD68^+$ TAMs were increased 1.7 or 1.3-fold in HBVHCC, compared to that from CHB or HBVcirrhosis, respectively (Fig. 4). There was no significant difference of Intrahepatic $CD68^+$ TAMs between HBVHCC patients and HCs.

Correlation between intrahepatic IL34, MCSF and CD68 + TAMs in HBVHCC and clinical parameters

Associations between clinical pathological parameters of HBVHCC and IL34, MCSF or $CD68^+$ TAMs were listed (Table 3). IL34 was associated with HBVDNA, HBeAg, tumor differentiation and tumor size of HBVHCC patients (Fig. 5). IL34 was 28% lower in the group of patients with low HBVDNA level compared to patients with high level ($p < 0.05$). Nearly 50% reduced intra-hepatic IL34 was also observed in $HBeAg^+$ compared to $HBeAg^-$ HBVHCC patients ($p < 0.05$). In addition, there was a significant inverse correlation between IL-34 and differentiation or tumor size of HCC. IL34 was increased by 1.3-fold in low differentiated HCC compared to that of high differentiation group ($p < 0.05$); as well as, 1.3-fold in intrahepatic IL-34 production from small tumor size ($\leq$ 5cm) than that from big size tumor group ($p < 0.05$). However, there was no correlation between IL-34 and other parameters, including tumor number and AFP of HCC. Intrahepatic $CD68^+$ TAMs were associated with high HBVDNA, high tumor differentiation, small tumor size, abnormal AFP and more tumor number. MCSF was associated with low HBVDNA, $HBeAg^-$, abnormal AFP, little tumor number of HBVHCC patients, except tumor differentiation and tumor size.

Table 3

Correlations between intrahepatic IL34, MCSF and $CD68^+$ TAMs and clinical features in patients with HBVHCC (n = 30)

Characteristics		N	IL-34		MCSF		$CD68^+$ TAMs	
			Median	*p* value	Median	*p* value	Median	*p* value
HBV-DNA (IU/mL)	$< 5*10^2$	15	35.7	< 0.001	122.3	< 0.01	21.5	< 0.05
	$\geq 5*10^2$	11	49.4		56.3		26.3	
HBeAg	$HBeAg^-$	10	45.3	< 0.01	134.7	< 0.05	25.8	ns
	$HBeAg^+$	3	24.6		50.0		23.0	
AFP	normal	14	44.4	ns	57.2	< 0.05	19.2	< 0.0001
	abnormal	16	43.0		101.2		27.2	
Differentiation	≤II	10	54.9	< 0.05	90.3	ns	16.5	< 0.0001
	>II	19	34.8		75.1		26.1	
Tumor number	1	22	43.4	ns	89.1	< 0.05	20.1	< 0.0001
	≥ 2	7	39.1		46.0		31.7	
Tumor size	≤ 5	12	53.9	< 0.0001	66.0	ns	25.0	< 0.001
	> 5	17	34.8		88.1		21.0	

All datum were $*10^3$ image unites. The reference range of AFP is 0–9 μg/L. The histopathological classification is well described in the published Literature.

Discussion

In the present study, we evaluated circulating and intra-hepatic IL34 in HBV related liver diseases. Circulating IL34 of HBVHCC patients was significantly higher than that of CHB, HBVcirrhosis and HCs. The highest AUC was detected from AFP plus serum IL-34, suggesting the combination boosts sensitivity and specificity than AFP alone. Furthermore, circulating IL34 was suppressed with antitumor TACE treatment in HBVHCC, further confirmed the potential role of IL34 during the development of HBV-HCC. Consistent with circulating IL34, it was also detected that upregulated intrahepatic IL34 from HBV-HCC, compared to that of CHB, HBVcirrhosis and HCs. Intrahepatic IL34 was associated with high HBVDNA, $HBeAg^-$, poor tumor differentiation and small tumor size of HBV-HCC patients. Intrahepatic $CD68^+$ TAMs were upregulated in HBVHCC compared to that from CHB and HBVcirrhosis. Intrahepatic $CD68^+$ TAMs were associated with high HBVDNA, high tumor differentiation, small tumor size, abnormal AFP and more tumor number. Our current data suggests that IL-34 probably contributes to the development of HBV-HCC, i.e. promoting the disease progression from CHB, HBV cirrhosis and then HBV-HCC. The current

observation is supported by the findings from the Zhou group, showing that IL-34 is a key regulator for the growth of HCC in nude mice, probably is *via* miR-28-5p mediated activation of TAM [20]. Thus our finding is an extension and validation of the important role of IL-34 during the development of HBV-HCC in human tissues.

In our current study, circulating IL34 from HBVHCC patients was significantly higher than that of CHB, HBVcirrhosis, and HCs, suggesting that IL34 may contribute to tumorigenesis of HCC, enhancing progression from CHB patients to cirrhosis and finally HCC. Furthermore, intra-hepatic IL34 expression was consistent with circulating IL-34, which is in line with previous studies, showing that high IL34 in autoimmune diseases [15, 16]. More specifically, IL34 is overexpressed in the inflamed synovium of rheumatoid arthritis patients, where it perhaps acting synergistic with TNF and IL1β, induces osteoclastogenesis and contributes to tissue inflammation and bone erosion [27]. In addition, upregulated circulating and intra-hepatic IL-34 in HBV-HCC from the current study is supported by the others, showing that the circulating IL34 markedly increased in HBVHCC patients, compared to those in CHB and HBV-negative HCC patients [28], and the HBX gene of HBV upregulates IL-34 expression in hepatoma [28]. However, our previous study demonstrates that IL-34 is inversely correlated with differentiation, metastasis and invasion of gastric cancer [14], which is rather controversial with our current discovery in HBV-HCC disease. Our explanation for such discrepancy between HBV-HCC and gastric cancer is more likely related to the different carcinogenesis between gastric cancer and HBV-HCC, as well as completely different microenvironments, despite of gastric cancer and HBV-HCC are all belonging to gastrointestinal system.

The development of tumor is closely related to the microenvironment, including tumor cells, monocytes/macrophages, cytokines and neovasculisation. TAMs are mixed phenotype, expressing M1 or M2 markers [5], and may be influenced by different microenvironments in different regions and/or in different individuals. In our current study, intrahepatic $CD68^+$ TAMs were increased gradually with the order from CHB, HBVcirrhosis to HBVHCC patients. Our current observation invites speculation that the increased infiltrating $CD68^+$ TAMs may be M2 dominant, contributing to stimulate tumor growth activity [5]. This is in line with Zhou *et. al.* showing that IL-34 induced TAMs can inhibit miR-28-5p, which is one of the miRNAs that decrease IL-34 production, in HCC cells *in vitro via* TGFβ1, suggesting a paracrine factor fashion among miR-28-5p, IL-34 and macrophage. In clinical HCC study, lower miR-28-5p is correlated with high IL-34 and TAMs in HCC patients with a poor overall survival and recurrence [20].

IL34 is upregulated in HCV infection and inhibited the production of IFNγ [12], and IL34 may also be associated with inflammatory activity and liver fibrosis in CHB [21]. Moreover, baseline circulating IL34 seems to serve as a prognostic factor for progression in such patients. The high serum IL-34 level is associated with poor prognosis in non-viral HCC patients compared with the patients with low serum IL-34 level [29]. In our current study, there was only circulating IL34 significantly decreased in postantitumor treatment compared to that of pre-treatment of HBVHCC, suggesting IL-34 is closely related the weight of HBV-HCC or partial source of IL-34 is coming from HCC cells. Thus, serum IL34 was significantly correlated with the incidence of HBVHCC. The sensitivity and specificity of AFP plus circulating IL-34

seems to be greatly improved, compared to that of AFP alone, suggesting that AFP plus serum IL-34 could improve the diagnostic accuracy of AFP for detecting HBV-HCC, as well as IL34 could be used as a diagnostic biomarker for HBVHCC, which will be further clarified *in vitro* and *in vivo*.

IL34 induces differentiation of leukemia cells into monocytelike, macrophagelike cells and mature macrophages [30], may also enhances differentiation of other cancers [14, 31]. These reports are consistent with our current finding that IL34 was correlated with the differentiation and tumor size of HBVHCC. In addition, intrahepatic IL34 was associated with HBVDNA, HBeAg, tumor differentiation and tumor size of HBVHCC patients. Our data may provide an explanation for the possible role of IL34 in the development of HBVHCC, i.e. IL34 also regulates differentiation of HBVHCC, which would have potential clinical relevance regarding IL34 as a therapeutic target for malignancy. Such speculation is in line with others, showing that IL-34 inhibits HBV replication *in vivo* and *in vitro* [32], and IL-34 is beneficially to the HBV-HCC patients for potential therapeutic target.

There are limitations in the current study. There is no kinetics of intrahepatic or circulating IL34 during the development and management of HCC, and no correlation between IL34 and prognosis is detected. These two interesting points will be determined in our future study. We also acknowledge that the sample size in the current study is relatively small, which may not perfectly reflect the real cases. However, the current experiment is just a proof of concept. We will explore the underlying mechanism with large sample size and multiple center studies in future.

In conclusion, the current study improves our understanding of the role of IL34 in HBV related liver disease. Increased IL34 may contribute to the transformation of HBV HCC, which is a potential predictor of HBVHCC. The underlying mechanism of IL34 in HBVHCC is being currently investigated.

Abbreviations

ACLF, acute-on-chronic liver failure; AFP: alpha-fetoprotein; AKP: alkline phosphatase; ALT, alanine aminotransferase; Alb: albumin; ANOVA, analysis of variance; anti-HBe, hepatitis B envelop antibody; anti-HBs, hepatitis B surface antibody; AST, aspartate aminotransferase; CHB, chronic hepatitis B; CSF-1, colony stimulating factor-1; HAV, hepatitis A virus; HBV, hepatitis B virus; HBV-HCC, hepatitis B virus related hepatocellular carcinoma; HBV-cirrhosis, hepatitis B virus related cirrhosis; HBeAg, hepatitis B envelop antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCs, healthy controls; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; MCSF, macrophage colony stimulating factor; M-CSFR, macrophage colony stimulating factor receptor; PT: prothrombin time; r-GT: gamma-glutamyl transpeptidase; TACE, trans-hepatic arterial chemoembolization; TAMs, tumor associated macrophage; TBil: total bilirubin.

Declarations

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Competing interests: The authors declare that there is no conflict of interest.

Availability of data and materials: All date generated or analyzed during this study were all included in this present article.

Code availability: Not applicable.

Authors' contributions: Hui Wang was fully responsible for the conduct of this study. Hui Wang and Wei Cai designed the experiment. Kehui Liu, Yezhou Ding, and Yun Wang coordinated the study. Kehui Liu performed the majority of the experiment and drafted the manuscript. Shisan Bao and Hui Wang interpretation data and revised the manuscript. Clinical data collection was completed by Qingqing Zhao, Lei Yan, Jingdong Xie, Yunye Liu and Qing Xie. All authors have read and approved this final version of the manuscript.

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Ethics approval and consent to participate: Informed consent was acquired from every participant who offered blood samples, liver tissues, and approval of the experimental protocol was obtained from the *Human Ethics Committee, Ruijin Hospital.*

Patient Consent for publication: Written informed consent has been obtained from the patients.

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Supplementary Table

Supplement Table 1 is not available with this version

Figures

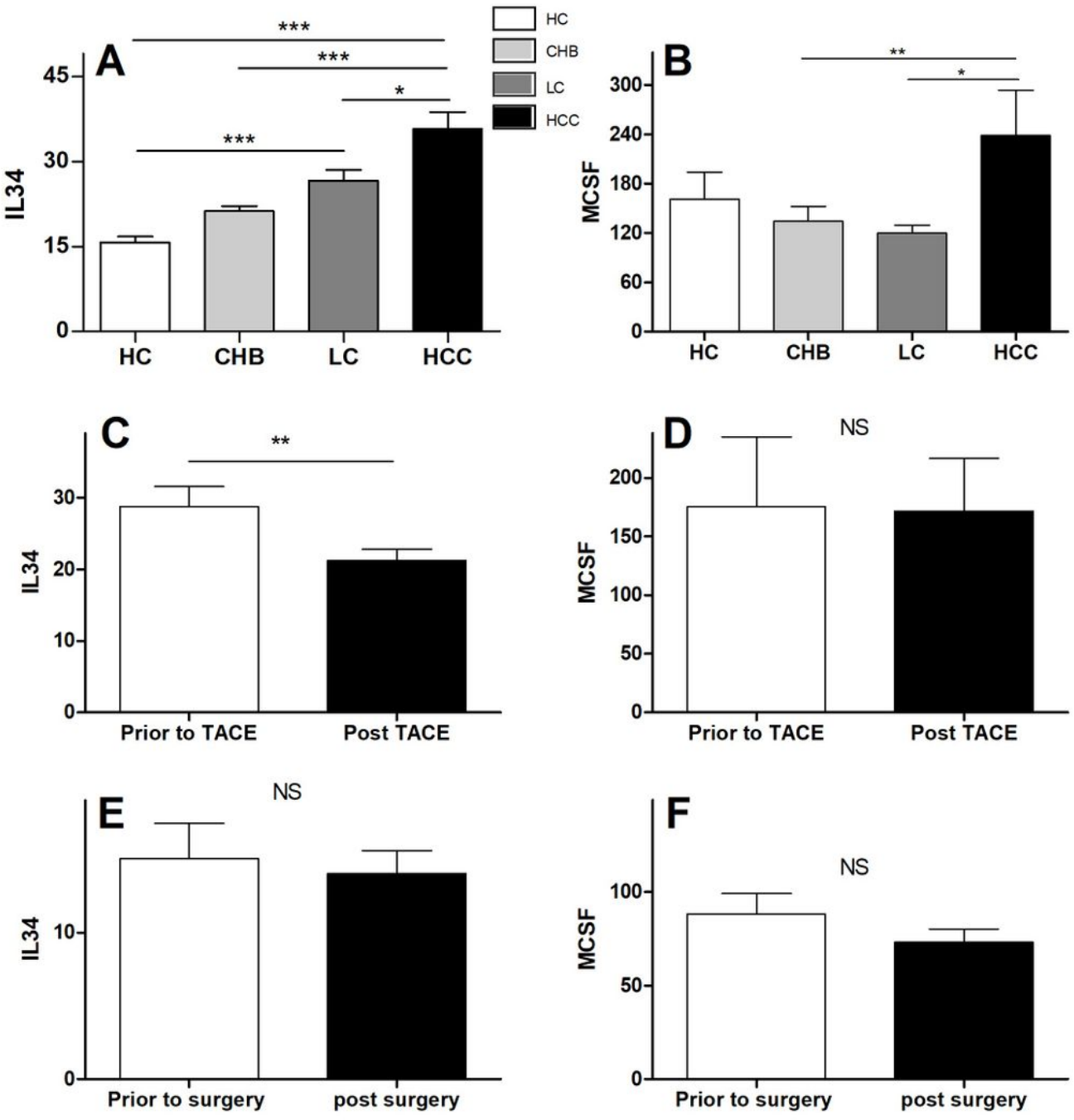
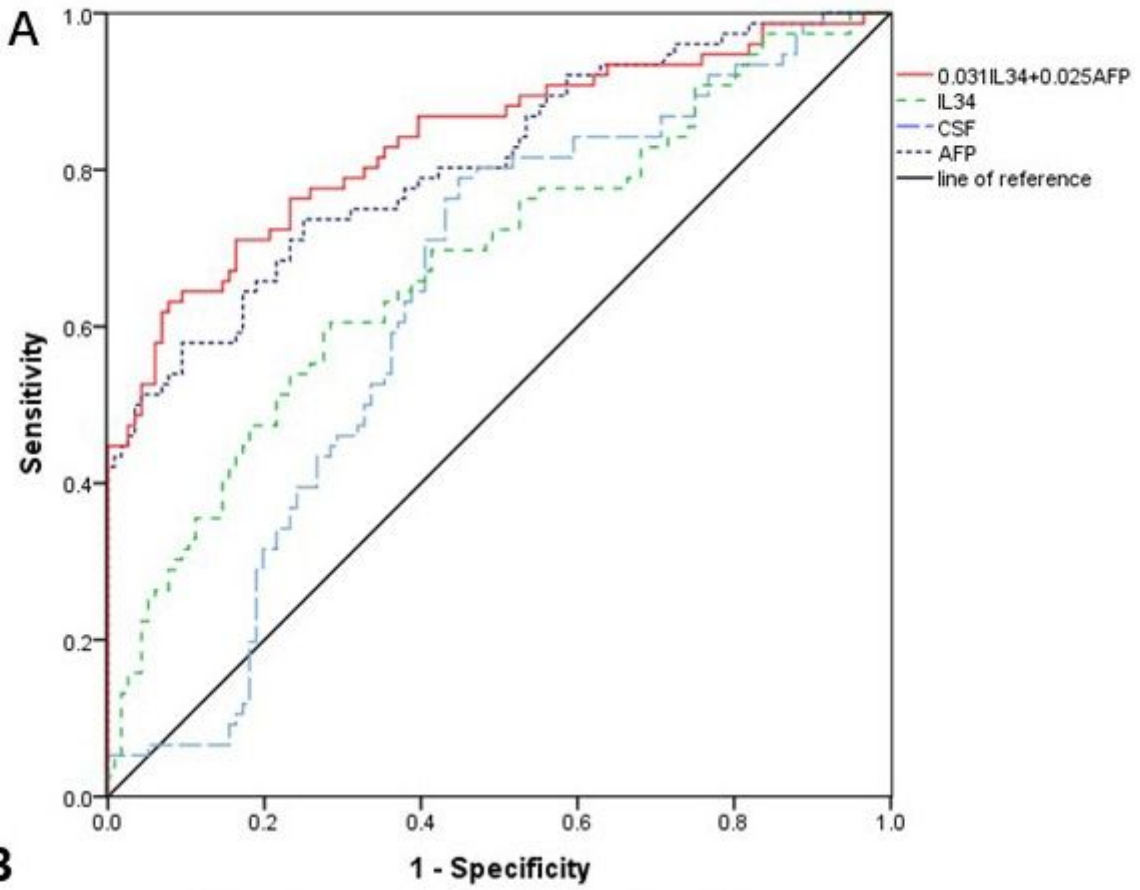


Figure 1

A. Serum IL 34 of HCs, CHB, HBV cirrhosis and HBV HCC; B. Serum MCSF of HCs, CHB, HBV cirrhosis and HBV HCC. (LC: liver cirrhosis); C. The serum IL 34 in HBV HCC patients prior to and post trans hepatic arterial chemotherapy and embolization (TACE); D. Serum MCSF in HBV HCC patients prior to and post TACE; E. Serum IL 34 in HBV HCC patients prior to and post surgery; F. Serum MCSF in HBV HCC patients prior to and post-surgery.



Marker	Cut-off value	AUC	Sensitivity (Sn)	Specificity (Sp)	Sn + Sp	LR+	LR-
IL-34(pg/ml)	27.22	0.683	0.605	0.716	1.321	2.13	0.55
MCSF(pg/ml)	95.19	0.635	0.789	0.552	1.341	1.72	0.38
AFP(ng/ml)	8.66	0.810	0.737	0.750	1.487	2.95	0.35
IL-34+AFP	1.88	0.837	0.632	0.922	1.554	8.14	0.40

AUC, area under the receiver operation characteristics curve; LR-, negative likelihood ratio; LR+, positive likelihood

Figure 2

ROC curves of AFP, IL-34, MCSF and AFP combined IL-34 diagnosis for HBV-HCC.

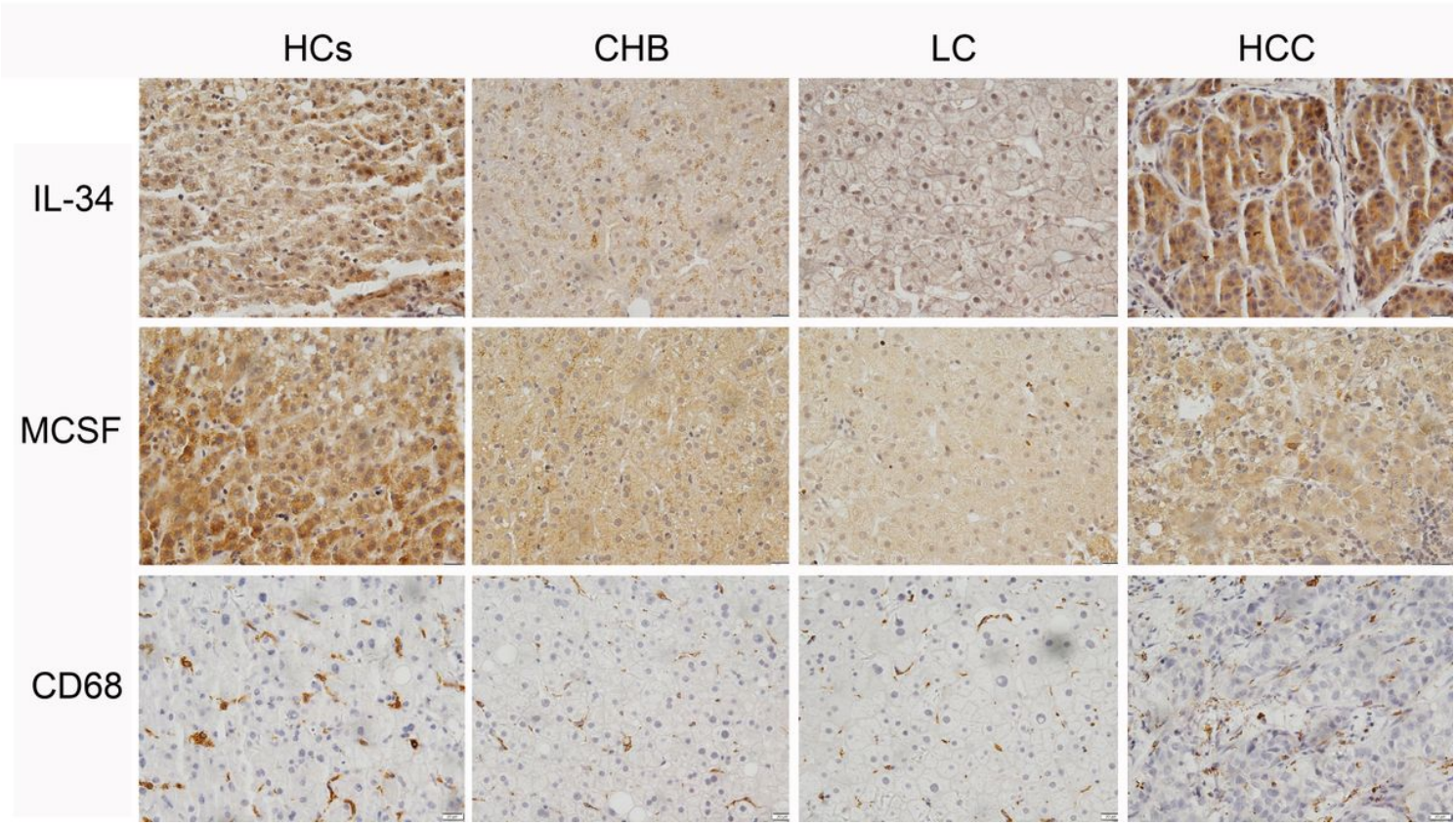


Figure 3

The immunohistochemistry of intra hepatic IL-34, MCSF, CD68+TAMs in HCs, CHB, HBV cirrhosis and HBV HCC. (LC: liver cirrhosis)

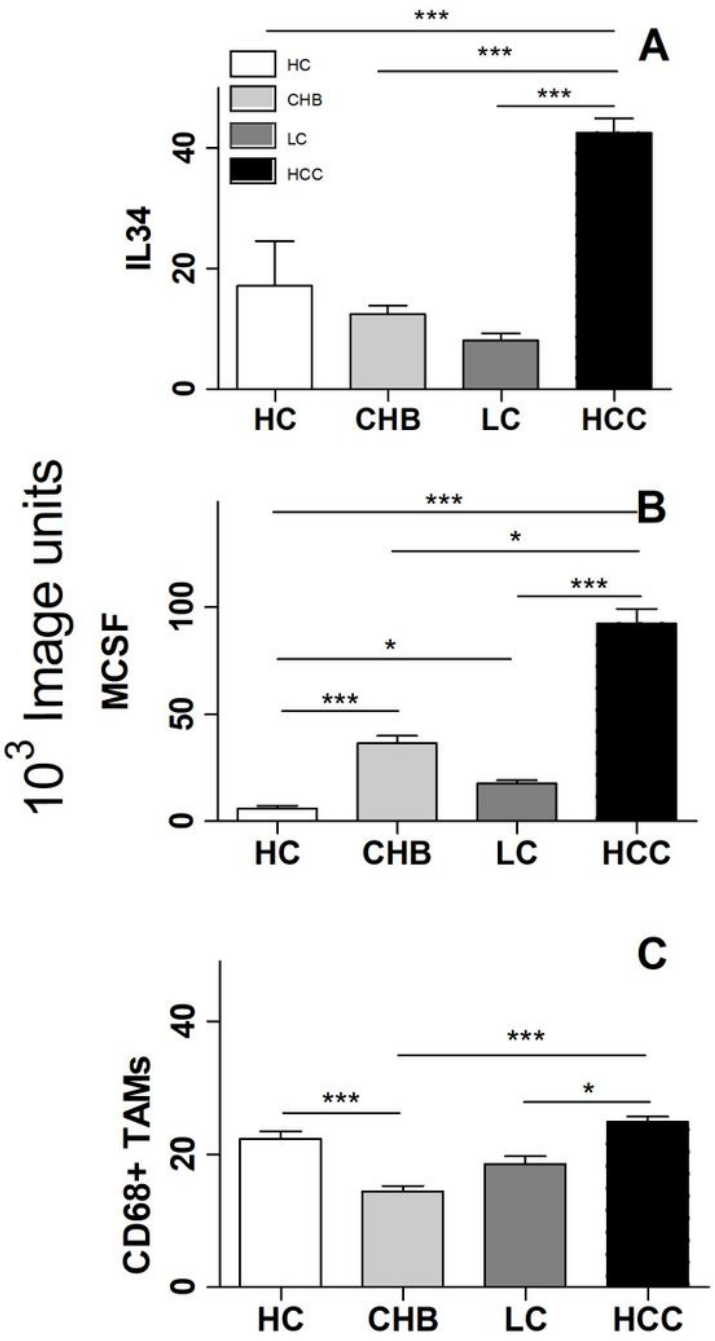


Figure 4

The corresponding quantification of immunohistochemical detection. (LC: liver cirrhosis)

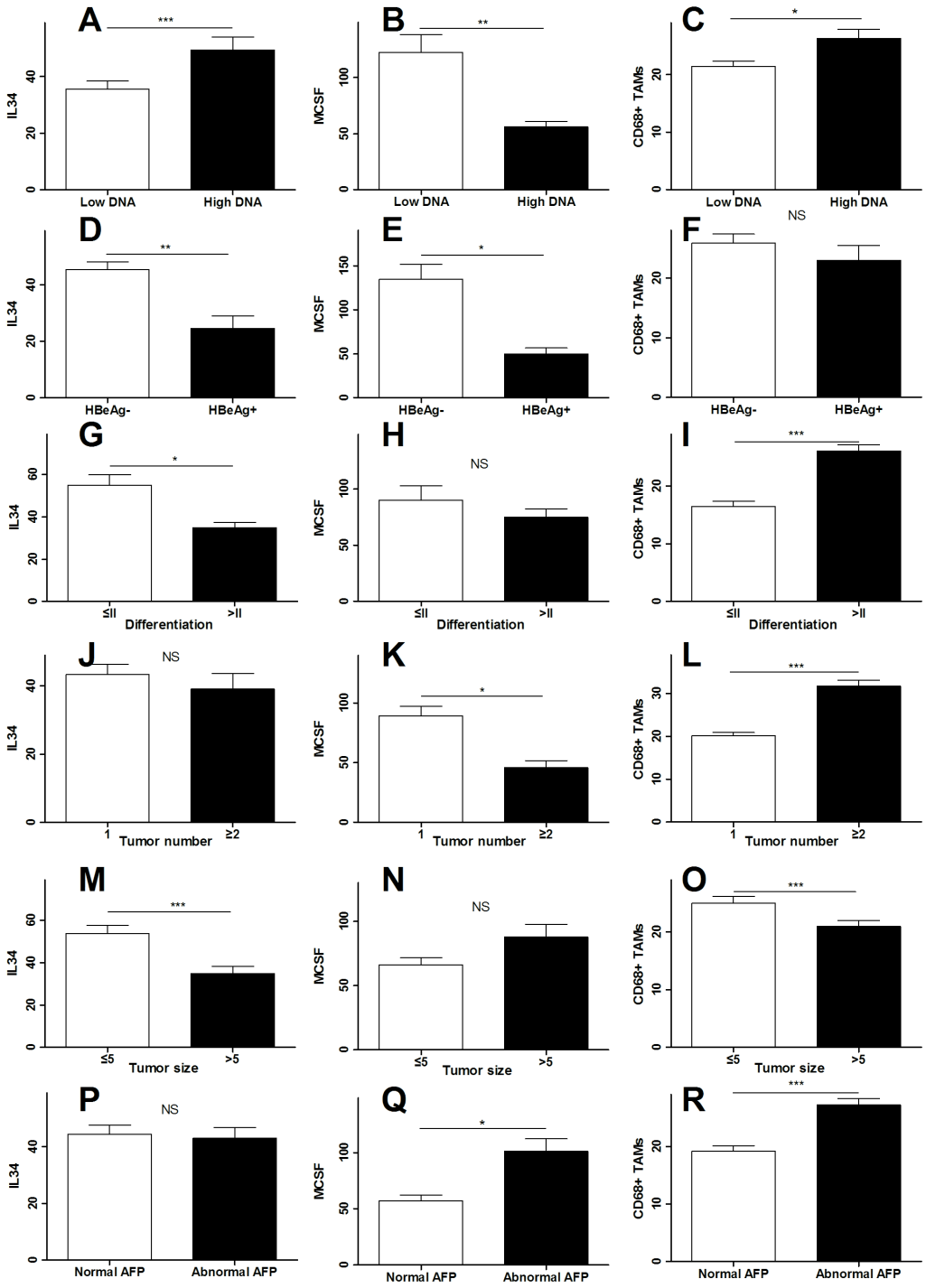


Figure 5

Correlation of intra hepatic IL 34, MCSF or CD68+ TAMs expression with HBV DNA, HBeAg and tumor differentiation subtypes. A. Correlation of intra hepatic IL 34 with HBV DNA; B. Correlation of intra hepatic MCSF with HBV DNA; C. Correlation of intra hepatic CD68+ TAMs expression with HBV DNA; D. Correlation of intra hepatic IL 34 with HBeAg; E. Correlation of intra hepatic MCSF with HBeAg; F. Correlation of intra hepatic CD68+ TAMs expression with HBeAg; G. Correlation of intra hepatic IL 34 with

tumor differentiation; H. Correlation of intra hepatic MCSF with tumor differentiation; I. Correlation of intra hepatic CD68+ TAMs expression with tumor differentiation; J. Correlation of intra hepatic IL 34 with tumor number; K. Correlation of intra hepatic MCSF with tumor number; L. Correlation of intra hepatic CD68+ TAMs expression with tumor number; M. Correlation of intra hepatic IL 34 with tumor size; N. Correlation of intra hepatic MCSF with tumor size; O. Correlation of intra hepatic CD68+ TAMs expression with tumor size; P. Correlation of intra hepatic IL 34 with AFP; Q. Correlation of intra hepatic MCSF with AFP; R. Correlation of intra hepatic CD68+ TAMs expression with AFP.