CLINICAL STUDIES

Increased PD-1 and decreased CD28 expression in chronic hepatitis B patients with advanced hepatocellular carcinoma

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Keywords
CD28 – hepatocellular carcinoma – PD-1 – tumour-infiltrating lymphocytes

Abbreviations:
HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

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Abstract

Background/aims: Hepatitis B infection is a well-known cause of hepatocellular carcinoma (HCC). This study aims to investigate the role that the co-stimulatory molecule CD28 and co-inhibitory molecule programmed death-1 (PD-1) play in compromising the function of tumour-infiltrating lymphocytes (TIL) in hepatitis B virus (HBV)-related HCC. *Methods:* A total of 45 patients with HBV-related HCC were enrolled during the period February 2008 to March 2010. The immune phenotype and the expression of PD-1, CD28 and CD127 in TIL in biopsy specimens and in peripheral blood lymphocytes (PBL) from the same patients were analysed by flow cytometry. *Results:* Among the 45 patients, there was a male predominance (80%) and the mean age was 50 ± 13.68 years (range: 29–71). The majority of TIL were $CD45RO^+CD69^+$. PD-1 expression was higher and CD28 and CD127 expression levels were lower in TIL than in PBL. The prevalence of portal vein thrombosis was 40%. Furthermore, tumour thrombosis invasion into the portal vein correlated with the expression level of the PD-1 co-inhibitory molecule. *Conclusion:* $PD\text{-}1^+$ tumour-infiltrating lymphocytes correlate with portal vein thrombosis and might serve as a potential prognostic marker of and a novel therapeutic target for HBV-related HCC.

Hepatitis B infection is a well-known cause of hepatocellular carcinoma (HCC). Globally, approximately one million people die each year from sequelae of hepatitis B virus (HBV) infections (1). However, the aetiology of persistent viral infection and the mechanism of poor viral clearance are not well known.

Cytotoxic T lymphocytes play critical roles in the clearance of HBV infection (2). Moreover, it has been proposed that viral clearance is strongly associated with the response of HCV-specific $CD8^+$ cytotoxic T lymphocytes. HCV-specific $CD8^+$ T cells significantly decline either in magnitude or in function in patients with chronic viral infection (3, 4). The activation of T cells by antigen-presenting cells requires the involvement of two signals. Signal 1 is produced by the interaction between T-cell receptors and major histocompatibility complex (MHC)-bound peptide on antigen-presenting cells. Signal 2 is generated by the binding of CD28 to its ligands B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells and is considered to be a positive signal for T-cell activation.

Recently, the programmed death-1 (PD-1) receptor and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) have attracted considerable attention. PD-1 is inducibly expressed on the surface of T cells, B cells, natural killer T cells and activated monocytes (5, 6). PD-1 is a member of the immunoglobulin superfamily and its cytoplasmic domain contains an immunoreceptor tyrosine-based inhibitory motif, which is associated with the inhibitory signalling (5, 7, 8). Apoptosis of T cells is induced upon binding of PD-1 to PD-L1 (B7-H1) or PD-L2 (B7-DC) (9, 10). The outcome of T-cell immune responses is, therefore, determined by the predominance between the positive and the negative signals (11).

*Contributed equally.

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T-cell response to foreign antigens is determined by antigen-specific signals from the TCRzeta/CD3 and CD4–CD8–p56lck complexes in combination with additional cosignals provided by coreceptors such as CD28, inducible costimulator (ICOS), cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-1. CD28 generates positive signals that promote and sustain T-cell responses, while PD-1 limits the responses. The balance between stimulatory and inhibitory cosignals is achieved without excess inflammation and autoimmunity, determines the ultimate nature of T-cell responses and controls T-cell responses to foreign pathogens (12). Therefore, the costimulating molecule CD28 and the co-inhibitory molecule PD-1 may play critical roles in the activation of T cells in patients with chronic viral hepatitis.

Manifestations of chronic viral infection include increased levels of exhausted T lymphocytes and a decrease in cytokine production and proliferative capacity. Recent reports indicate that PD-1 is markedly upregulated on the surface of exhausted virus-specific $CD8^+$ T cells in mice infected with lymphocytic choriomeningitis virus (13) and in humans infected with human immunodeficiency virus (14–16). Surprisingly, despite an altered function, HBV-specific $PD\text{-}1^+$–$CD8^+$ cells from chronic HBV patients display high levels of interleukin (IL)-7 receptor CD127, which has been described as a marker of functional memory T-cell differentiation (17–19).

Most studies on the phenotypes and functions of T cells have focused on the analysis of peripheral blood lymphocytes (PBL) and liver-infiltrating lymphocytes (20–23). However, the characterization of tumour-infiltrating lymphocytes can be more meaningful because the liver is the site of viral replication and cellular injury. Differences in T-cell phenotype and response between PBL and liver-infiltrating lymphocytes have been reported in chronic HCV infection (21–23), but not in chronic hepatitis B infection. To further define the role of T-cell exhaustion in the pathogenesis of chronic virus infection, we compared the expression of CD28, PD-1 and CD127 in tumour-infiltrating lymphocytes with that in PBL from patients with chronic HBV-related HCC.

Patients and Methods

Patients

During the period February 2008 to March 2010, we recruited 45 patients with HCC and HBV. All patients met the following criteria: (i) detection of HBsAg in serum for at least 6 months and (ii) histopathological evidence of HCC. Patients were excluded if they presented with antibody to hepatitis C virus (anti-HCV), co-infection or super-infection with hepatitis D virus (HDV), or human immunodeficiency virus or had a history of alcoholic liver disease. A total of three to four biopsy specimens of liver tumour were obtained from each patient. PBL from the same patients served as the control. Clinical stages of tumours were determined according to the TNM classification system of the International Union Against Cancer (edition 6). Tumour differentiation was graded according to the Edmondson–Steiner classification. Clinical characteristics of all patients are summarized in Tables 1 and 2. This study was approved by the Institutional Review Board of the China Medical University Hospital (Taichung, Taiwan).

Table 1. Disposition and baseline characteristics of HBV patients with hepatocellular carcinoma. ($n = 45$)

Male, number (%)	36 (80%)
Age (years), mean ± SD (range)	50 ± 13.68 (29–71)
ALT (IU/L), mean ± SD (range)	77.53 ± 61.82 (25–226)
AFP (ng/mL), mean ± SD (range)	8410.25 ± 21113.87 (2.99–80000)
HBV DNA (IU/mL), median (range)	4.8×10^4 ($112–1.1 \times 10^9$)
HBeAg(+), number (%)	23 (51.1%)

'+', positive; HBV, hepatitis B virus; ALT, alanine-aminotransferase; AFP, alpha-fetoprotein; mean ± SD, mean ± standard deviation.

Table 2. Clinicopathologic features of HBV patients with hepatocellular carcinoma. ($n = 45$)

PVT (positive/negative)	18/27
TNM Stage (I-II/III-IV)	30/15
Tumor differentiation (I-II/III-IV)	23/22
Tumor size, cm (≤ 5/ > 5)	24/21
Tumor multiplicity (solitary/multiple)	28/17
Cirrhosis (present/absent)	30/15
Child-Pugh classification (A/B)	42/3
Vascular invasion (present/absent)	29/16

PVT, portal vein thrombosis.

Isolation of tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes were isolated as described previously, with some modifications (24). Very few tumour-infiltrating lymphocytes (about 1×10^5 cells) could be recovered from a single biopsy specimen; therefore, tumour-infiltrating lymphocytes used in the assay were purified from 3–4 liver tumour biopsy specimens from each patient. The fresh tumour liver biopsy specimens were washed two times with sterile PBS and then transferred to a dish containing 5 ml complete DMEM (GIBOCL BRL, Gaithersburg, MD, USA) and 10% fetal calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml). The biopsy specimens were diced into 0.5 mm^3 pieces and then incubated for 2 h in [redacted] enzyme solution, 0.05% collagenase type IV (Sigma Co.) and 0.005% DNase (Sigma Co.) in complete DMEM medium. The cells were harvested by centrifugation, washed once with HBSS and then passed through a nylon mesh. Ficoll-Hypaque density gradient tubes were used to isolate liver-infiltrating lymphocytes. The tumour-infiltrating lymphocytes collected were resuspended in complete RPMI 1640 medium for cell number counting and further cell surface staining.

Flow cytometry analysis

For detection of specific surface markers on lymphocytes, tumour-infiltrating lymphocytes and PBL were prepared separately in FACS buffer solution at a concentration of $2\text{–}10 \times 10^5$/ml. The lymphocytes were treated with different surface marker antibodies for 30 min, including PE/Cy5-conjugated anti-CD3 mAb (Biolegend Co.), PE-conjugated anti-CD4 mAb (Biolegend Co.), FITC-conjugated anti-CD8 mAb (Biolegend Co.), FITC-conjugated anti-CD45RO mAb (Biolegend Co.), FITC-conjugated anti-CD69 mAb (Biolegend Co.), PE-conjugated anti-CD28 mAb (eBioscience Co.), PE-conjugated anti-PD-1 mAb (eBioscience Co.) and PE-conjugated anti-CD127 mAb (Pharmingen Co.). Lymphocytes were collected, washed and then subjected to flow cytometric analysis. The lymphocyte areas were gated with the help of the FFC and SSC dot plot and 20 000 cells were collected in the gated area. Normal isotype-matched irrelevant anti-mouse Ab was used as a staining control.

Statistical analysis

Differences in the expression of PD-1, CD28 and CD127 between tumour-infiltrating lymphocytes and PBL were compared using the non-parametric Mann–Whitney rank sum test. A P value of < 0.05 was regarded as indicating statistical significance.

Results

The majority of the tumour-infiltrating lymphocytes are activated memory T cells

Flow cytometric analysis revealed that the majority of the tumour-infiltrating lymphocytes mainly expressed CD8, CD69 and CD45RO (a marker of memory T cell) (Fig. 1), indicating that the majority of the tumour-infiltrating lymphocytes were activated memory $CD8^+$ T cells.

Increased programmed death-1 expression and decreased CD28 expression in tumour-infiltrating lymphocytes

To elucidate the roles of PD-1 and CD28 in HBV-related HCC, we evaluated the expression levels of PD-1 and CD28 molecules on tumour-infiltrating lymphocytes and PBL. As shown in Figure 2, the expression level of PD-1 was significantly higher and the expression level of CD28 was significantly lower in tumour-infiltrating lymphocytes than in PBL. These results indicate that the elevated expression of PD-1 in tumour-infiltrating lymphocytes is a crucial feature in HBV-related HCC.

The correlation between co-inhibitory and costimulatory molecules in liver-infiltrating lymphocytes and peripheral blood lymphocytes

To further elucidate the correlation between co-inhibitroy (PD-1) and costimulatory (CD28) molecules, the co-expression of PD-1 and CD28 in liver-infiltrating lymphocytes and PBL was evaluated. As seen in Fig. 3A, the expression of PD-1 was higher in $CD28^+CD3^+$ tumour-infiltrating lymphocytes than in $CD28^+CD3^+$ PBL. In contrast, the expression of CD28 was lower in $PD\text{-}1^+CD3^+$ tumour-infiltrating lymphocytes than in $PD\text{-}1^+CD3^+$ PBL. As shown in Fig. 3B, the expression of PD-1 was significantly higher and that of CD28 was significantly lower in tumour-infiltrating lymphocytes than in PBL. Also, the co-expression of PD-1 and CD28 was higher in $CD3^+$ tumour-infiltrating lymphocytes than in $CD3^+$ PBL. All of these data further indicate that the over expression of PD-1 and the under expression of CD28 are a characteristic of tumour-infiltrating lymphocytes in HBV-related HCC.

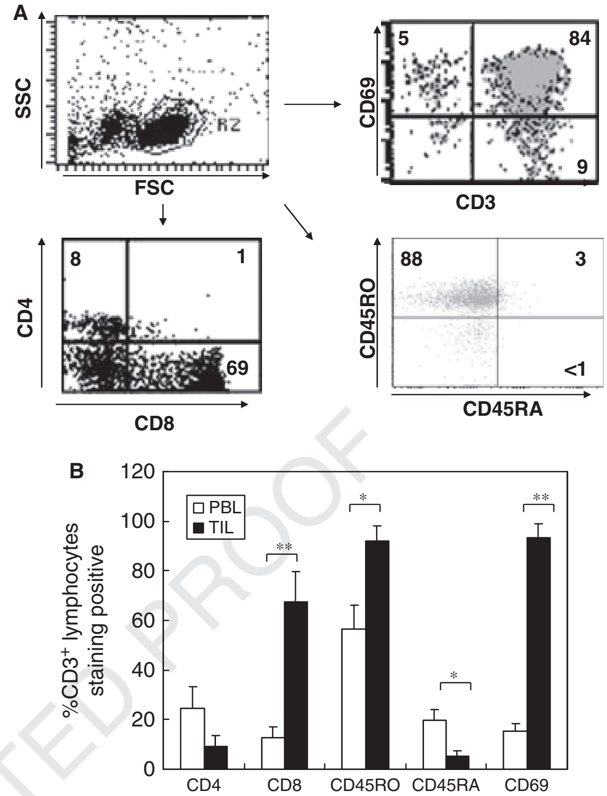

Fig. 1. Characterization of tumour-infiltrating lymphocytes isolated from tumour tissues of hepatitis B virus-related hepatocellular carcinoma patients and peripheral blood lymphocytes by flow cytometry (A). Suspensions of mononuclear cells were isolated from tumour tissue specimens as described in Materials and Methods. Immediately after isolation, cells were stained for CD3, CD4, CD8, CD69, CD45RA and CD45RO, and analysed by flow cytometry. The numbers represent the percentage of cells detected in each respective quadrant in the flow cytometric scatter plots. These data are representative of the phenotype observed in tumour-infiltrating lymphocytes isolated from 45 subjects. (B) Percentages of CD4, CD8, CD45RO, CD45RA and CD69 expression in $CD3^+$ tumour-infiltrating T cells (TIL) from the above subjects. Values represent the mean ± SD. ($^*P < 0.05$, $^{**}P < 0.01$; Mann–Whitney rank sum test).

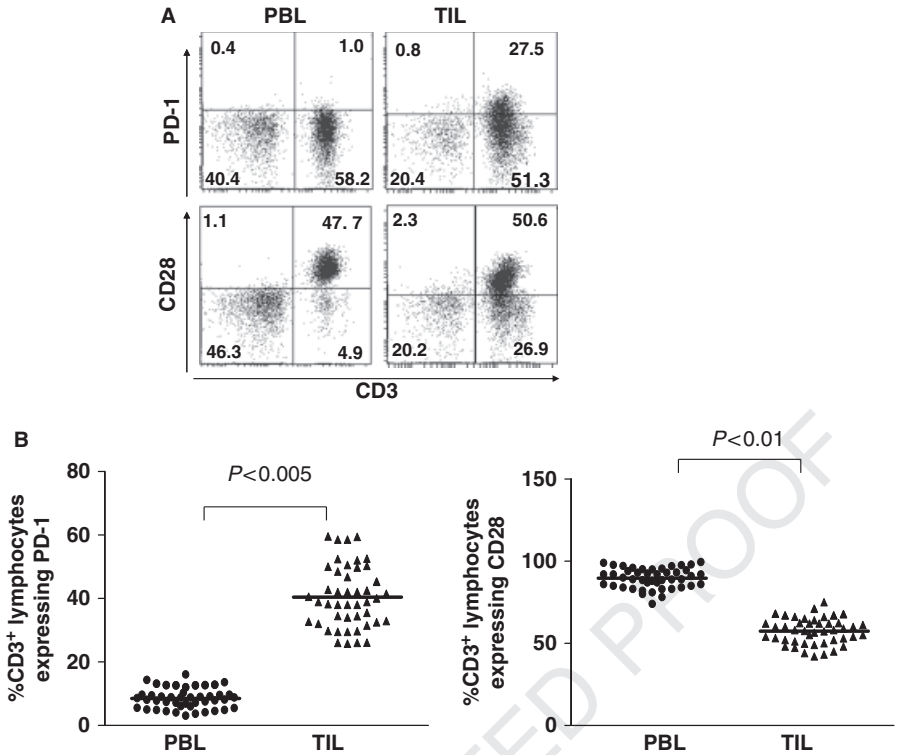

Fig. 2. The expression of PD-1 and CD28 in tumour-infiltrating lymphocytes. (A) Tumour-infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) were incubated with anti-CD28 and anti-PD-1 monoclonal antibody (mAb) and analysed by flow cytometry. These data are representative of the phenotype observed in TIL and PBL isolated from 45 subjects (B) Percentages of $CD3^+$ lymphocytes expressing CD28 or PD-1 in tumour-infiltrating lymphocytes and peripheral blood lymphocytes isolated from the same subjects.

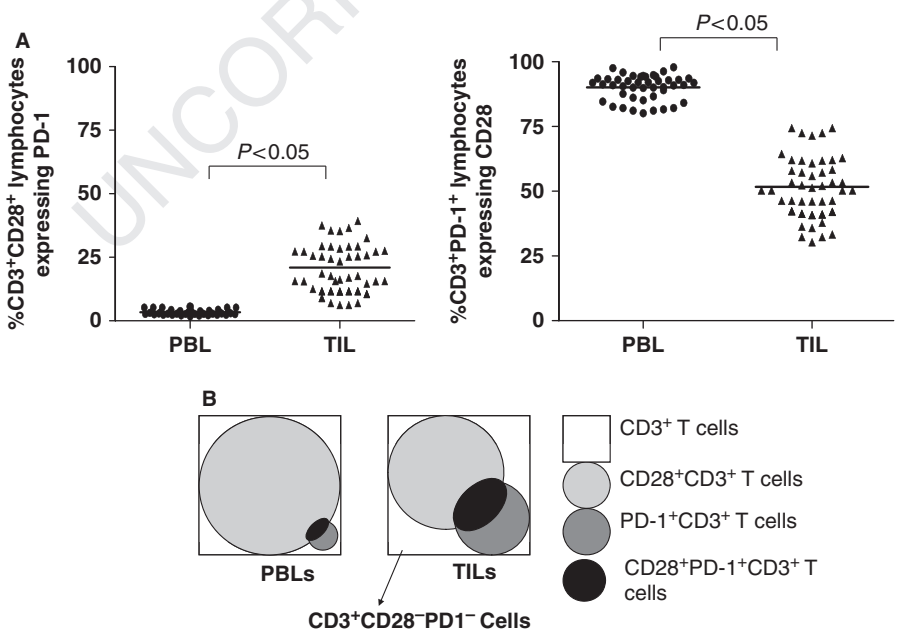

Fig. 3. Co-expression of CD-28 and PD-1 in $CD3^+$ T cells in tumour-infiltrating lymphocytes and peripheral blood lymphocytes from hepatitis B virus-related hepatocellular carcinoma (HCC) patients. (A) Percentages of $CD3^+$ lymphocytes expressing both CD28 and PD-1 in tumour-infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) isolated from the same subjects. (B) The correlation of CD28 and PD-1 expression in $CD3^+$ T cells in TIL and PBL from hepatitis B virus-related HCC patients.

The expression of CD127 decreased in the tumour-infiltrating lymphocytes

It has been shown that down-regulation of CD127 (IL-7 receptor) correlates with the exhausted phenotype of lymphocytes (23). In addition, high levels of PD-1 and low levels of CD127 are linked to a functionally 'exhausted' phenotype of HCV-specific $CD8^+$ T cells in chronic infection (18). Therefore, it is of interest to analyse the expression of CD127 in tumour-infiltrating lymphocytes in patients with HBV-related HCC. Figure 4 clearly shows that the expression of CD127 in tumour-infiltrating lymphocytes was lower than that in PBL. Combined with the aforementioned results of high PD-1 expression in tumour-infiltrating lymphocytes (Fig. 2), this evidence highly suggests that the tumour-infiltrating lymphocytes in HBV-related HCC exhibit an exhausted phenotype.

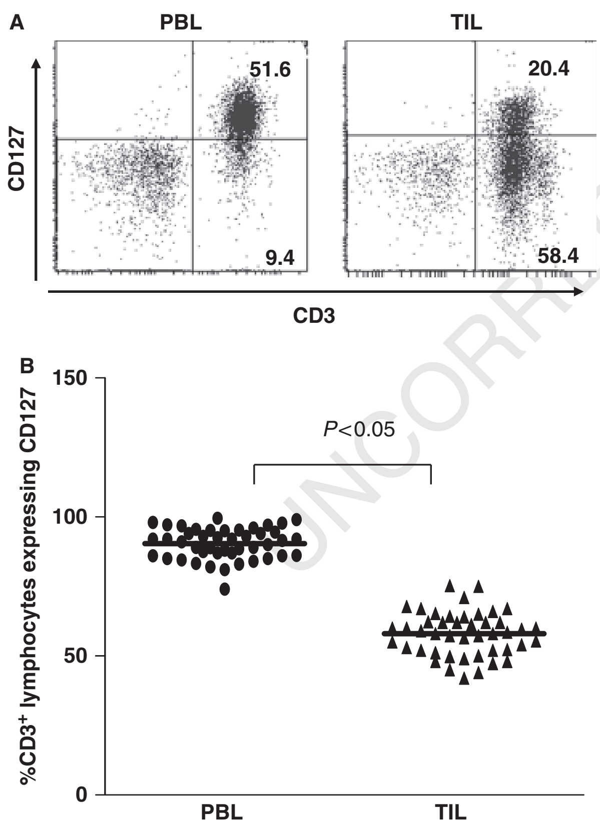

Fig. 4. The expression of CD127 and CD3 in tumour-infiltrating lymphocytes and peripheral blood lymphocytes. (A) Tumour-infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) were incubated with the anti-CD3 and anti-CD127 mAbs and analysed by flow cytometry. These data are representative of the phenotype observed in TIL and PBL isolated from 45 subjects. (B) Percentages of $CD3^+$ lymphocytes expressing CD127 in tumour-infiltrating lymphocytes and peripheral blood lymphocytes isolated from the same subjects.

The correlation between clinical data and the expression of co-inhibitory programmed death-1 molecules

We investigated the relationship between the clinical data and the expression levels of co-inhibitory PD-1 molecules *in vitro*. Patients with decreased PD-1 expression were more likely to exhibit aggressive clinicopathological features. In HCC, vascular invasion includes tumour thrombosis of the portal vein as well as the hepatic vein. In this study, vascular invasion was evaluated using contrasted abdominal computed tomography (CT) and color Doppler imaging with conventional grey-scale ultrasound. Portal vein thrombosis was differentiated from hepatic vein thrombosis by both anatomic position and a specific filling defect in the portal phase of the dynamic CT imaging. The expression levels of PD-1 were lower in tumour-infiltrating lymphocytes in patients with vascular invasion, but this was not statistically significant ($P = 0.08$). Meanwhile, decreased PD-1 expression was significantly associated with portal vein tumour thrombosis invasion (Fig. 5). However, we found no significant correlation between PD-1 and other clinicopathological parameters (Fig. 6).

Discussion

We found that the majority of the tumour-infiltrating T cells were $CD45RO^+CD69^+$ cells (Fig. 1), indicating that activated memory $CD8^+$ T cells accumulate during hepatitis B virus-related HCC. Similar findings have been reported in liver-infiltrating lymphocytes in previous studies (3, 4, 22). This implies that both liver-infiltrating lymphocytes and tumour-infiltrating lymphocytes are primed and have the memory to respond to the antigen exposed previously.

Many studies have shown that T-cell dysfunction contributes to the development of viral persistence. For example, virus-specific T-cell responses have been shown to be significantly abated in chronic virus hepatitis B (2). In addition, patients with chronic viral infection present with increased levels of exhausted T lymphocytes and a decrease in cellular proliferation and cytokine secretion (20). However, the precise mechanisms governing dysfunction of the cytotoxic T-lymphocyte effector response remain poorly defined. The PD-1/PD-L1 inhibitory pathway has been proposed to induce functional impairment or exhaustion of cytotoxic T lymphocytes and, then, to render persistent infection in chronic viral hepatitis (9–16, 21, 22, 25, 26, 27). In this study, tumour-infiltrating lymphocytes from patients with HBV-related HCC exhibited increased PD-1 expression and decreased CD28 expression.

The percentage of $CD28^+$ in $PD\text{-}1^+CD3^+$ of tumour-infiltrating lymphocytes is low and the percentage of $PD\text{-}1^+$ percentage in $CD28^+CD3^+$ tumour-infiltrating lymphocytes is high relative to PBL (Fig. 3), which highly suggests that the expression of CD28 and PD-1 are mutually affected. Higher expression of PD-1 seems to accompany a lower expression of CD28 and vice versa.

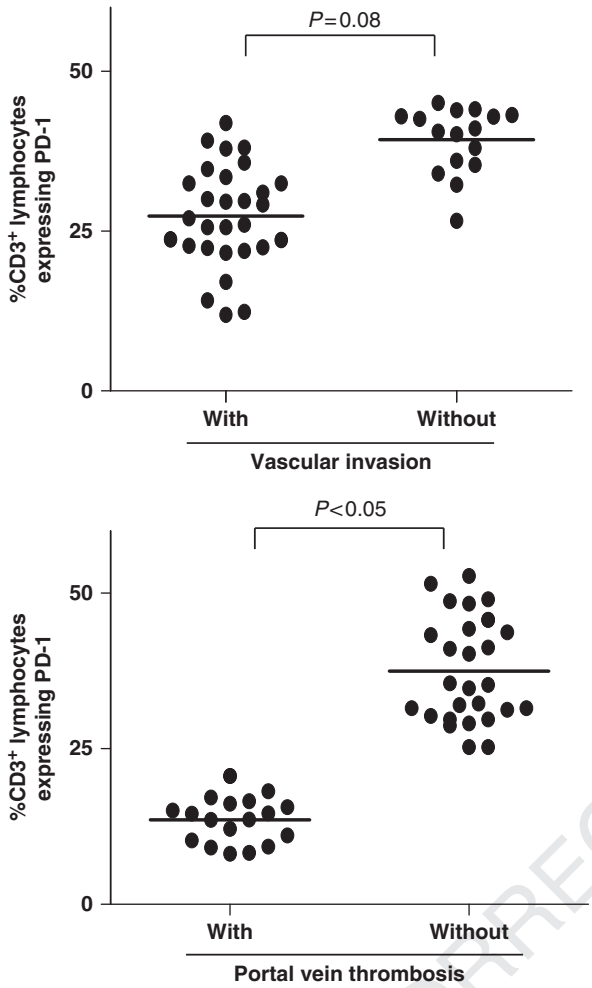

Fig. 5. Decreased expression of PD-1 molecules in tumour-infiltrating lymphocytes in patients with portal vein tumour thrombosis invasion. The expression levels of PD-1 were lower in tumour-infiltrating lymphocytes in patients with vascular invasion, but this was not statistically significant ($P = 0.08$). In contrast, decreased PD-1 expression was significantly associated with portal vein tumour thrombosis invasion. ($P < 0.05$; Mann–Whitney rank sum test).

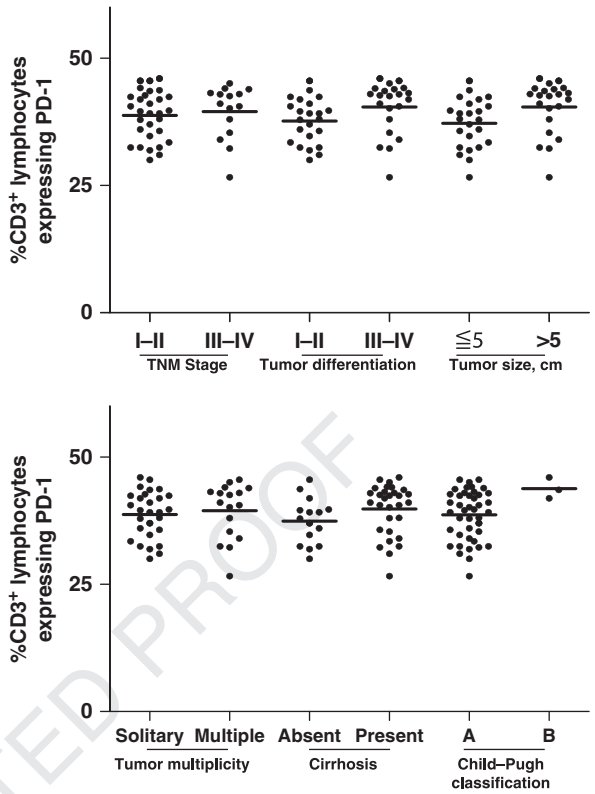

Fig. 6. There was no significant difference in expression of PD-1 in tumour-infiltrating lymphocytes in patients with other clinicopathological parameters. There was no significant correlation between PD-1 and TNM stage, tumour differentiation, tumour size, tumour multiplicity, cirrhosis or Child–Pugh classification. ($P > 0.05$; Mann–Whitney rank sum test).

Therefore, a balance between the co-inhibitory pathway (PD-1) and the costimulatory pathway (CD28) seems to determine the fate of viral-infected liver cells. Viral clearance is less effective if tumour-infiltrating lymphocytes overexpress PD-1.

It has been shown that CD127 (IL-7R alpha chain) expression plays an important role in chronic viral infection (25). Boettler and colleagues showed that overexpression of CD127 on liver-infiltrating antigen-specific $CD8^+$ T cells correlated with a loss of PD-1 expression and acquisition of CCR7 expression in the clearance of acute viral hepatitis infection. These results suggest that the expression of CD127 is a marker for the development of functionally and phenotypically defined antigen-specific $CD8^+$ memory T cells (28). Another study revealed that in chronic human hepatitis C virus infection, liver-infiltrating lymphocytes display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression (21). To confirm whether the tumour-infiltrating lymphocytes of our enrolled patients showed the exhausted phenotypes, we checked the expression of CD127 in the tumour-infiltrating lymphocytes and PBL. Our results show that the level of CD127 expression was lower in the tumour-infiltrating lymphocytes than in the PBL, which is consistent with the finding reported by Radziewics *et al.* (21) who showed that liver-infiltrating lymphocytes were mostly exhausted T lymphocytes.

All of our enrolled patients had HBV-related HCC. Clinically, we use ALT as a surrogate marker of intrahepatic immune activity. Most of the patients showed increased levels of ALT; however, there was no correlation between ALT level and PD-1 expression. We found a negative correlation between portal vein tumour thrombosis invasion and the expression level of PD-1 co-inhibitory molecule, i.e. the expression of PD-1 in tumour-infiltrating lymphocytes decreases in patients with portalvein thrombosis compared with those without portal vein thrombosis. This implies that low levels of PD-1 in tumour-infiltrating lymphocytes in HCC

patients might serve as a marker of tumour progression. Previous studies showed that elevated PD-L1 expression in HCC was significantly associated with tumour aggressiveness and enhanced risk for post-operative recurrence (29). It may be possible that PD-L1 on tumour cells induces the apoptosis of PD-1^{+} T cells, thereby reducing their numbers, as it has been shown that PD-L1 is induced in tumour cells by viral infection and mediates T-cell apoptosis (30).

In this study, we compared the expression of PD-1, CD28 and CD127 in tumour-infiltrating lymphocytes from HBV-related HCC patients with that in PBL from the same patients. The tumour-infiltrating lymphocytes were characterized by high levels of PD-1 expression, and low levels of CD28 and CD127 may contribute to persistent chronic viral infection. Our findings provide a better understanding of the pathogenesis of hepatitis B virus-related HCC.

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During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers clearly on the query sheet if there is insufficient space on the page proofs. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

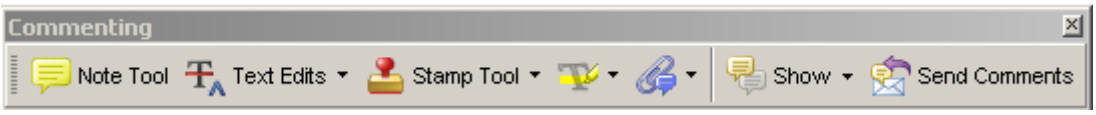
Query No.	Description	Author Response
Q1	Author: Please provide the full name of author P.-N. Hsu in the author group.	**Ping-Ning Hsu**
Q2	Author: Please provide the address information for Sigma Co.: town, state (if USA) and country.	Sigma Chemical Co., St Louis, MO, USA
Q3	Author: Please provide the address information for Biolegend Co.: town, state (if USA) and country.	BioLegend, San Diego, CA
Q4	Author: Please provide the address information for eBioscience Co.: town, state (if USA) and country.	eBioscience, San Diego, CA, USA
Q5	Author: Please provide the address information for Pharmingen Co.: town, state (if USA) and country.	BD PharMingen (La Jolla, San Diego, CA, USA)
Q6	Author: "It may be ... apoptosis". The sentence has been reworded for clarity. Please check.	ok
Q7	Author: If this is not a one-page article, please provide the first and last page for reference [1]. Please check the author name and also provide the article title.	Screening and diagnosis of hepatocellular carcinoma. Colombo M. Liver Int. 2009 Jan;29 Suppl 1:143-7. Review.

USING E-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required Software

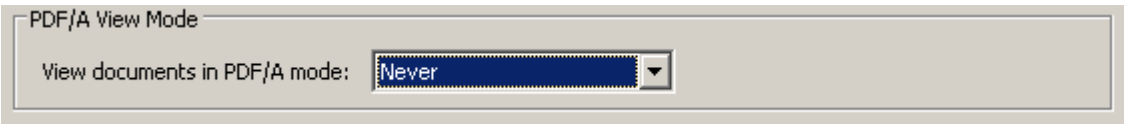
Adobe Acrobat Professional or Acrobat Reader (version 7.0 or above) is required to e-annotate PDFs. Acrobat 8 Reader is a free download: http://www.adobe.com/products/acrobat/readstep2.html

Once you have Acrobat Reader 8 on your PC and open the proof, you will see the Commenting Toolbar (if it does not appear automatically go to Tools>Commenting>Commenting Toolbar). The Commenting Toolbar looks like this:



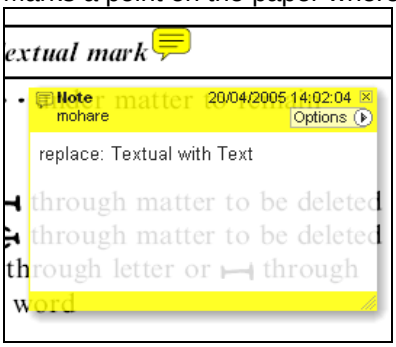
If you experience problems annotating files in Adobe Acrobat Reader 9 then you may need to change a preference setting in order to edit.

In the “Documents” category under “Edit – Preferences”, please select the category ‘Documents’ and change the setting “PDF/A mode:” to “Never”.



Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.

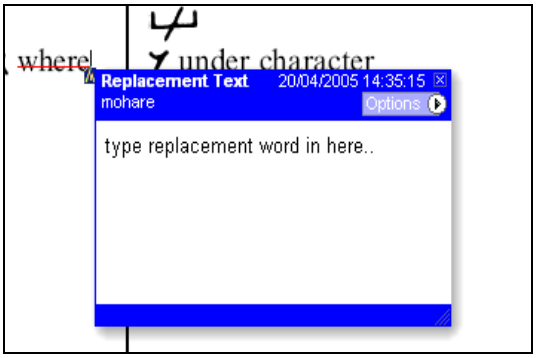


How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.

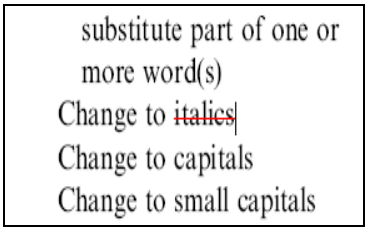


How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

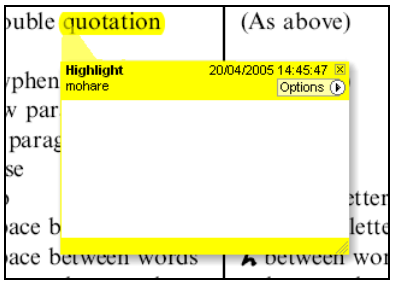
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

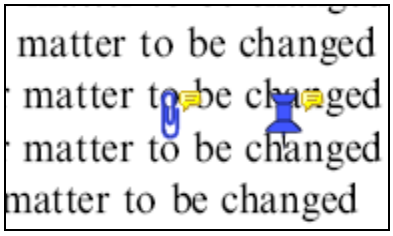


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

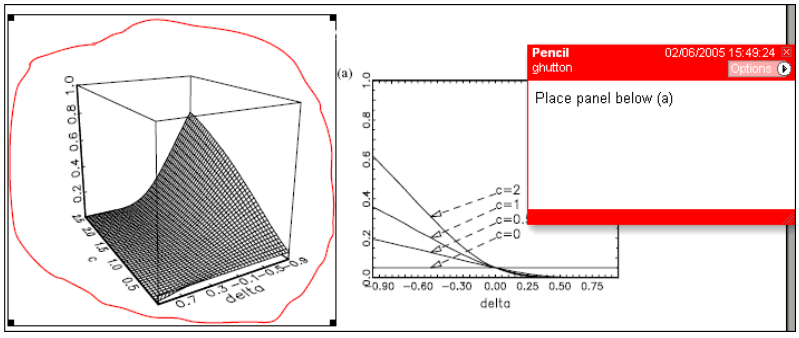


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: