FULL PAPER

BJC

British Journal of Cancer (2013) 109, 1031–1039 | doi: 10.1038/bjc.2013.390

Keywords: tumour-associated antigen; immune infiltration; prognosis; hepatocellular carcinoma

Expression pattern of tumour-associated antigens in hepatocellular carcinoma: association with immune infiltration and disease progression

J Liang[1,2,5], T Ding[1,3,5], Z-W Guo[2], X-J Yu[1], Y-Z Hu[1,4], L Zheng[1,2] and J Xu[*,1]

[1]State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-sen (Zhongshan) University, Guangzhou 510 060, PR China; [2]State Key Laboratory of Biocontrol, School of Life Science, Sun Yat-sen (Zhongshan) University, Guangzhou 510 031, PR China; [3]Department of Cell Biology, Nanjing Medical University, Nanjing 210 029, PR China and [4]Bank of Tumor Resources, Cancer Center, Sun Yat-sen (Zhongshan) University, Guangzhou 510 060, PR China

Background: The distinct expression pattern of tumour-associated antigens (TAAs) might be a critical reason for the inefficacy of immunity-based treatments and heterogeneous postsurgical recovery in patients with solid tumours, including hepatocellular carcinoma (HCC). However, little is known about the clinical value of the coexpression patterns of multiple TAAs.

Methods: We determined the expression of multiple TAAs with identified immunogenicity (GPC3, AFP, SSX-2, NY-ESO-1, EpCAM, midkine) and the density of tumour-infiltrating immune cells by immunohistochemistry in a panel of 362 primary HCC patients. We evaluated the association between the TAAs, immune cell infiltration, clinicopathological parameters, and prognosis.

Results: Patients who coexpressed more TAAs had better prognosis ($P<0.00001$, overall survival). The integrated pattern of TAA was associated with good differentiation and small tumour size, and with more $CD57^+$ natural killer and $CD20^+$ B-cell infiltration ($P<0.05$). Multivariate Cox proportional hazards analysis identified the TAA index as an independent prognostic indicator (hazard ratio 0.625; 95% confidence interval 0.467–0.837; $P=0.002$), and could further predict patient prognosis in collaboration with local immune infiltration.

Conclusion: Our results could provide new evidence for the improvement of prognostic molecular signatures in HCC, and a novel rationale for patient enrolment in future immunotherapeutic trials and/or clinical treatments.

Hepatocellular carcinoma (HCC) is among the world's leading cancer threats, and its incidence is increasing (Fan *et al*, 2011; Jemal *et al*, 2011). Despite improved diagnostic and treatment strategies, the overall survival (OS) of patients with HCC remains poor (Bruix and Llovet, 2009; Villanueva *et al*, 2012). Hepatocellular carcinoma is a highly complex disease that is generally resistant to commonly used chemotherapy and radiotherapy. Only a small proportion of newly diagnosed patients are eligible for potential curative therapies, including resection and liver transplantation. However, these therapeutic procedures most often do not provide a complete cure, and half of the treated patients experience tumour recurrence within 3 years (Villanueva *et al*, 2010; Yang and Roberts, 2010; Gao *et al*, 2012).

Hepatocellular carcinoma is usually present in inflamed fibrotic and/or cirrhotic liver with extensive leukocyte infiltration. Thus, the immune status at a tumour site can largely influence the

*Correspondence: Dr J Xu; E-mail: xujing@sysucc.org.cn
[5]These authors contributed equally to this work.

Received 24 April 2013; revised 14 June 2013; accepted 24 June 2013; published online 18 July 2013

biologic behaviour of HCC (Grivennikov *et al*, 2010; Fridman *et al*, 2012; Qin, 2012). Although it is commonly believed that innate and adaptive immunity would inhibit cancer growth, solid tumour cells, which often arise from chronic inflammation and survive immunoediting, can escape from immunosurveillance through different mechanisms (Disis, 2010; Vesely *et al*, 2011). After decades of substantial efforts, the success of several recent proof-of-concept clinical trials targeting immune regulators (e.g., ipilimumab and MDX-1106) suggests that active immunotherapy represents a path to obtain a durable and long-lasting response in cancer patients. However, their effects on the regression of human tumours remain limited, and only a relatively small fraction of patients derives clinical benefits (Mellman *et al*, 2011; Topalian *et al*, 2011). The reconstruction of immune surveillance for aberrant cells requires adequate tumour-associated antigen (TAA) expression, and the distinct expression pattern of TAAs in different tumours may be a critical reason for heterogeneous therapeutic effectiveness. Severe deficiency in TAAs is a common trick of tumour immune escape in primary cancer and/or during postsurgical relapse (Schreiber *et al*, 2011; Fu, 2012). However, little is currently known about the clinical significance of the coexpression pattern of multiple TAAs in solid tumours, including HCC.

In view of the present situation, we aimed to determine the expression of several TAAs with identified immunogenicity and potential therapeutic value in HCC and to evaluate their clinicopathological roles. In general, our results showed that the expression patterns of these TAAs (TAA index) were associated with local immune infiltration, disease progression, and prognosis.

MATERIALS AND METHODS

Patients and tissue samples. Archived formalin-fixed, paraffin-embedded tissues were obtained at the Cancer Center of Sun Yat-sen University, from 362 patients who had all undergone curative resection for HCC between January 2002 and December 2005. There were 317 (87.6%) patients with hepatitis B virus infection. We defined curative resection for HCC as a resection margin of at least 1 cm, complete resection of all tumour nodules, and leaving the cut surface free of tumour based on histological examination. Intra-operative ultrasound and postsurgical contrast-enhanced computed tomography (CT) were routinely used to ensure the complete removal of the HCC (Gao *et al*, 2007; Kuang *et al*, 2011; Xu *et al*, 2012b). No patient received anti-cancer therapies or had distant metastasis prior to the surgery. The clinical stage of tumours was determined according to the tumor-nodes-metastasis (TNM) classification system of the International Union Against Cancer (edition 6). The Institutional Review Boards of the Cancer Center approved the study. Written informed consent was obtained from all patients. Clinicopathological characteristics are summarised in Table 1.

Table 1. Patient characteristics

Characteristics	No. of patients (%)
Age, year	
Median	48.5
Range	20–78
Gender	
Male	324 (89.5)
Female	38 (10.5)
Hepatitis B virus infection	
No	45 (12.4)
Yes	317 (87.6)
Hepatitis C virus infection	
No	356 (98.3)
Yes	6 (1.7)
Alpha-fetoprotein	
$\leq 25\,ng\,ml^{-1}$	107 (29.6)
$>25\,ng\,ml^{-1}$	255 (70.4)
Child-Pugh class	
A	339 (93.6)
B	23 (6.4)
Differentiation	
I–II	289 (79.8)
III–IV	73 (20.2)
Tumour number	
Single	274 (75.7)
Multiple	88 (24.3)
Tumour size	
≤ 5 cm	128 (35.4)
>5 cm	234 (64.6)
Vascular invasion	
Absent	309 (85.4)
Present	53 (14.6)
TNM stage	
I	259 (71.5)
II–III	103 (28.5)

Abbreviation: TNM = tumour-lymph node metastasis.

Follow-up of patients and postoperative treatment. Patients were followed postoperatively at the outpatient clinic with regular surveillance for recurrence using the serum alpha-fetoprotein (AFP) level, abdominal ultrasonography, and chest radiography at 2- to 4-month intervals (Gao *et al*, 2007; Ding *et al*, 2011; Kuang *et al*, 2011; Xu *et al*, 2012b). When tumour recurrence or metastasis was suspected, further examinations, including CT and hepatic angiography, were performed. Biopsies were obtained when necessary. Patients with confirmed recurrence received further treatment, including a second surgical resection, transcatheter arterial chemoembolisation, radiofrequency ablation or percutaneous ethanol injection. The median follow-up was 34.5 months (range 1–95 months). Of the 362 patients examined during the follow-up period, 190 patients (52.5%) died, 193 (53.3%) were diagnosed with tumour recurrence, and 106 (29.3%) remained alive without recurrence. Overall survival was defined as the interval between surgery and death or between surgery and the last observation for surviving patients. The time to recurrence was defined as the interval between surgery and recurrence or between surgery and the last observation for patients without recurrence.

Tissue microarray and immunohistochemistry. The tissue microarray (TMA) was constructed as described previously (Xu *et al*, 2012b). Briefly, blocks containing the advancing edges of tumoural and peri-tumoural HCC tissue were used for TMA construction. Haematoxylin and eosin (H&E)-stained slides were reviewed without the knowledge of the patient clinical characteristics and outcomes. Duplicate 1.0-mm tissue cores were obtained from

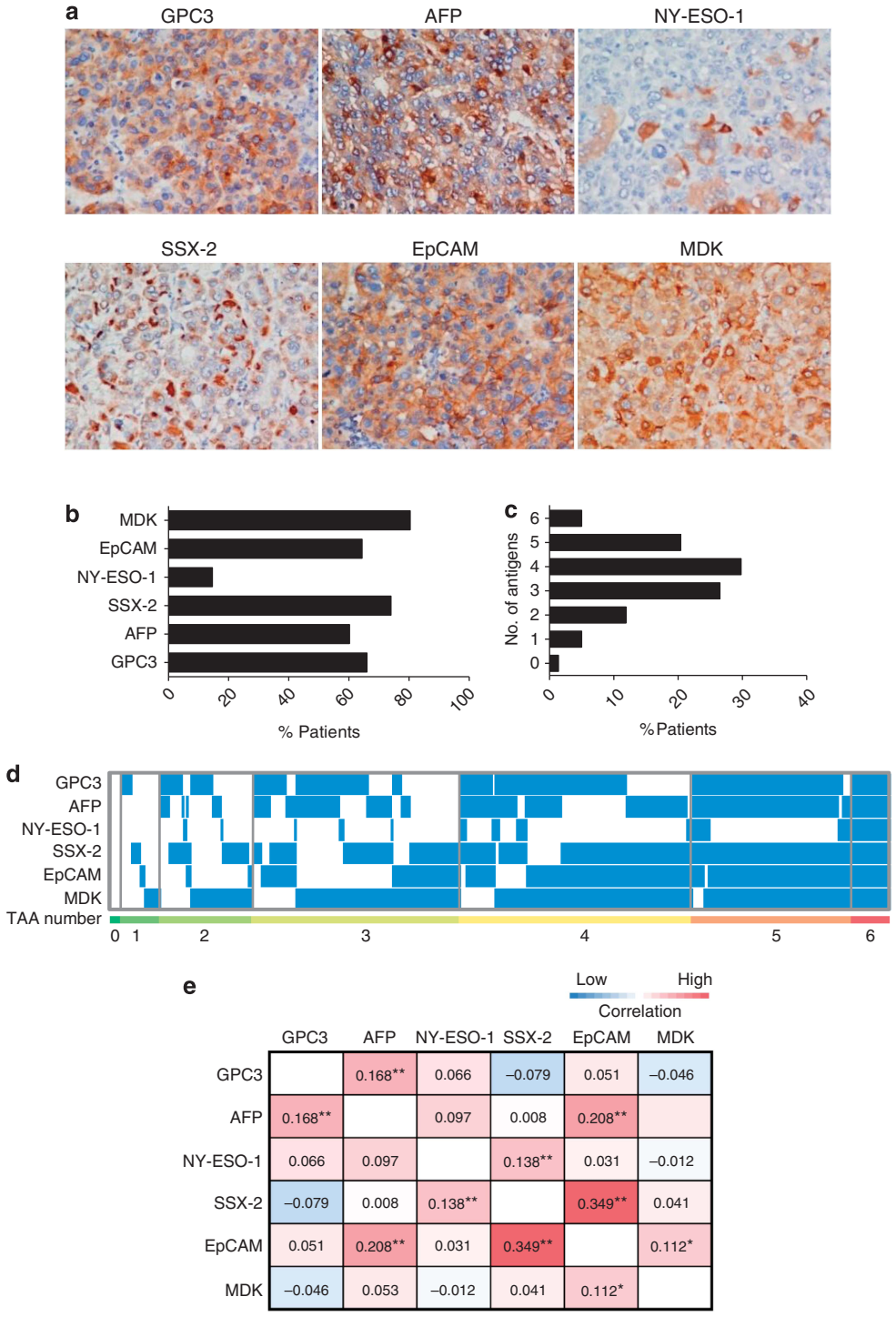


Figure 1. Tumour-associated antigen (TAA) expression in hepatocellular carcinoma (HCC). (**A**) Immunohistochemical detection of GPC3, AFP, NY-ESO-1, SSX-2, EpCAM, MDK in HCC tumour tissue (× 400 magnification). (**B**) Frequency of expression of the six TAAs in HCC tumour tissue determined by immunohistochemistry. (**C**) Frequency of coexpression of the indicated numbers of TAA (in any combination) in HCC tumour tissue. (**D**) TAA expression in each sample. Each column represents the TAA expression in an individual patient. Blue bar, positive expression. (**E**) The inter-relationship between each TAA in HCC tumour tissue. Values denote the Pearson correlation coefficients; values closer to 1 indicate a better correlation. *$P<0.05$; **$P<0.001$.

two regions (total, four punches) in the paraffin-embedded tissue blocks. Tissue microarrays containing the tissue cores were then cut into 5 μm sections for immunohistochemistry (IHC) staining.

The IHC of the paraffin sections was carried out using a two-step protocol (DakoCytomation, Glostrup, Denmark). Briefly, 5 μm sections were deparaffinised, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide containing double-distilled water for 10 min. Antigen retrieval was performed by microwave treatment in 0.01 mol l^{-1} citrate buffer, pH 6.0. Primary antibodies (Abs) were applied and incubated at 4 °C overnight. Antigen retrieval was developed with peroxidase and 3,3′-diamino-benzidine tetrahydrochloride. The sections were then counterstained with haematoxylin (Zymed Laboratories Inc.) and mounted in nonaqueous mounting medium. Antibodies information is

summarised in Supplementary Table 1. The appropriate negative controls were used, in which the primary Abs were replaced by irrelevant, isotype-matched Abs at the same concentration. If necessary, positive control tissues were applied as suggested by the manufacturers.

Evaluation of immunohistochemical variables. All TMA cores were screened and evaluated with a computerised image analysis platform constructed using the TMAJ Image application (http://tmaj.pathology.jhmi.edu). The expression of each antigen was scored according to the proportion of expression, as previously described (Xu *et al*, 2012b).

We initially investigated eight TAAs with identified immunogenicity and potential therapeutic function (Breous and Thimme, 2011): AFP (Thimme *et al*, 2008), GPC3 (Sawada *et al*, 2012), SSX-2 (Bricard *et al*, 2005), NY-ESO-1 (Korangy *et al*, 2004; Xu *et al*, 2012a), EpCAM (Yamashita *et al*, 2008), MDK (Jia *et al*, 2007; Kerzerho *et al*, 2010), melanoma antigen gene-A (MAGE-A) (Bricard *et al*, 2005), and telomerase reverse transcriptase (hTERT) (Mizukoshi *et al*, 2011). However, the expression of MAGE-A was below 10% and the immunodetection of hTERT was unsatisfactory due to nonspecific IHC staining in HCC (data not shown). For the six TAAs we eventually examined (AFP, GPC3, SSX-2, NY-ESO-1, EpCAM, and MDK), any proportion of any positive degree of intensity was considered positive. For the densities of lymphocytic infiltration, median cut-off points were used for the definition of subgroups.

Statistical analysis. Kaplan–Meier estimates were calculated and compared using the log-rank test. A multivariate Cox proportional hazard regression model was applied to estimate the adjusted hazard ratio (HR) and 95% confidence interval (CI) and to identify independent prognostic factors. The association between variables was evaluated using the χ^2 test or Fisher's exact test when appropriate. $P<0.05$ was considered to indicate statistical significance. Statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Expression of TAAs in HCC tissue. Immunohistochemistry was performed to analyse the expression of TAAs in HCC parenchyma. Of eight initial TAAs, six (GPC3, AFP, SSX-2, NY-ESO-1, EpCAM, and MDK; Figure 1A) were eventually included and evaluated in a panel of 362 HCC patients. Most of the TAAs were frequently expressed and were detected in over 50% of the samples, except for NY-ESO-1, which was detected in only 14.6% (53/362) of the samples (Figure 1B). The frequency for coexpression of two to five TAA was 11.9% (43/362), 26.5% (96/362), 29.8% (108/362), and 20.4% (74/362), respectively. Only 1.4% (5/362) of the samples revealed no expression of any TAA, and 5% (18/362) of the samples contained coexpression of all six TAAs (Figure 1C and D). Relevance analysis showed that several antigens were significantly associated with one another, indicating that different antigens might be regulated simultaneously during immunoediting (Figure 1E).

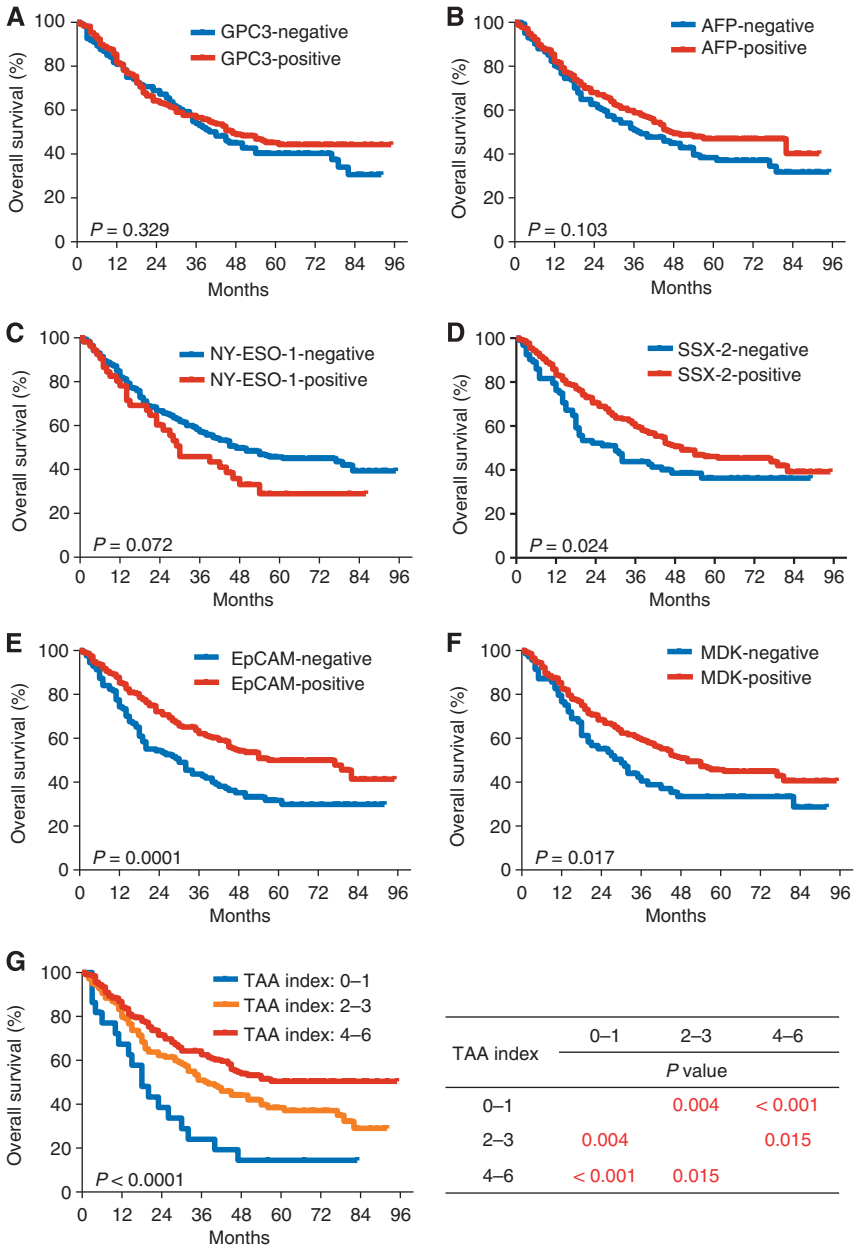
Association between TAAs and disease progression. Patients were categorised into three groups according to the number of positive TAAs (TAA index) in the tumour tissue. As summarised in Table 2, tumour differentiation and tumour size were significantly associated with TAA expression ($P=0.009$ and 0.018, respectively). In cases with less TAA expression, patients tended to have larger tumours and poor differentiation. For example, only 4.8% (14/289) of patients with grade I-II tumours expressed none or one of the six TAAs as compared with 12.3% (9/73) of patients with grade III-IV tumours, indicating that TAA deficiency might be another special feature of poorly differentiated tumours.

Table 2. Association between TAA expression and patients' clinical characteristics

	TAA index			
Characteristics	**0–1**	**2–3**	**4–6**	***P*-value**
Age, year				0.115
Median Range	43 25–62	50 20–77	48 21–78	
Gender				0.609
Male Female	22 1	124 15	178 22	
Hepatitis B virus infection				0.089
No Yes	4 19	23 116	18 182	
Hepatitis C virus infection				0.579
No Yes	22 1	137 2	197 3	
Alpha-fetoprotein				0.097
$\leqslant 25\,ng\,ml^{-1}$ $>25\,ng\,ml^{-1}$	9 14	48 91	50 150	
Child-Pugh class				0.242
A B	20 3	133 6	186 14	
Differentiation				**0.009**
I–II III–IV	14 9	120 19	155 45	
Tumour number				0.568
Single Multiple	18 5	101 38	155 45	
Tumour size				**0.018**
$\leqslant 5$ cm >5 cm	2 21	49 90	77 123	
Vascular invasion				0.239
Absent Present	17 6	118 21	174 26	
TNM stage				0.366
I–II III	15 8	95 44	149 51	

Abbreviations: TAA = tumour-associated antigen; TNM = tumour-lymph node metastasis. Bold values indicate statistical significance.

Kaplan–Meier survival curves were plotted to investigate the correlation between the expression of each TAA and patient survival. The log-rank statistic was used to compare survival rates. The expression of SSX-2, EpCAM, and MDK was positively correlated with better OS ($P=0.024$, 0.0001, and 0.017, respectively; Figure 2). Interestingly, survival was better in the patients with a higher TAA index (Figure 2G). The OS of patients who expressed more than four TAAs (median survival >60 months) was prolonged as compared with patients who expressed zero or one TAA (median survival, 18 months). The five-year OS rate in patients with 0-1 TAA expression was only 13.6% as compared with the 37.2% of those who expressed 4–6 TAAs. However, there was no significant association between TAA expression and recurrence in this cohort (Supplementary Figure 1).



TAA index	0–1	2–3	4–6
	P value		
0–1		0.004	< 0.001
2–3	0.004		0.015
4–6	< 0.001	0.015	

Figure 2. TAA expression and overall survival (OS). Kaplan–Meier survival estimates of the OS were performed according to the expression of (**A**) GPC3, (**B**) AFP, (**C**) NY-ESO-1, (**D**) SSX-2, (**E**) EpCAM, (**F**) MDK, and (**G**) the TAA index in hepatocellular carcinoma (HCC) tumour tissue.

We next assessed whether the TAA index could serve as an independent predictor of OS. A multivariate Cox proportional hazards analysis was performed, and the variables that were associated with survival by univariate analysis were adopted as covariates (summarised in Table 3). Serum AFP level, tumour differentiation, tumour size, and vascular invasion remained associated with OS in the multivariate Cox proportional hazards analysis ($P = 0.019$, 0.002, 0.001, and < 0.0001, respectively). The TAA index could still predict OS independent of these clinical factors (HR, 0.625; 95% CI, 0.467–0.837; $P = 0.002$; Table 3).

Association between TAA expression and local immune cell infiltration. Recent studies by our and other groups have shown that local immune cell status could influence HCC progression (Gao *et al*, 2007; Ding *et al*, 2009; Zhou *et al*, 2009; Wu *et al*, 2013). Therefore, we also assessed the infiltration of different type of immune cells (Supplementary Figure 2) in the tumour microenvironment, and their relationship with TAA expression. As shown in Figure 3 and Supplementary Table 2, there was a positive correlation between AFP or EpCAM and $CD20^+$ B cell or $CD57^+$ natural killer (NK) cell densities, but a negative correlation between SSX-2 and $FoxP3^+$ regulatory T (Treg) cells. However, NY-ESO-1 expression was significantly positively correlated with $CD8^+$ T, B, NK, and Treg cells. Moreover, patients who coexpressed more TAAs tended to have more B and NK cells, but not $CD15^+$ neutrophils, $CD68^+$ macrophages, or Treg cells infiltrating in the tumour. In addition, there was no correlation between the TAA index and immune cell infiltration in the peri-tumour region. Taken together, a higher TAA index could indicate a microenvironment with more anti-tumour immune cell infiltration but not tumour-educated regulatory cells in the HCC nest.

Prognostic values with combination of TAA index and immune infiltration. We next evaluated the combined influence of the TAA index and immune cell densities ($CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, $CD57^+$, and $FoxP3^+$ cells; Figure 4). Patients with a high TAA index (4–6 TAA coexpression) and high density of T (including $CD3^+$, $CD4^+$, and $CD8^+$ cells) and $CD20^+$ B or

Table 3. Cox proportional Hazard regression analysis of patients' overall survival

	Univariable			Multivariable		
Variables	**Hazard ratio**	**95% CI**	***P*-value**	**Hazard ratio**	**95% CI**	***P*-value**
Gender (male *vs* female)	1.073	0.842–1.368	0.568			NA
Hepatitis B virus infection (absent *vs* present)	1.218	0.781–1.901	0.384			NA
Hepatitis C virus infection (absent *vs* present)	1.852	0.687–4.994	0.223			NA
Alpha-fetoprotein ($\leq 25\,ng\,ml^{-1}$ *vs* $>25\,ng\,ml^{-1}$)	1.66	1.189–2.316	**0.003**	1.505	1.070–2.118	**0.019**
Child-Pugh class (A *vs* B)	1.17	0.679–2.016	0.572			NA
Differentiation (I–II *vs* III–IV)	1.749	1.254–2.441	**0.001**	1.716	1.219–2.416	**0.002**
Tumour number (single *vs* multiple)	1.233	0.890–1.707	0.208			NA
Tumour size (≤ 5 cm *vs* >5 cm)	2.109	1.516–2.934	**<0.0001**	1.796	1.266–2.547	**0.001**
Vascular invasion (absent *vs* present)	3.935	2.770–5.591	**<0.0001**	3.399	2.061–5.605	**<0.0001**
TNM stage (I–II *vs* III)	2.132	1.583–2.871	**<0.0001**	0.926	0.595–1.442	0.735
TAA index (0–3 *vs* 4–6)	0.626	0.471–0.833	**0.001**	0.625	0.467–0.837	**0.002**

Abbreviations: CI = confidence interval; NA = not applicable; TAA = tumour-associated antigen; TNM = tumour-lymph node metastasis. Note: Cox proportional hazards regression model; variables associated with survival by univariate analysis were adopted as covariates in multivariate analyses. Bold values indicate statistical significance.

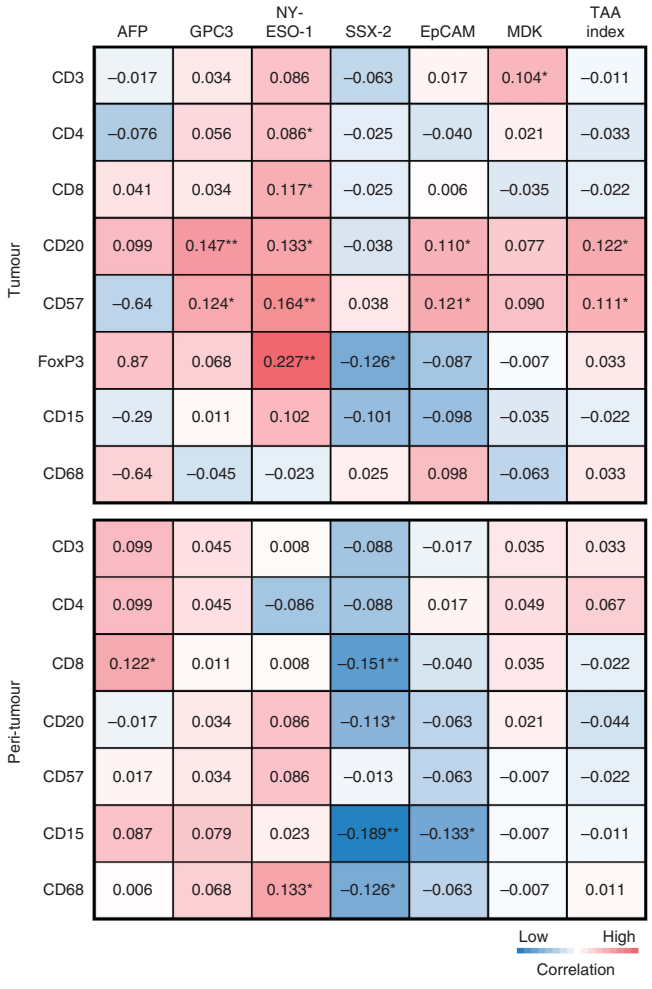


Figure 3. The inter-relationship between tumour-associated antigen (TAA) expression and immune cell infiltration in tumoural and peri-tumoural regions. Values denote the Pearson correlation coefficients; values closer to 1 indicate a better correlation. *$P<0.05$; **$P<0.001$.

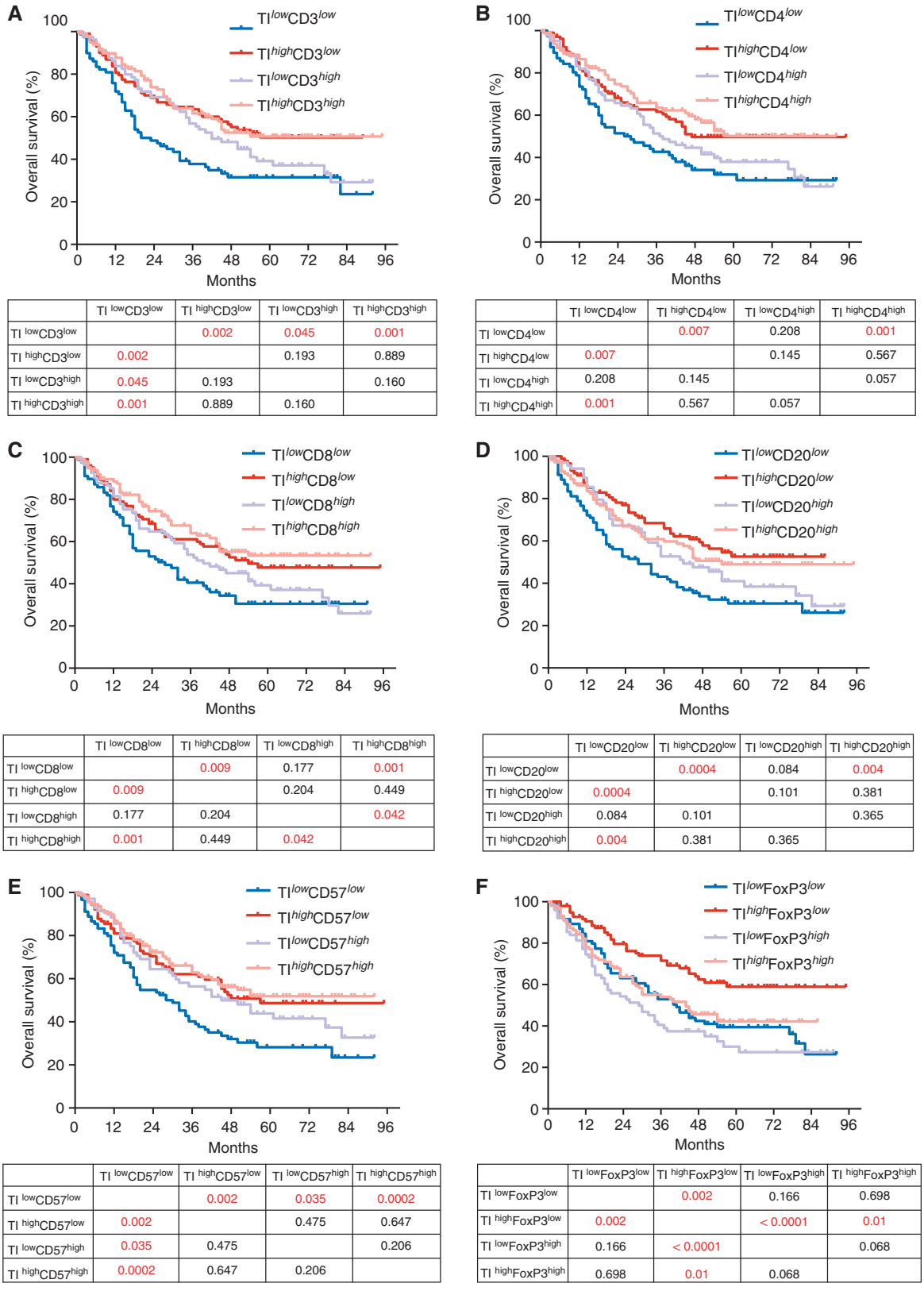
$CD57^+$ NK cells all had longer survival time (median survival >60 months). Patients with a low TAA index (0–3 TAA coexpression) and low density of these effective immune cells had shorter survival time (median survival ≤28 months; Figure 4). Supporting the general view that Treg cells suppress tumour immunity, low $FoxP3^+$ Treg cell infiltration with a high TAA index predicted better OS (median survival >60 months) as compared with any other subgroup ($P<0.01$, Figure 4F). Patients with high Treg density and a low TAA index had the shortest survival time (median survival, 29 months). These results demonstrated that the TAA index and immune cell infiltration could together determine tumour progression and patient survival.

DISCUSSION

It is commonly believed that poorly immunogenic transformed cells that escape immune surveillance would lead to the primary appearance of overt cancer and/or post-treatment relapse; however, little is known about the clinical value of the immunogenic features of malignant cells in solid tumours, including HCC. This study provides the first evidence that the expression pattern of multiple TAAs is associated with cancer progression and postsurgical prognosis in HCC patients.

Several other groups have reported the expression frequencies of single TAA, mostly on an mRNA level (Yamashita *et al*, 2008; Wang *et al*, 2009). In the present study, we further demonstrated the expression and clinical value of multiple antigens on a protein level, which should be the critical reason for heterogeneous effectiveness of immunotherapy and provide supportive evidence for 'immunoediting' in human cancers. The host immune system not only controls tumour quantity, but also shapes tumour immunogenicity. The finding that the coexpression of more TAAs was associated with better-differentiated and/or smaller tumours and the association between the expression of several antigens can aid in our understanding of the nature of immunoedited tumour progression.

Therefore, the expression patterns of TAAs in human tumours should be a result of long-term co-evolution between the immune system and malignant cells, and would be closely related to patient prognosis after surgical interventions and/or other treatments such as immunotherapies. Further analysis of the immune cell infiltration provided more evidence for the association between TAAs and tumour progression. Generally, the expression of single TAAs was positively associated with the infiltration of different lymphocyte subsets, including $CD3^+$ (Galon *et al*, 2006), $CD8^+$



	TI low CD3low	TI high CD3low	TI low CD3high	TI high CD3high
TI low CD3low		0.002	0.045	0.001
TI high CD3low	0.002		0.193	0.889
TI low CD3high	0.045	0.193		0.160
TI high CD3high	0.001	0.889	0.160	

	TI low CD4low	TI high CD4low	TI low CD4high	TI high CD4high
TI low CD4low		0.007	0.208	0.001
TI high CD4low	0.007		0.145	0.567
TI low CD4high	0.208	0.145		0.057
TI high CD4high	0.001	0.567	0.057	

	TI low CD8low	TI high CD8low	TI low CD8high	TI high CD8high
TI low CD8low		0.009	0.177	0.001
TI high CD8low	0.009		0.204	0.449
TI low CD8high	0.177	0.204		0.042
TI high CD8high	0.001	0.449	0.042	

	TI low CD20low	TI high CD20low	TI low CD20high	TI high CD20high
TI low CD20low		0.0004	0.084	0.004
TI high CD20low	0.0004		0.101	0.381
TI low CD20high	0.084	0.101		0.365
TI high CD20high	0.004	0.381	0.365	

	TI low CD57low	TI high CD57low	TI low CD57high	TI high CD57high
TI low CD57low		0.002	0.035	0.0002
TI high CD57low	0.002		0.475	0.647
TI low CD57high	0.035	0.475		0.206
TI high CD57high	0.0002	0.647	0.206	

	TI low FoxP3low	TI high FoxP3low	TI low FoxP3high	TI high FoxP3high
TI low FoxP3low		0.002	0.166	0.698
TI high FoxP3low	0.002		< 0.0001	0.01
TI low FoxP3high	0.166	< 0.0001		0.068
TI high FoxP3high	0.698	0.01	0.068	

Figure 4. The combination of tumour-associated antigen (TAA) expression and immune cell infiltration correlates with overall survival (OS). Kaplan–Meier curves illustrate the duration of OS according to the TAA index (TI) and the density of (**A**) $CD3^+$, (**B**) $CD4^+$, (**C**) $CD8^+$, (**D**) $CD20^+$, (**E**) $CD57^+$, or (**F**) $FoxP3^+$ cells in the tumour region. Red values indicate statistical significance.

(Gao *et al*, 2007), $CD20^+$ (Nielsen *et al*, 2012), and $CD57^+$ cells (Wu *et al*, 2013), which have all been reported to facilitate anti-tumour immunity, and was negatively associated with $FoxP3^+$ Treg cells (Zhou *et al*, 2009). Recently, Chew *et al* (2012) reported that a given set of chemokines was correlated with lymphocyte infiltration and prognosis in HCC, which also support the protective role of anti-tumour immune milieu in HCC progression. Tumours coexpressing more TAAs tended to have more $CD20^+$ B

and $CD57^+$ NK cells, but not $FoxP3^+$ Treg cells or other inflammatory cells, including $CD15^+$ neutrophils (Kuang *et al*, 2011) and $CD68^+$ macrophages (Ding *et al*, 2009), which are often exploited by solid tumours to establish a tumour-promoting microenvironment. Interestingly, the unexpected result of relationship between NY-ESO-1 and FoxP3 indicated that self-TAAs might also be involved in immune homeostasis and limiting acute inflammation. Taken together, the expression of more TAAs might promote anti-tumour immune reaction or surveillance and facilitate the postsurgical recovery of HCC patients.

It is conventionally believed that the adaptive immune response mediated by tumour-infiltrating lymphocytes (TILs) influences the behaviour of human cancers (Abastado, 2012). High densities of $CD3^+$ cells in the centre of a tumour and the invasive margin of colorectal tumours predict better clinical outcomes (Galon *et al*, 2006). For HCC, however, results by ours (Supplementary Figure 3) and other have shown that the infiltration of total T lymphocytes was not associated with postsurgical prognosis (Gao *et al*, 2007). In the present study, we stratified the patients according to the TAA index, and the density of $CD3^+$ cells was positively associated with the OS in patients with a low TAA index (Figure 4A). Subsequently, apart from the type and density of immune cells, features of the tumour itself, such as TAA expression, should also be important factors of immune regulation in HCC. Therefore, the combination of the TAA index and the densities of immune cells could further predict HCC prognosis. Recently, we developed an immune-based *in situ* molecular classifier that could aid in the identification of patients who are at greatest risk for postsurgical recurrence of HCC (Xu *et al*, 2012b). The predictive values of TAAs could provide more parameters to optimise molecular classifiers for HCC outcomes. Of course, other tumour cell features (such as proliferation) should also be considered important during early cancer evolution and later progression. In tumours with weak proliferation (low Ki-67), the TAA index was closely associated with better prognosis, while all of the patients with intensive proliferation had poor prognosis (Supplementary Figure 4). In general, the coactions of immunoediting and the vital power of tumour cells could continue shaping malignancies and influence patient survival after treatments, including resections and/or biological therapies.

Although clinical trials involving immunotherapy with T-cell clones specific for a single antigen have provided a foundation for proof-of-principle studies, reduced clinical efficacy has been encountered in contrast to the substantial therapeutic impact of transfer with polyclonal TIL cultures. The outgrowth of antigen-loss tumour variants in treated patients indicates the ability of rapidly adaptable tumour cells to evade narrowly focused therapies (Mellman *et al*, 2011; He *et al*, 2012). Recently, new therapies based on sophisticated knowledge of the suppressive tumour immune microenvironment were designed to overcome tolerance and reactivate anti-tumour immunity to induce potent, long-lasting responses (Mellman *et al*, 2011). For example, in early-phase clinical trials involving patients with advanced solid tumours such as metastatic melanoma, renal cell carcinoma, colorectal cancer, and non–small-cell lung cancer, monoclonal Abs against immune-checkpoint proteins (such as ipilimumab, tremelimumab, and MDX-1106) could induce a state of equilibrium between the immune system and cancer, resulting in prolonged disease stabilisation. Nevertheless, only a relatively small fraction of patients exhibited an objective response and derived clinical benefits (Topalian *et al*, 2011). In view of this, the discrepancies in the TAA profiles should be a critical reason for heterogeneous therapeutic efficacy. At present, immunotherapies that interrupt the tolerogenic pathways and reactivate endogenous immunity are being evaluated, appearing to be a promising HCC treatment option (primary or adjuvant for chemotherapy and/or surgery). To prevent overtreatment and to achieve more convincing results, molecular classification based on TAA expression patterns should also be an important strategy in clinical trials of immunotherapy.

In brief, TAA expression patterns could serve as important prognostic factors in HCC. Tumour-associated antigen expression should be associated with anti-tumour immune infiltration, and particularly, involved in disease progression and the reconstitution of immune surveillance after surgical intervention. Moreover, our results could provide a new evidence for improvement of the prognostic molecular signatures in HCC, and a potential rational consideration for patient enrolment in future immunotherapeutic trials and/or clinical treatments.

ACKNOWLEDGEMENTS

This work was supported by Project Grants from the Ministry of Health of China (2012ZX10002-011).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)