FULL PAPER

BJC

British Journal of Cancer (2015) 112, 1911–1920 | doi: 10.1038/bjc.2015.92

Keywords: tumour antigens; hepatocellular carcinoma; vaccination; immunohistochemistry; MAGE-C1; MAGE-C2; Glypican-3; Annexin-A2

Tumour antigen expression in hepatocellular carcinoma in a low-endemic western area

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Background: Identification of tumour antigens is crucial for the development of vaccination strategies against hepatocellular carcinoma (HCC). Most studies come from eastern-Asia, where hepatitis-B is the main cause of HCC. However, tumour antigen expression is poorly studied in low-endemic, western areas where the aetiology of HCC differs.

Methods: We constructed tissue microarrays from resected HCC tissue of 133 patients. Expression of a comprehensive panel of cancer-testis (MAGE-A1, MAGE-A3/4, MAGE-A10, MAGE-C1, MAGE-C2, NY-ESO-1, SSX-2, sperm protein 17), onco-fetal (AFP, Glypican-3) and overexpressed tumour antigens (Annexin-A2, Wilms tumor-1, Survivin, Midkine, MUC-1) was determined by immunohistochemistry.

Results: A higher prevalence of MAGE antigens was observed in patients with hepatitis-B. Patients with expression of more tumour antigens in general had better HCC-specific survival ($P=0.022$). The four tumour antigens with high expression in HCC and no, or weak, expression in surrounding tumour-free-liver tissue, were Annexin-A2, GPC-3, MAGE-C1 and MAGE-C2, expressed in 90, 39, 17 and 20% of HCCs, respectively. Ninety-five percent of HCCs expressed at least one of these four tumour antigens. Interestingly, GPC-3 was associated with SALL-4 expression ($P=0.001$), an oncofetal transcription factor highly expressed in embryonal stem cells. SALL-4 and GPC-3 expression levels were correlated with vascular invasion, poor differentiation and higher AFP levels before surgery. Moreover, patients who co-expressed higher levels of both GPC-3 and SALL-4 had worse HCC-specific survival ($P=0.018$).

Conclusions: We describe a panel of four tumour antigens with excellent coverage and good tumour specificity in a western area, low-endemic for hepatitis-B. The association between GPC-3 and SALL-4 is a novel finding and suggests that GPC-3 targeting may specifically attack the tumour stem-cell compartment.

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death, with over half a million deaths per year worldwide (El-Serag *et al*, 2001; Jemal *et al*, 2011). HCC is more prevalent in eastern Asia (Jemal *et al*, 2011) where hepatitis-B (HBV) accounts for 65% of HCC cases (Perz *et al*, 2006). In contrast, western Europe is a low-endemic area where HBV is not the main cause of HCC (Perz *et al*, 2006), and HCC is often diagnosed in non-cirrhotic livers (Verhoef *et al*, 2004; Witjes *et al*, 2012). However, it is estimated that the incidence of HCC is expected to continue to rise significantly in western Europe and North America due to the hepatitis C virus infections during the 1960s and 1970s (IARC, 2011).

Primary treatment for early-stage disease includes resection, local ablation and, in selected cases, liver transplantation. However,

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Received 9 October 2014; revised 21 January 2015; accepted 12 February 2015

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only 20% of patients are candidates for curative procedures (El-Serag *et al*, 2008). Once the cancer is advanced cure is no longer possible and median survival is a dismal 6–8 months, which can be extended to 10–13 months with the addition of sorafenib, a tyrosine kinase inhibitor (Llovet *et al*, 2008; Abou-Alfa *et al*, 2010).

Recognition of the important role of the immune system in cancer surveillance and elimination (Zou, 2005) has led to the development of various immunotherapeutic strategies against cancer (Mellman *et al*, 2011). One such strategy, cancer vaccination, holds great promise, as has been recently demonstrated in prostate cancer (Kantoff *et al*, 2010a,b). In HCC, cancer vaccine trials have shown promising results, in particular after local therapy to prevent relapses (Kuang *et al*, 2004; Lee *et al*, 2005; Peng *et al*, 2005a). However, despite the promise of cancer vaccines, success has been limited due to a number of factors. One of these is the proper identification of tumour antigens. Important requirements for inclusion of tumour antigens in therapeutic vaccines are immunogenicity, prevalence of expression within the cancer population, tumour tissue specificity and biologic significance (Cheever *et al*, 2009; Lang *et al*, 2009; Kvistborg *et al*, 2013).

Multiple studies have described the expression of tumour antigen panels in HCC, but the vast majority of these studies were conducted in east Asian populations (Chen *et al*, 2001; Luo *et al*, 2002; Peng *et al*, 2005b; Nakamura *et al*, 2006; Sera *et al*, 2008; Shirakawa *et al*, 2009; Yan *et al*, 2011; Yorita *et al*, 2011; Liang *et al*, 2013; Xia *et al*, 2013) where the aetiology of HCC is predominately related to HBV. Very few such studies have been performed in western, low-endemic areas (Riener *et al*, 2009).

Tumour tissue specificity refers to the predominant, most preferably exclusive, expression of the tumour antigen in cancer and not in normal tissues (Kvistborg *et al*, 2013). A strict interpretation of this requirement would limit tumour antigens to antigens resulting from somatic mutations, chromosomal translocations resulting in neo-antigens, or viral-derived antigens. However, exome sequencing has recently shown that somatic mutation patterns in HCC are strongly variable between individual patients and therefore not suitable for design of off-the-shelf therapeutic vaccines (Fujimoto *et al*, 2012). The most promising alternative tumour antigens are cancer-testis antigens, which are exclusively expressed in germ cells but not in other normal tissues (Hofmann *et al*, 2008), and oncofetal antigens, expressed primarily during embryogenesis but not broadly in adult humans (AFP, Glypican-3). Both types of antigens are aberrantly expressed in various types of cancer. In addition, self-antigens that are overexpressed in cancer (Survivin, Wilms tumor-1, Midkine and Annexin-A2) are also considered tumour antigens, and several clinical trials are underway in HCC and other cancers, targeting these types of overexpressed self-antigens. Many of the existing studies in HCC do not include tumour antigen expression in the corresponding surrounding tumour-free liver (TFL) compartment and thus tissue specificity cannot be assessed.

In this study, we used immunohistochemistry on tissue microarrays (TMAs) to examine, on the protein level, the expression pattern in HCC of a comprehensive panel of 15 tumour antigens belonging to different categories, including the cancer testis antigens MAGE-A1, MAGE-A3/4, MAGE-A10, MAGE-C1, MAGE-C2, NY-ESO-1, Sperm Protein 17 (SP17) and SSX-2, the oncofetal proteins AFP and Glypican-3 (GPC-3), the overexpressed tumour antigens Annexin-A2, Wilms tumour-1 (WT-1), Survivin, Midkine (MDK) and the glycoprotein MUC-1. All these antigens have previously demonstrated immunogenicity in human studies. In addition, we tested for the expression of SALL-4, a transcription factor involved in the maintenance of embryonic and cancer stem cells (Zeng *et al*, 2014). SALL-4 has recently been shown to be expressed in an HCC subtype with stem-cell like features and to be associated with poor prognosis (Oikawa *et al*, 2013; Yong *et al*, 2013b; Zeng *et al*, 2014). The goal of the study was to identify a panel of biologically relevant tumour antigens with (a) broad expression in a western European population of HCC patients and (b) specific expression in the tumour tissue with no, or little, expression in surrounding TFL tissue.

MATERIALS AND METHODS

Patient population and tissue samples. Archived formalin-fixed paraffin-embedded tissue samples from 133 patients who underwent hepatic resection ($n = 94$) or liver transplantation ($n = 39$) for HCC in our centre, between July 2004 and October 2013, were used for this study. Clinicopathologic characteristics are shown in Table 1. All patients had undergone procedures with curative intent and none had received systemic therapy before resection or transplantation. Patients with evidence of residual cancer after resection were excluded. Informed consent for the use of tissue for research purposes was obtained from all patients.

TMA construction. Three 0.6-mm cores were taken from the tumorous area of 133 patients and two 0.6-mm cores were taken from the corresponding TFL tissue of 105 of these patients. The tumorous as well as the TFL areas with vital tissue were marked by an experienced pathologist using archived H&E glass slides. In each TMA we included cores of testis, placenta, tonsil, ovary, stomach, prostate, bladder, kidney, lung and liver as control tissues. The TMAs were made using a Beecher automated tissue-arrayer ATA-27 (Beecher Instruments, Sun Prairie, WI, USA).

Immunohistochemistry. Immunohistochemistry was performed on 4-μm thick sections mounted on Superfrost Plus slides (Erie Scientific LLC, Portsmouth, NH, USA). The sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 15 min. Antigen retrieval was performed in a microwave for 10 min using the appropriate antigen retrieval buffer for each antigen (Table 2). After serum block, primary antibodies were applied at 4°C overnight. The primary antibodies (Table 2) were carefully selected to be monoclonal (with the exception of AFP and SP17) and to have been validated in scientific literature. HRP-conjugated anti-mouse or anti-rabbit polymer secondary antibody (Envision, DAKO, Glostrup, Denmark) was then applied for 1 h, followed by diaminobenzadine (DAB) as the chromogen detection method. The slides were stained with haematoxylin followed by dehydration. The above protocol was used for all antibodies with the exception of GPC-3 and AFP where an automated BenchMark ULTRA instrument (Ventana Medical Systems, Inc, Tuscon, AZ, USA) was used in a clinical laboratory setting. Scoring was performed by two independent investigators and differences resolved by mutual agreement. Intensity was scored as either none, weak, moderate or strong, while percentage of positive cells was scored as $<5\%$, 5–25%, 25–75% and $>75\%$. For a staining to be considered positive at least 5% of cells had to be stained. Negative controls consisted of omission of the primary antibody and appropriate positive control tissues were used for all antibodies. H-scores were calculated by multiplying the intensity score (0 to 3) with the level of % of positive cells where $1 = <5\%$, $2 = 5$–25%, $3 = 25$–75% and $4 = >75\%$.

Statistical analysis. The association of the expression level of tumour antigens with the various subgroup populations was analysed using the χ^2-test. The association of the tumour antigen expression with the clinicopathologic parameters was analysed using the χ^2-tests for categorical variables and the Student's *t*-test for continues variables. Survival analyses were performed using the Kaplan–Meier method and the log-rank test. Univariate and multivariate hazard ratios, 95% confidence intervals and

Table 1. Patient characteristics

Characteristics	No. of patients 133 (%)
Age (years)	
Median	60.4
Range	22.9–86.6
Gender	
Male	95 (71.4)
Female	38 (38.6)
Ethnicity	
Western-European	103 (77.4)
Non western-European[a]	30 (22.7)
Aetiology[b]	
No known liver disease	37 (27.8)
Hepatitis B	24 (18.0)
Alcohol abuse	22 (16.5)
Hepatitis C	18 (13.5)
Cryptogenic cirrhosis	10 (7.5)
NASH	9 (6.8)
Hemochromatosis	5 (3.8)
Primary biliary cirrhosis	3 (2.3)
Other	5 (3.8)
Viral hepatitis status[c]	
Hepatitis B positive[d]	30 (22.6)
Hepatitis C positive[e]	19 (14.3)
Cirrhosis present	
Yes	69 (51.9)
No	64 (48.1)
Tumour differentiation	
Good	43 (32.6)
Moderate	66 (50.0)
Poor	23 (17.4)
Vascular invasion	
Yes	71 (62.3)
No	43 (37.7)
Number of lesions	
Single	90 (67.7)
Multiple	43 (32.3)
Size of largest lesion	
Median	4.5 cm
Range	0.5–25
AFP level before resection	
Median	8 $\mu g\,l^{-1}$
Range	1–63 000

[a]Non-western European patients are from East-Europe ($n=3$), Suriname ($n=7$), Middle-East ($n=8$), Sub-Sahara Africa ($n=3$) and South-East Asia ($n=9$). See Supplementary Table 1.
[b]Patients with more than one aetiologic factor were listed based on the most dominant cause of liver disease.
[c]Three patients had both hepatitis B and hepatitis C.
[d]HBsAg(+) and/or anti-HBc positive.
[e]Anti-HCV positive.

corresponding *P*-values were obtained using Cox regression analysis. The statistical analysis was performed using the SPSS 21 software (IBM Corp., Armonk, NY, USA).

RESULTS

Tumour antigen expression in HCC and TFL tissue. The expression of the 15 tumour antigens in both tumour and TFL tissue is shown in Table 3. No expression of SSX-2 and MUC-1 was observed, although the antibodies properly stained testis (seminiferous duct cells) and gastric control tissue, respectively. The prevalence of expression of MAGE-A3/4, NY-ESO-1, AFP, MAGE-A1 and MAGE-A10 was low (<10% of patients), while increasing numbers of HCC showed expression of MAGE-C1, MAGE-C2, GPC-3, MDK, Survivin, WT-1, SP17 and Annexin-A2 (prevalence ranging from 17 to 90%, Table 3). However, the overexpressed self-antigens MDK, Survivin, WT-1 and SP17 showed equal expression in tumours and in TFL tissues. Thus, the tumour antigens with the highest differential expression level between tumour tissue and TFL tissue are Annexin-A2 (90.2 *vs* 37.1%), GPC-3 (39.1 *vs* 0%), MAGE-C2 (19.5 *vs* 0%) and MAGE-C1 (17.3 *vs* 0%). This conclusion did not change when we analysed only the 105 patients with paired tumour and TFL tissue as compared to the entire cohort of 133 patients. Representative immunohistochemical stainings of these four tumour antigens in HCC and TFL tissue are shown in Figure 1, while representative immunohistochemical stainings of all the tumour antigens can be seen in Supplementary Figure 1. The distribution of intensity and the percentage of stained cells in tumour tissue, and in the case of Annexin-A2 in TFL tissue, are shown in Figure 2. MAGE-C1 and GPC-3 showed cytoplasmic expression in tumour cells, while MAGE-C2 showed nuclear expression in tumour cells. Annexin-A2 showed membranous and cytoplasmic expression in hepatocytes in HCC and TFL tissue, and stained sinusoidal endothelium. These expression patterns are in agreement with previous observations in HCC (Riener *et al*, 2009; Longerich *et al*, 2011; Liang *et al*, 2013). Only hepatocyte and not sinusoidal staining was scored for Annexin-A2. Moreover, Annexin-A2 expression showed a weaker intensity in the hepatocytes of the TFL tissue than the corresponding tumour cells (Figures 2D and E). Looking at aetiologic factors, there was a significantly higher prevalence of expression of MAGE-A3/4 ($P=0.011$), MAGE-A1 ($P=0.034$) and MAGE-C1 ($P=0.008$) in patients with HBV infection compared with patients without HBV infection, while MAGE-C2 ($P=0.264$) and GPC-3 ($P=0.334$) showed a statistical trend towards higher expression in patients with HBV infection (Figure 3).

Tumour antigen index. As in previous studies (Liang *et al*, 2013) a tumour antigen index (TAA) was calculated based on the total number of antigens co-expressed in a given tumour tissue. Patients were grouped based on whether they co-expressed 0–2 tumour antigens, 3–6 tumour antigens or 7–9 tumour antigens. No patients co-expressed more than 9 out of the 15 tumour antigens. The higher the TAA index the better the HCC-specific survival was ($P=0.020$, Supplementary Figure 2). In multivariable analysis this was an independent prognostic factor for HCC-specific survival (Table 4).

Co-expression patterns of MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. Further analysis was performed on the four antigens with the greatest differences in expression between tumour and TFL tissue, namely MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. Expression of at least one of these antigens was observed in tumour tissues of 95% of patients, while 48% of patients expressed only one antigen, 30% expressed two antigens, 11% expressed three antigens and 6% expressed four antigens (Figure 4A). Co-expression of these antigens in individual patients is shown in Figure 4B. Ninety-two percent of patients express, individually, or in combination, Annexin-A2 and GPC-3. Of the patients that do not express Annexin-A2 or GPC-3, 3% express MAGE-C1 or MAGE-C2. In 11% of patients MAGE-C1 and MAGE-C2 add a second tumour antigen to patients that otherwise express only one antigen, either GPC-3 or Annexin-A2. Finally, in 10% of patients MAGE-C1 and MAGE-C2 add a third antigen to patients that co-express GPC-3 and Annexin-A2. Interestingly, MAGE-C1 and MAGE-C2 are significantly and strongly co-expressed in tumours of our HCC patients ($P<0.001$, Pearson's correlation coefficient = 0.68). This panel of four antigens also covers tumours of most HBV-negative patients (95%), of which 52% expressed one antigen, 29% expressed two antigens, 9%

Table 2. Primary antibodies

Antigens	Primary antibody source	Clone	Retrieval buffer	Dilution	References
MAGE-A1	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	MA454	Tris EDTA	1 : 50	(Jungbluth *et al*, 2000)
MAGE-A3/4	Professor G.C. Spagnoli[a]	57B	Tris EDTA	1 : 100	(Landry *et al*, 2000)
MAGE-A10	Professor G.C. Spagnoli[a]	3GA11	Citric acid	1 : 10	(Schultz-Thater *et al*, 2011)
NYESO-1	Santa Cruz Biotechnology	E978	Tris EDTA	1 : 50	(Vaughan *et al*, 2004)
SSX-2	Professor A.G. van Kessel[b]	E3AS	Tris EDTA	1 : 25	(dos Santos *et al*, 2000)
MAGE-C1	Santa Cruz Biotechnology	CT7-33	Tris EDTA	1 : 50	(Xia *et al*, 2013)
MAGE-C2	Professor Boquan Yin[c]	CT-10	Tris EDTA	1 : 100	(Zhuang *et al*, 2006)
MUC-1	Sanbio (Uden, The Netherlands)	MA695	Citric acid	1 : 100	(Langner *et al*, 2004)
AFP	Dako	Polyclonal	Tris EDTA	1 : 400	Dako[d]
GPC-3	Santa Cruz Biotechnology	1G12	Tris EDTA	1 : 200	(Shirakawa *et al*, 2009)
Annexin-A2	BD Biosciences (Breda, The Netherlands)	5	Tris EDTA	1 : 200	(Yee *et al*, 2007)
WT-1	Novus Biologicals (Abingdon, UK)	6F-H2	Tris EDTA	1 : 400	(Nakatsuka *et al*, 2006)
Survivin	Santa Cruz Biotechnology	D-8	Tris EDTA	1 : 50	(Brennan *et al*, 2008)
MDK	GeneTex (Irvine, CA, USA)	EP1143Y	Citric acid	1 : 400	(Liang *et al*, 2013)
SP17	Proteintech (Manchester, UK)	Polyclonal	Citric acid	1 : 100	Proteintech[e]
SALL-4	Santa Cruz Biotechnology	EE-30	Tris EDTA	1 : 50	(Yong *et al*, 2013b)

[a]MAGE-A3/A4 and MAGE-A10 antibodies graciously provided by Professor Giulio Spagnoli, Department of Surgery, Research Laboratory, University Hospital Basel, Basel, Switzerland (Landry *et al*, 2000; Schultz-Thater *et al*, 2011).
[b]SSX-2 antibody graciously provided by Professor Ad Geurts van Kessel, Department of Human Genetics University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands (dos Santos *et al*, 2000).
[c]MAGE-C2 antibody graciously provided by Professor Boquan Yin, Department of Immunology, Fourth Military Medical University, Xi'an 710032, PR China (Zhuang *et al*, 2006).
[d]http://www.dako.com/nl/ar38/p102130/prod_products.htm Accessed 8-9-14.
[e]http://www.ptglab.com/PView/SPA17-Antibody-13367-1-AP-PVIEW.htm Accessed 8-9-14.

Table 3. Tumour antigen expression

Antigens	% Positive stainings in tumour tissue ($n = 133$)	% Positive stainings in TFL tissue ($n = 105$)
SSX-2	0	0
MUC-1	0	0
MAGE-A3/4	3.0	0
NYESO-1	3.8	0
AFP	6.8	0.9
MAGE-A10	7.5	0
MAGE-A1	9.8	0
MAGE-C1	17.3	0
MAGE-C2	19.5	0
GPC-3	39.1	0
MDK	57.7	64.4
Survivin	79.5	91.1
WT-1	85.6	84.6
SP17	87.0	88.0
Annexin-A2	90.2	37.1

Abbreviation: TFL = tumour-free liver

expressed three antigens and 5% expressed four antigens. Together, these data show that this panel of four tumour antigens may be suitable for vaccination studies in HCC patients in western low-endemic areas.

Expression of SALL-4 and co-expression with GPC-3. SALL-4 is a transcription factor involved in the maintenance of embryonic and cancer stem cells (Zeng *et al*, 2014) and has recently been shown to be expressed in an HCC subtype with stem-cell like features associated with poor prognosis (Oikawa *et al*, 2013; Yong *et al*, 2013b; Zeng *et al*, 2014). In our study SALL-4 nuclear expression was seen in 26% of tumours and in none (0%) of the TFL samples. Like in previous studies (Zeng *et al*, 2014), SALL-4 was more frequently expressed in tumours of patients with HBV infection (40 *vs* 22%, $P = 0.05$) and its expression was correlated with poor differentiation ($P = 0.002$) and higher AFP levels before surgery ($P = 0.007$), while there was a trend towards correlation with vascular invasion ($P = 0.081$). Interestingly, there was a significant correlation between SALL-4 expression and GPC-3 expression ($P = 0.001$, Pearson's correlation coefficient = 0.29, Figure 5). While neither SALL-4 or GPC-3 were individually associated with HCC-specific survival, there was a trend towards worse HCC-specific survival in patients co-expressing both GPC-3 and SALL-4 ($P = 0.190$, Supplementary Figure 3a). In addition, when the strength of the staining was taken into consideration in the form of the H-score (intensity x % of positive cells) patients who co-expressed high levels of both SALL-4 (H-score > 2) and GPC-3 (H-score > 3) had a significantly worse HCC-specific survival ($P = 0.018$, Supplementary Figure 3b). This was an independent prognostic factor in multivariate analysis (Table 4).

Relationship of individual tumour antigen expression with known prognostic markers. Of all the tumour antigens tested, GPC-3 was the one most strongly associated with known prognostic factors. Specifically, GPC-3 was associated with poor tumour differentiation ($P = 0.004$), the presence of vascular invasion ($P = 0.002$) and higher AFP before resection ($P = 0.03$).

DISCUSSION

The aim of this study was to identify a panel of tumour antigens suited for immunotherapeutic approaches, such as vaccination, for HCC in western-European, low-endemic areas, where HBV infection is not the main aetiology of HCC and where the diagnosis is often made in non-cirrhotic livers (Verhoef *et al*, 2004; Witjes *et al*, 2012). In our cohort, only 23% of patients were HBV

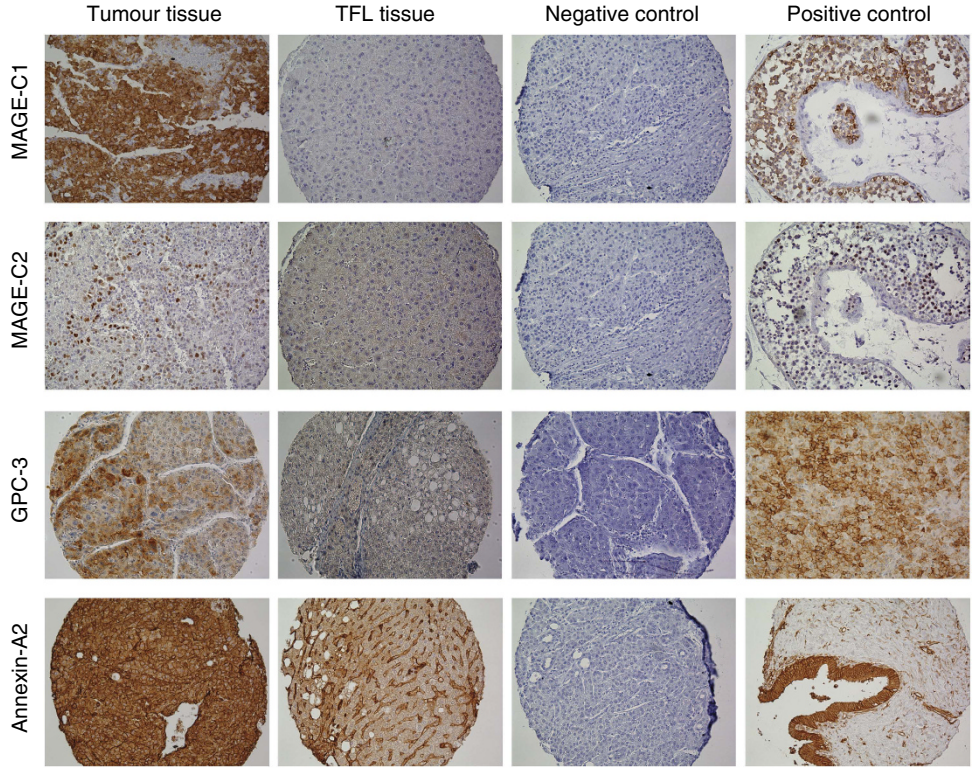


Figure 1. Representative stainings for tumour tissue and TFL tissue with negative and positive controls for MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. Strong tumour cell stainings for MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2 are seen in the leftmost column. The second column shows lack of staining in the corresponding TFL tissues with the exception of Annexin-A2 where staining of sinusoids is seen. The third column shows the corresponding negative controls and the last column shows the corresponding positive controls, which are testis tissue for MAGE-C1 and MAGE-C2, fetal liver tissue for GPC-3 and pancreatic cancer tissue for Annexin-A2.

positive, 14% HCV-positive and 48% had no liver cirrhosis. In addition, 77.4% of our patients are of western-European decent. Supplementary Table 1 describes the hepatitis-B status of patients per patient region of origin. Groups other that western-European are too small for subgroup analysis of antigen expression.

The observed prevalence of expression of testis and oncofetal antigens was generally lower than previous studies reported. Most of these previous studies have been conducted in East Asia where HBV infection is the most prevalent cause of HCC and the majority of HCC patients have liver cirrhosis. For example, a previous east Asian study reported that 36% of patients expressed MAGE-C1 (Xia *et al*, 2013), while we found only 17% expression. Supporting the association between cancer testis antigens and HBV infection, we found increased prevalence of MAGE-A1, MAGE-A3/4 and MAGE-C1 expression in HBV-positive patients. The prevalence of MAGE-C1 expression in HCC tissues in our HBV positive patients was similar to that reported by Xia *et al* (2013) (32% in HBV positive *vs* 13% in HBV negative patients). The only other large western study that has examined several of these antigens by immunohistochemistry is by Riener *et al* (2009) who studied 146 HCC patients from Switzerland, of which only 12% had HBV. In that study MAGE-C1 expression was found expressed in 12% of patients, NY-ESO-1 in 2% and MAGE-A3/4 in 0%, results that are similar to our findings. Likewise, expression of the oncofetal protein GPC-3 was found in 61–84% of patients in four Asian studies with HBV positivity ranging between 25 and 85% (Shirakawa *et al*, 2009; Yan *et al*, 2011; Yorita *et al*, 2011; Liang *et al*, 2013), and all these studies showed evidence of increased GPC-3 expression in the HBV-positive patients compared with the HBV-negative patients. In our study GPC-3 expression was found in 39% of all patients but in 48% of HBV-positive patients.

Another explanation for the relatively low prevalence of tumour antigen expression observed in our study is that many previous studies have used RT-PCR measuring mRNA expression (Chen *et al*, 2001; Luo *et al*, 2002; Peng *et al*, 2005b), while we have measured protein expression by immunohistochemistry. In fact, large discrepancies between tumour antigen expression in HCC by RT-PCR and immunohistochemistry have been reported. For example, Nakamura *et al* (2006) found 18/41 of HCC samples (43%) expressing NY-ESO-1 by RT-PCR while only 3 (7%) expressed the protein. It is likely that protein expression rather than RNA expression is a reliable predictor of suitability of tumour antigens for vaccination studies.

While the absence of MUC-1 expression in HCC is in agreement with previous work (Cao *et al*, 1999), the absence of SSX-2 in our study (0%) is in contrast to the study by Liang *et al* (2013) where a prevalence of 75% was reported. The use of different antibody clones may be one explanation. Clone 4D10, used in the Liang *et al* (2013) study, was not tested in TFL samples to examine tumour specificity. In addition, two studies (Luo *et al*, 2002; Wu *et al*, 2006) using RT-PCR have shown expression in 2/21 and 13/36 HCC-patients, respectively, indicating that it is unlikely that the true protein expression level of SSX-2 in HCC is very high. Finally, despite the lack of staining in tumour or TFL tissue, antibody clone E3AS, which we used, showed proper staining of positive control seminiferous duct cells in testis tissue (Supplementary Figure 1).

AFP was found to be expressed in few HCC samples (7%) in our study. While an incidence as low as 2% has been reported (Ferrandez-Izquierdo and Llombart-Bosch, 1987) most studies show expression of AFP in around 17–50% of HCC tumours (Ganjei *et al*, 1988; Brumm *et al*, 1989; Minervini *et al*, 1997; Tsuji

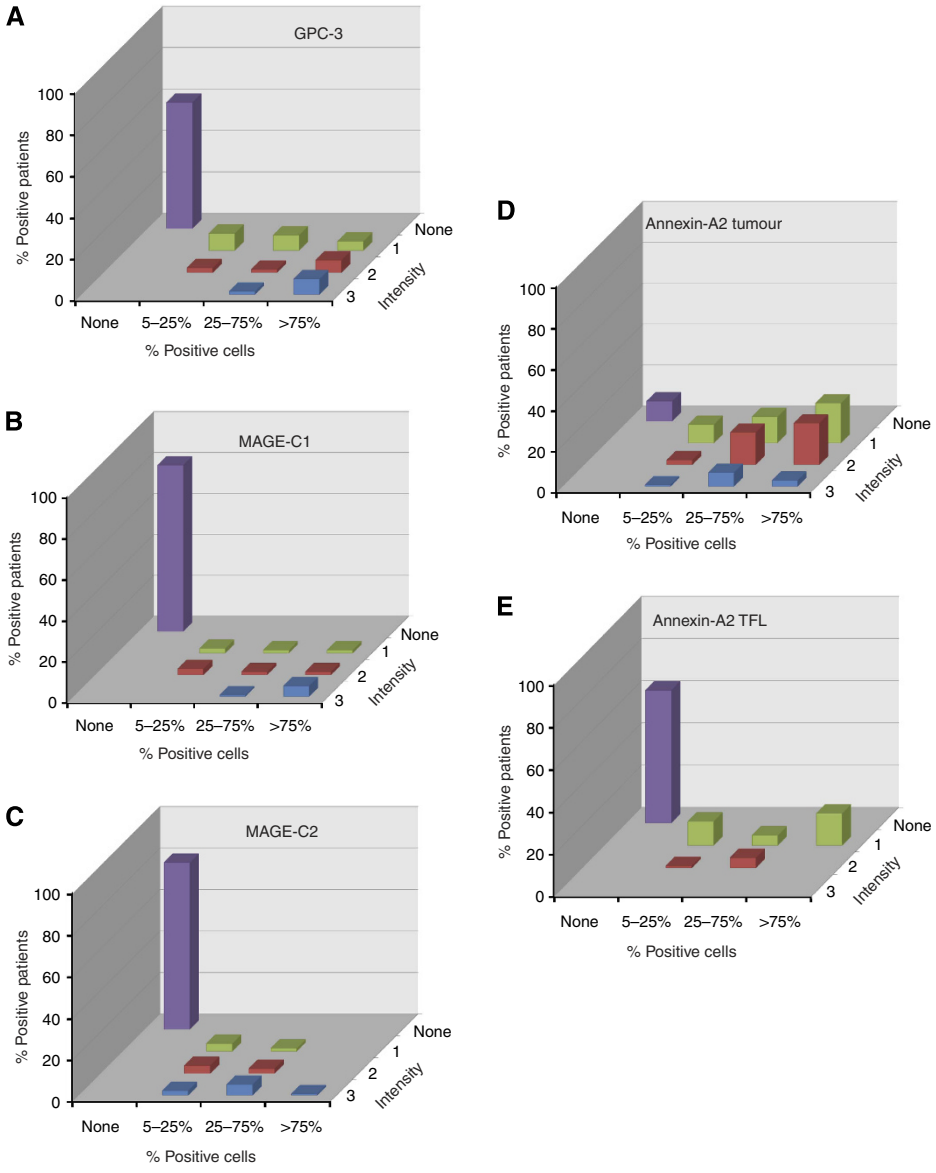


Figure 2. Distribution of staining intensity and percentage of positive cells for MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. GPC-3 cancer staining (**A**), MAGE-C1 cancer staining (**B**), MAGE-C2 cancer staining (**C**), Annexin-A2 cancer staining (**D**), Annexin-A2 TFL staining (**E**). Intensity 1 = weak; 2 = moderate; 3 = strong.

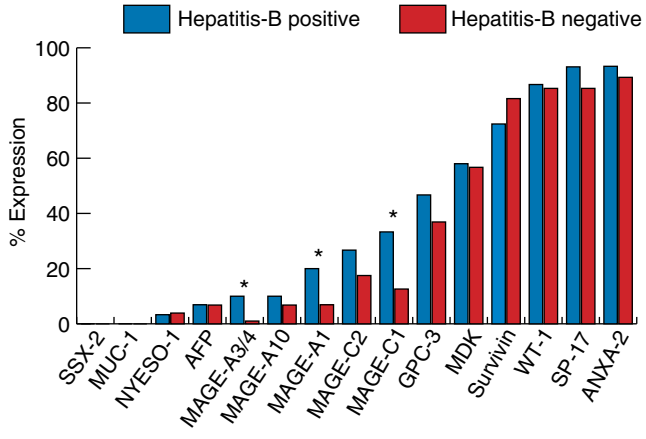


Figure 3. Antigen expression based on hepatitis-B status.
*$P<0.05$.

et al, 1999; Lau *et al*, 2002). To ensure accuracy of AFP staining in our study AFP expression was determined twice, under clinical laboratory conditions (automated BenchMark ULTRA instrument), which yielded identical results. On further examination AFP expression was strongly correlated with serum AFP level before resection ($P<0.001$). Of the patients with a serum AFP $>400\,\mu g\,l^{-1}$ 29% expressed AFP in their tumours *vs* only 3% in patients with a serum AFP $<400\,\mu g\,l^{-1}$ ($P=0.001$). This correlation of AFP serum levels with tumour AFP expression has been demonstrated before (Li *et al*, 2011). In our cohort, however, only 17% of our patients had an AFP value above $400\,\mu g\,l^{-1}$. Thus, one possible explanation for the low incidence of AFP staining is the relative low number of patients with high serum AFP levels. Indeed, most contemporary series report high AFP serum levels ($>400\,\mu g\,l^{-1}$) in 27 to 45% of patients undergoing resection (Wang *et al*, 2009; Ma *et al*, 2013; Liu *et al*, 2014).

While MDK, SP17, WT-1 and Survivin were expressed in the majority of tumours, we observed similar expression in adjacent TFL tissues, suggesting they might be unsuitable for vaccination studies in HCC due to lack of tumour-tissue specificity.

Table 4. Cox proportional Hazard regression analysis of patients' overall survival

		Univariate			Multivariate		
Variables		**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
AFP $>400\,\mu g\,l^{-1}$		2.867	1.176–6.992	**0.021**	2.682	0.947–7.601	0.063
>3 *vs* $\leqslant 3$ lesions		4.438	1.594–12.353	**0.004**	3.771	1.276–11.141	**0.016**
TAA index	0–2 *vs* 3–6 antigens	0.266	0.076–0.925	**0.042**	0.238	0.062–0.909	**0.033**
	0–2 *vs* 7–9 antigens	0.070	0.007–0.711		0.048	0.004-0.557	
High H-score for both GPC-3 and SALL-4		3.119	1.154–8.430	**0.025**	3.674	1.120–12.055	**0.032**

Abbreviations: CI = confidence interval; HR = hazard's ratio; TAA = tumour antigen index.

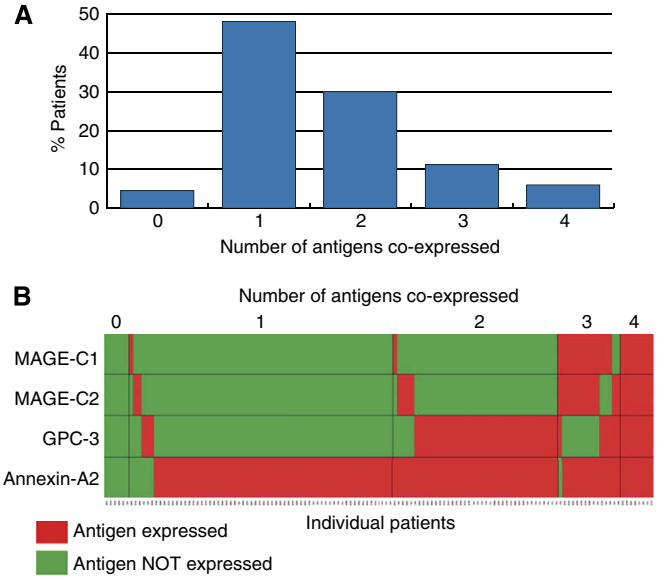


Figure 4. Co-expression of MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2 antigens. Distribution of total number of antigens expressed in the tumours of HCC-patients (**A**). Heat-map representation of 133 individual patients with expression of each antigen per patient (**B**).

Indeed these four antigens have been shown to be expressed in tissues other than cancer (Scharnhorst *et al*, 2001; Deguchi *et al*, 2002; Kannangai *et al*, 2005; Monma *et al*, 2013), or other than cancer and testis in the case of SP17 (Lacy and Sanderson, 2001; Frayne and Hall, 2002). Although in some reports the expression of MDK, SP17 and WT-1 has been shown to be lower in TFL tissue than in HCC tissue (Koide *et al*, 1999; Sera *et al*, 2008; Xia *et al*, 2013; Zhu *et al*, 2013), in the case of Survivin another report corroborates the equal or higher TFL, compared with tumour, expression (Chau *et al*, 2007).

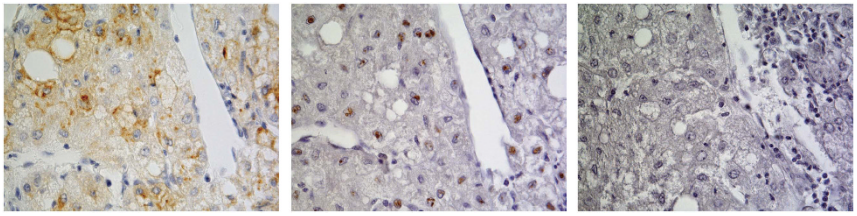
It has been previously shown by Liang *et al* (2013), that the higher the number of tumour antigens expressed by a given tumour the better the survival is. The hypothesis is that the higher the number of tumour antigens present the more the immunologic targets available to the immune system. In our study, in agreement with Liang *et al* (2013), we show that the higher the number of tumour antigens present in a given tumour the better the HCC-specific mortality is (Table 4, Supplementary Figure 2). While our findings are supportive of the above hypothesis, further validation and experimentation is necessary to prove the concept.

Therapeutic vaccination with a panel of tumour antigens, as opposed to a single antigen, would have the advantage of better coverage of the target tumour cell population as well as covering patients who express different antigens in their tumours. The panel that we selected (MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2) covers 95% of patients, with nearly 50% of them expressing at least two antigens. In addition, as in many patients expression of each individual antigen is limited to only 5–25% of tumour cells (Figure 2), targeting multiple antigens per patient may be needed to realise a successful clinical outcome. In addition, our antigen panel lacks, for the most part, expression in TFL tissue, which is an advantage, as it may reduce unwanted side effects. Even in the case of Annexin-A2, where a sizable proportion of TFL samples expressed the antigen (37%), the level of expression was much lower than that in the corresponding tumour samples (Figure 2D and E).

Biologically, GPC-3, a heparin sulphate proteoglycan expressed during embryogenesis, has been shown to be a poor prognostic factor in multiple studies (Shirakawa *et al*, 2009; Yan *et al*, 2011; Yorita *et al*, 2011; Liang *et al*, 2013). We confirm that GPC-3 expression is associated with higher serum AFP level (Yorita *et al*, 2011; Liang *et al*, 2013), worse tumour differentiation (Shirakawa *et al*, 2009; Yan *et al*, 2011; Yorita *et al*, 2011) and the presence of vascular invasion (Yorita *et al*, 2011). The immunogenicity of GPC-3 has been well demonstrated, and a phase I clinical cancer vaccine trial has already demonstrated tolerability and biologic efficacy (Sawada *et al*, 2012). In addition, gene expression profiling has shown that GPC-3 is significantly overexpressed in CD90 + HCC stem cells (Ho *et al*, 2012), suggesting that targeting GPC-3 may enable eradication of the tumour stem cell compartment. Our newly reported association of GPC-3 with SALL-4 strengthens the notion that GPC-3 is involved in stem cell biology in HCC. In fact, we show that patients who co-express high levels of both GPC-3 and SALL-4 have worse HCC-specific survival (Supplementary Figure 3b), indicating that the co-expression is biologically significant. However, it should be noted that strong co-expression is a relatively infrequent event occurring in 7.5% of patients. While other studies (Yan *et al*, 2011; Yong *et al*, 2013a) have shown worse overall survival for patients expressing individually GPC-3 or SALL-4 we did not show such an association. This is likely due to the fact that our study is smaller in size and was not designed to test the presence of biomarkers in HCC. In fact, when considering patients with higher GPC-3 staining, or patients with higher SALL-4 staining, statistical trends towards worse HCC-specific survival are apparent and consistent with the smaller size of our cohort (Supplementary Figure 3c and d). Finally, although both GPC-3 and SALL-4 are considered possible therapeutic targets in HCC (Filmus and Capurro, 2013; Yakaboski *et al*, 2014), information on immunogenicity of SALL-4 is lacking. Therefore, further research on immunogenicity of SALL-4 is needed before we can suggest to include SALL-4 in a therapeutic vaccine.

Annexin-A2, a calcium-dependent phospholipid binding protein, is involved in membrane formation, exocytosis and interaction with the extracellular matrix (Gerke and Moss, 2002). It is overexpressed in HCC (Yu *et al*, 2007; Mohammad *et al*, 2008) and multiple other cancers (Zhang *et al*, 2012), is involved in invasion and metastasis (Zhao *et al*, 2010), and immunogenicity has been demonstrated (Liu *et al*, 2011; Zheng and Jaffee, 2012).

A



B

$P = 0.001$ Pearson's coefficient = 0.29		Sall-4		
		–	+	Total
GPC-3	–	68 (51.1%)	13 (9.8%)	81
	+	30 (22.5%)	22 (16.5%)	52
	Total	98	35	133

Figure 5. Co-expression of GPC-3 and SALL-4. Representative case co-expressing GPC-3 and SALL-4 (**A**). 2 × 2 table of expression status of GPC-3 and SALL-4 in the entire cohort (**B**).

Our results are in agreement with Liu *et al* (2013) in that Annexin-A2 is expressed in the majority of patients with HCC and expression is significantly more pronounced in the tumour cells as compared with the surrounding TFL tissue. Our study is one of the very few to examine the protein level expression of Annexin-A2 in a 'western' cohort. Longerich *et al* (2011) demonstrated Annexin-A2 expression in HCC in a small western European patient cohort, but did not study expression in TFL tissue.

MAGE-C1 and MAGE-C2 are involved in embryogenesis, their expression is known to be reactivated in various cancers, and they are known immunogens (Li *et al*, 2004). The strong co-expression between MAGE-C1 and MAGE-C2 was not surprising, as the two genes are located close to each other on chromosome X (q27) and are likely translated together. However, despite their strong co-expression, a little less than half of positive cases expressed either one of the two antigens alone indicating a potential value in including both of these antigens in a tumour vaccine.

In conclusion, we show that there are aetiological differences in tumour antigen expression in HCC. In addition, we describe a panel of four antigens, MAGE-C1, MAGE-C2, GPC-3 and Anexxin-A2, which combine several favourable characteristics for future vaccination studies in patients in western low-endemic areas, such as combined coverage for the majority of patients, as well as tumour specificity. Finally, we demonstrate for the first time a relationship between GPC-3 and SALL-4 expression, which further substantiates that targeting GPC-3 may enable eradication of the HCC tumour stem-cell compartment.

ACKNOWLEDGEMENTS

We graciously thank the following professors for providing as with valuable monoclonal antibodies for our research: Professor Giulio Spagnoli, Department of Surgery, Research Laboratory, University Hospital Basel, Basel, Switzerland, for providing monoclonal antibodies against MAGE-A10 (clone 3GA11) (Schultz-Thater *et al*, 2011) and MAGE-A3/4 (clone 57B) (Landry *et al*, 2000). Professor Ad Geurts van Kessel, Department of Human Genetics, University Hospital Nijmegen, 6500 HB Nijmegen, the Netherlands, for providing a monoclonal antibody against SSX-2 (clone E3AS) (dos Santos *et al*, 2000). Professor Boquan Yin, Department of Immunology, Fourth Military Medical University, Xi'an 710032, PR China, for providing a monoclonal antibody against MAGE-C2 (clone CT-10) (Zhuang *et al*, 2006). The study was financially supported by the grant 2012-17 from the Gastrostart Foundation of the Netherlands Society of Gastroenterology to Jaap Kwekkeboom and the VENI grant 916-13-032 from the Netherlands Organization for Scientific Research (NWO/ZonMw) to Q Pan.

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