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ORIGINAL ARTICLE

Circulating biomarkers in hepatocellular carcinoma

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Abstract
Purpose Our aims are to determine levels of circulating cellular and protein biomarkers in hepatocellular carcinoma (HCC) patients and to analyse any relationships with clinical parameters.
Methods Fifty-four consenting patients were recruited. Circulating tumour cells (CTCs) were enumerated (by CellSearch) and characterised via filtration [by isolation by size of epithelial tumour cells (ISET)] with downstream immunohistochemistry (IHC). Glypican-3 (GPC3) expression in tumour biopsies and CTCs (by IHC) was compared, and levels of circulating caspase-cleaved and full-length cytokeratin 18 (CK18, measured using M30 and M65 ELISAs) were examined as a putative prognostic factor and marker of tumour burden.
Results CTCs were identified in 14 out of 50 (28 %) patients by CellSearch and in 19 out of 19 (100 %) patients by ISET. The presence of GPC3-positive CTCs by ISET was 100 % concordant with the presence of GPC3-positive cells in the original tumour ($n = 5$). No statistically significant correlations were observed between CTC number and clinical characteristics, although trends were noted between CTC subtypes, Child–Pugh score and tumour node metastasis stage. Serum M30 and M65 levels (as continuous variables) significantly correlated with overall survival (OS) in a univariate analysis ($p = 0.003$ and $p < 0.001$, respectively); M65 levels remained statistically significant in a multivariate analysis ($p = 0.029$).
Conclusions This is the first study to detect GPC3-positive CTCs in HCC, important for drug development with this target. The significant association of circulating CK18 with OS in HCC further exemplifies the utility of circulating biomarkers in cancer.

Keywords Circulating tumour cells (CTCs) · Hepatocellular carcinoma (HCC) · Cytokeratin 18 (CK18) · Prognostic biomarker · Glypican-3 (GPC3)

Introduction

Hepatocellular carcinoma is the third most common cause of cancer-related deaths worldwide [1]. HCC incidence continues to rise, particularly in developed Western countries where there is a strong association with non-alcoholic fatty liver disease [2]. The majority of patients present with advanced and/or non-resectable disease, and consequently the prognosis for HCC patients remains very poor (OS 12 % at 5 years) [1].

Arguably, the most important factor contributing to poor prognosis is the inability to diagnose the disease early in the majority of cases. In this regard, the development of sensitive and robust circulating biomarkers is critical. Glypican-3 is a heparan sulphate glycoprotein that is highly expressed in

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approximately 70 % of HCC cases [3], but poorly expressed in pre-neoplastic lesions and in normal liver tissue [4]. GPC3 is considered a potential therapeutic target for HCC, and a recombinant humanised monoclonal antibody to GPC3 has been evaluated in a Phase I trial with advanced HCC patients, indicating an association of disease stabilisation with GPC3 expression in tumours [5]. In addition, circulating tumour cells (CTCs) are being increasingly evaluated as 'liquid biopsies', and we have shown previously the number of CTCs measured using the Veridex CellSearch platform in small cell and non-small cell lung cancer patients is an independent prognostic biomarker [6, 7].

In the present study, we explore the relationships between circulating biomarkers (CTCs, GPC3 and other serum proteins) and clinical parameters of HCC patients. We compare the utility of CellSearch with a marker-independent (isolation by size of epithelial tumour cells) approach for CTC capture, enumeration and analysis in HCC. Finally, we characterise and compare GPC3 expression in tumour samples and in CTCs with a view to evaluating the potential of a GPC3 CTC assay to assist in drug development.

Materials and methods

Patients

Sequential patients with a diagnosis of HCC were recruited to the study at two centres: The Christie NHS Foundation Trust, Manchester, UK, and North Manchester General Hospital, Manchester, UK. Patients either had no anti-tumour therapy within 4 weeks, or had evidence of progressive disease if prior therapy had been received and were awaiting further therapy. Clinical data collected included age, sex, Child–Pugh score, serum alpha-fetoprotein (AFP), performance status (PST), viral status, radiological extent of disease, time to progression and overall survival (OS). In addition, treatment history, tumour node metastasis (TNM) staging, Okuda stage, Barcelona stage (BCLC) and aetiological risk factors were collated.

The study was performed in accordance with the ethical principles in the Declaration of Helsinki, ICH Good Clinical Practice, Good Clinical Practice for Laboratories, Human Tissue Act 2004, Medical Research Council (MRC) operational and ethical guidelines for research using human tissue and biological samples, and other applicable regulatory requirements.

Samples

After informed consent, a single blood draw of up to 30 ml was taken for biomarker analysis at baseline (defined as 4 weeks after any previous treatment, or evidence of progressive disease if therapy had been received and awaiting further therapy or supportive care only). Serum was isolated from 10 ml coagulated whole blood (collected in serum gel tubes) for analysis of the levels of full-length and/or caspase-3-cleaved cytokeratin 18 (CK18) using the M65 and M30 ELISAs, respectively; 10 ml blood was sampled into a CellSave tube (Veridex) for CTC enumeration by CellSearch, and wherever possible 10 ml (EDTA) was sampled for analysis using the filtration-based ISET device [8]. Tumour biopsies were also obtained from 12 patients in order to compare GPC3 expression levels in CTCs with tumour at the time of diagnosis.

Cell lines and antibodies

SK-HEP-1 HCC cells (GPC3-negative) that had been transfected with human GPC3 cDNA were used as positive controls: SK-03 was a high expresser of GPC3, and SK-PCA13a had intermediate expression of GPC3 [9]. These cell lines were provided by Chugai Pharmaceutical Co., Ltd. Human Vascular Endothelial Cells (HUVEC) were used as endothelial cell controls to allow exclusion of circulating endothelial cells (CECs) in the CTC assay following CTC enrichment by ISET. All cell lines were cultured according to standard protocols. A mouse monoclonal antibody against GPC3 (mGC33) for immunohistochemical (IHC) analysis of tissue biopsies and of CTCs captured on ISET filters was provided by Chugai Pharmaceutical Co., Ltd. [10]. The cell lines and mGC33 antibody were used to validate GPC3 immunohistochemical assays in tissue and in cells.

Enumeration and assessment of GPC3 status of CTCs

CTCs were enumerated using the CellSearch platform in blood samples from 50 patients. The CellSearch methodology has been described previously [11, 12] and is based on the immunomagnetic capture of epithelial adhesion molecule (EpCAM) positive cells followed by immunophenotyping of cytokeratin, DAPI and CD45 in cells isolated from a 7.5-ml blood volume. CTCs are classed as CK^+, $DAPI^+$ and $CD45^-$. In order to establish GPC3 expression in CTCs, an AF-488-conjugated antibody against GPC3 (mGC33) was also added to the samples in the fourth channel of the CellSearch at a concentration of 4 μg/test. The exposure time for signal detection of GPC3 was 0.4 s. This enabled the enumeration of GPC3-positive CTCs as a proportion of the total CTC count in 7.5 ml blood.

ISET sample filtration and immunohistochemical staining for GPC3

Blood samples from 19 patients were evaluable by ISET. The ISET process has also been described previously

in detail [8]. Immunohistochemical staining for GPC3 was carried out on four ISET membrane 'spots' using the mGC33 antibody at a concentration of 1 μg/ml. Whereas 10 ISET membrane 'spots' per sample are generated, qualitatively similar data are obtained when either 4 spots per sample or all 10 spots are analysed (our unpublished observations). Individual membrane spots were stained and imaged as described previously [13]. Image review and scoring of tumour cells were performed independently by two analysts, and candidate cells were then reviewed by a pathologist (P.S.).

CTCs present on the membranes were classified into three types. Type 1: GPC3-positive cells <16 μm in diameter with hypochromatic, irregular-shaped nuclei and a high nuclear to cytoplasmic ratio; Type 2: GPC3-positive cells >16 μm with hypochromatic, irregular-shaped nuclei and a high nuclear to cytoplasmic ratio, and Type 3: GPC3-negative cells >16 μm with hypochromatic, irregular-shaped nuclei and a high nuclear to cytoplasmic ratio. CTC counts in the 3 categories were normalised to 7.5 ml for comparison with CellSearch.

Immunohistochemical staining for GPC3: tissue biopsies

Tissue biopsy sections were firstly stained with Haematoxylin and Eosin and examined by a pathologist (P.S.) to determine the presence and prevalence of tumour. Tissue biopsy sections containing tumour (5 μm) were then stained for GPC3 using the mGC33 antibody on a Leica Bond Max autostainer (Leica, Germany) using the bond polymer refine detection reagent kit (Cat no. DS9800). Slides were counterstained with haematoxylin and imaged using the Leica SCN400 scanner at 40× magnification.

Circulating biomarkers of epithelial cell death

Full-length and caspase-cleaved CK18 levels in the serum of 48 patients were quantified by the M65 and M30 ELISAs, respectively (Peviva, Bromma, Sweden), according to previously published methods [14, 15].

Statistical analysis

Statistical analysis was carried out in GraphPad Prism® version 5 (GraphPad, La Jolla, USA) and Statistical Package for the Social Sciences version 19 (SPSS, Chicago, USA). The Mann–Whitney *U* test was used to determine significance of biomarker assays with the patients' clinical factors tabulated in Table 1. Kaplan–Meier analysis was applied to determine significance between clinical factors, biomarkers and OS. COX linear regression analysis was applied to determine significance of clinical factors and biomarkers in a univariate analysis (see Supplementary Table 1).

Results

Fifty-four patients with a median age of 67 years (range 29–80), were recruited between June 2009 and October 2011. Two patients were excluded due to alternative diagnosis and Hepatitis C infection. The demographic characteristics of the patients are summarised in Table 1. Biopsies were obtained from 21 patients out of the 52 in the study.

Of the 52 patients included in the study, 50 underwent CTC analysis by CellSearch and 19 by ISET. Of the 12 tumour biopsies deemed to contain tumour cells by an accredited pathologist (P.S.), all had matched CellSearch samples and 5 had matched ISET samples (Table 2). Fourteen patients had CTCs detectable by the EpCAM-dependent CellSearch system (28 %) and the number ranged from 1 to 8 per 7.5 ml of blood (Table 2); thirty-six patients (72 %) had no detectable CTCs by CellSearch, whereas all patients (100 %) had detectable CTCs by the EpCAM-independent ISET method. To determine whether CTCs expressed the GPC3 protein, four cell lines expressing varying levels of GPC3 were used to establish the GPC3 staining parameters (SK-HEP-1, HUVEC, SK-PCA13a, and SK-03). Figure 1 shows GPC3 staining by IHC in these cell lines and in white blood cells (WBCs).

Figure 2a, b shows GPC3 staining in patient tumour biopsies and putative CTCs. A matched tumour biopsy was available for three patients where CTCs were found by CellSearch. In each sample, a single CTC was identified, of which one stained positively for GPC3. Of the 12 biopsies analysed in total, 10 were GPC3-positive and 2 were GPC3-negative. The presence of GPC3-positive CTCs by ISET was 100 % concordant with the presence of GPC3-positive cells in the original tumour ($n = 5$).

Enumeration of the GPC3-stained CTCs revealed that in all cases, the Type 1 cells were the least abundant, and in 16 out of 19 patients, the GPC3-negative Type 3 cells were the most abundant (Table 2). In the three remaining patients, GPC3-positive Type 2 cells were the most abundant. Although Type 1 cells stained positively for GPC3, the subsequent statistical analysis was restricted to the larger Type 2 and Type 3 cells. This analysis revealed a trend towards greater numbers of large GPC3-positive (Type 2) CTCs by ISET with more advanced disease stage (TNM) and Child–Pugh score B. Patients with Child–Pugh score B also tended to have fewer GPC3-negative (Type 3) CTCs. These trends, however, were not statistically significant according to the Mann–Witney *U* test. No other trends or significant relationships could be established between CTC number and characteristics and clinical parameters.

The levels of circulating CK18 detected by M30 and M65 ELISAs were determined in serum from 48 patients (Table 2). The Mann–Whitney *U* test was applied to assess statistically significant relationships between the M30 and

Table 1 Patient demographics and clinical details

Characteristic	Evaluable patients ($n = 52$)	
	Number of patients	%
Age (years)		
Median	67	
Range	29–80	
Sex		
Female	6	12
Male	46	88
Tumour stage		
I	6	11.5
II	6	11.5
III	21	40
IV	18	35
Unknown	1	2
Okuda stage		
1	25	48
2	15	29
3	1	2
Unknown	11	21
BCLC (Barcelona) stage		
A	2	4
B	4	8
C	34	65
D	7	13
Unknown	5	10
Child–Pugh score		
A	43	83
B	9	17
Performance status		
0	6	12
1	20	38
2	14	27
3	7	13
Unknown	5	10
Aetiology		
Alcohol	17	32
Hepatitis B	3	6
Diabetes	2	4
Hemochromatosis	1	2
Hepatitis B and diabetes	1	2
Hemochromatosis and alcohol	2	4
Diabetes and alcohol	3	6
Diabetes/NASH	1	2
Hemochromatosis and diabetes	1	2
Diabetes/NAFLD	1	2
Diabetes/α antitrypsin deficiency/ alcohol	1	2
Autoimmune hepatitis	1	2
None	4	7
None recorded	17	27

M65 data and all clinical parameters in Table 1, with the exception of disease aetiology.

Significant associations ($p \leq 0.05$) between the concentration in serum of caspase-cleaved and full-length CK18 concentration (M65) and four of the clinical parameters were observed (Fig. 3a), specifically tumour node metastases (TNM; stages 1 vs. 2, 1 vs. 3, 1 vs. 4 and 2 vs. 3), BCLC stage (A, B, C vs. D), serum AFP levels (normal vs. elevated) and PS (0 vs. 3, 1 vs. 3). There was no such relationship with Okuda staging or Child–Pugh score (data not shown). Similarly, there were significant associations observed between the concentration of circulating caspase-cleaved CK18 (by M30 ELISA) and the same four clinical parameters (Fig. 3b), except that here the association with TNM stage was only able to significantly discriminate stages 1 versus 3 and 1 versus 4.

Discussion

The development of diagnostic, prognostic, predictive and pharmacodynamic biomarkers to aid drug development is of critical importance to the improvement of outcomes for patients with HCC.

A number of studies have reported the detection of CTCs in HCC patients and associated CTC numbers with poor patient prognosis (see [16] for review). The commonly used CellSearch method for CTC detection relies on the expression of the epithelial markers EpCAM and CK. Inter- and intra-tumoural expression of EpCAM in HCC is variable, and a minority (15–30 %) of HCC tumours are EpCAM-positive by IHC [17, 18]. Consistent with these observations is the generally low number of CTCs isolated from HCC patients using CellSearch, and the reported variation between HCC patient cohorts. For example, Sun et al. reported that 51 of 123 (41 %) pre-operative HCC patients had ≥2 CTCs/7.5 ml blood (range 1–34 CTCs/7.5 ml blood) using CellSearch [19], and Schulze et al. [20] reported 18 of 59 (31 %) patients with ≥1 CTC/7.5 ml blood (range 1–5 CTCs/7.5 ml blood) in patients across a range of disease stages.

We performed a direct comparison between the ISET and CellSearch methods for CTC isolation. Consistent with previous studies using CellSearch, we identified ≥1 CTC/7.5 ml blood in 14/50 (28 %) patients, with a range of 1–8 CTCs/7.5 ml blood (Table 2). However, using the ISET technique, we identified ≥1 CTC/7.5 ml blood in 19 of 19 (100 %) samples, with a range of 13–158 (Type 3) CTCs/7.5 ml blood. Of these 19 samples evaluated by ISET, 18 had matched CellSearch samples. Of these 18 samples, 6 (33 %) had ≥1 CTC/7.5 ml blood as determined by CellSearch, indicating as anticipated poor concordance between the two techniques. This again

Table 2 Numbers of CTCs in patient blood samples as determined by CellSearch and by ISET

Patient number	CellSearch		ISET		Tissue	
	Total number of CTCs/7.5 ml	Number of GPC3-positive CTCs/7.5 ml	Number of Type 1 cells/7.5 ml	Number of Type 2 cells/7.5 ml	Number of Type 3 cells/7.5 ml	GPC3 status (pos/neg)
301	4	4	N/A	N/A	N/A	N/A
302	0	0	N/A	N/A	N/A	N/A
303	0	0	N/A	N/A	N/A	N/A
304	0	0	N/A	N/A	N/A	N/A
306	0	0	N/A	N/A	N/A	N/A
307	0	0	N/A	N/A	N/A	N/A
308	1	1	N/A	N/A	N/A	POS
309	1	0	N/A	N/A	N/A	N/A
310	2	2	N/A	N/A	N/A	N/A
311	0	0	N/A	N/A	N/A	N/A
312	0	0	N/A	N/A	N/A	N/A
313	1	0	N/A	N/A	N/A	N/A
314	1	N/A	N/A	N/A	N/A	NEG
315	0	0	N/A	N/A	N/A	N/A
316	1	0	N/A	N/A	N/A	N/A
318	0	0	N/A	N/A	N/A	N/A
319	0	0	N/A	N/A	N/A	N/A
320	2	0	N/A	N/A	N/A	N/A
321	0	0	N/A	N/A	N/A	N/A
322	0	0	N/A	N/A	N/A	POS
323	0	0	N/A	N/A	N/A	N/A
324	0	0	N/A	N/A	N/A	N/A
325	0	0	N/A	N/A	N/A	N/A
326	0	0	N/A	N/A	N/A	N/A
327	0	0	N/A	N/A	N/A	N/A
328	0	0	N/A	N/A	N/A	N/A
329	0	0	N/A	N/A	N/A	NEG
330	0	0	N/A	N/A	N/A	POS
331	0	0	N/A	N/A	N/A	POS
332	0	0	N/A	N/A	N/A	POS
333	1	1	0	9	41	N/A
334	1	0	17	28	52	N/A
335	8	0	4	8	19	N/A
336	0	0	47	66	90	POS
337	1	0	4	13	51	N/A
338	1	0	0	17	158	POS
340	0	0	N/A	N/A	N/A	N/A
341	0	0	N/A	N/A	N/A	N/A
342	0	0	6	9	34	N/A
343	0	0	5	8	50	N/A
344	0	0	0	9	43	N/A
345	0	0	2	21	26	POS
346	0	0	4	28	34	POS
347	0	0	0	0	19	N/A
348	0	0	0	23	13	N/A
349	0	0	9	9	73	POS

Table 2 continued

Patient number	CellSearch		ISET		Tissue	
	Total number of CTCs/7.5 ml	Number of GPC3-positive CTCs/7.5 ml	Number of Type 1 cells/7.5 ml	Number of Type 2 cells/7.5 ml	Number of Type 3 cells/7.5 ml	GPC3 status (pos/neg)
350	0	0	2	6	38	N/A
351	2	0	2	19	30	N/A
352	0	0	13	32	107	N/A
353	0	0	2	36	34	N/A
354	N/A	N/A	9	45	17	N/A

The GPC3 status as determined by IHC is shown, together with the GPC3 status of the matched tumour biopsy where available

N/A sample/result not available

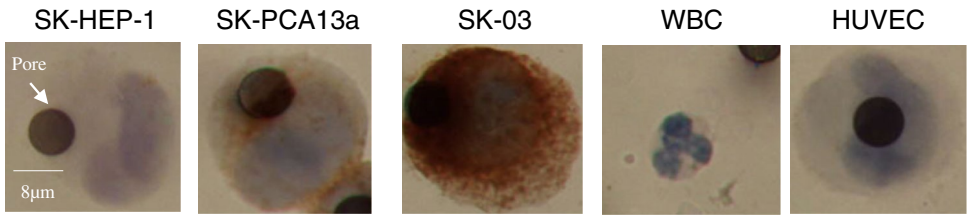

Fig. 1 GPC3 staining using IHC, in control cell lines and in WBCs. The pore in the ISET filter is indicated, and the *scale bar* is 8 µm

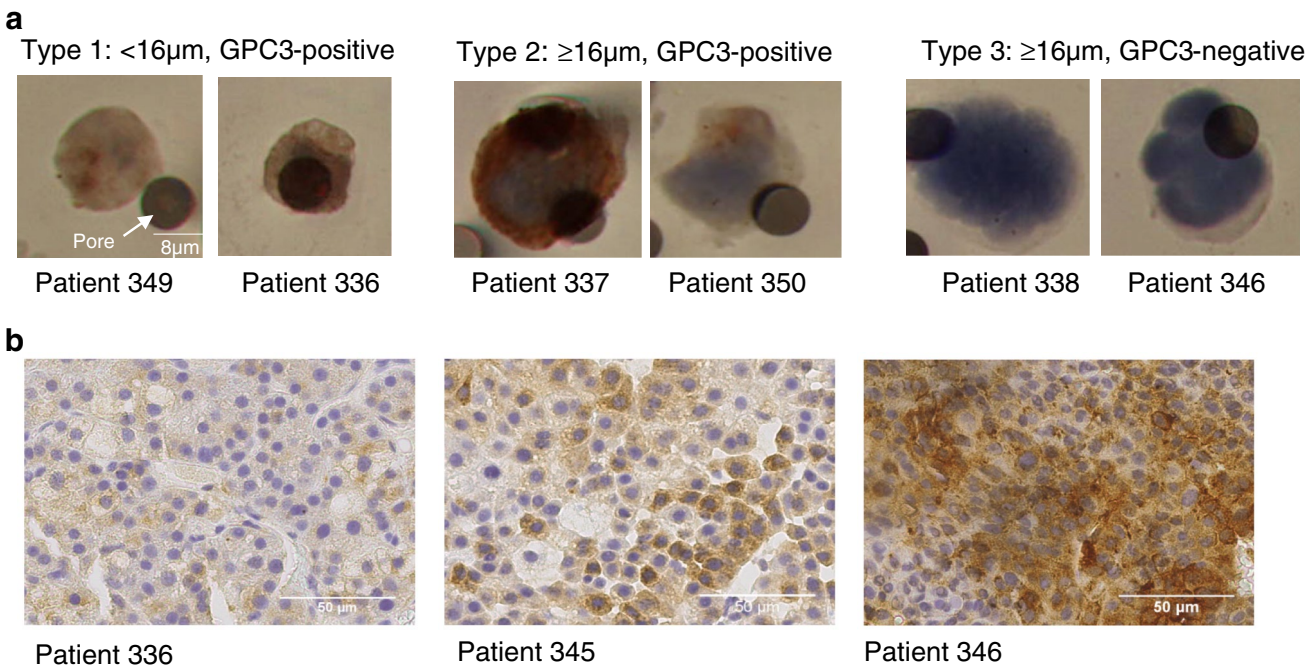

Fig. 2 GPC3 staining of patient CTC samples and tumour biopsies. **a** CTCs isolated from patient blood samples by ISET. Examples of Types 1, 2 and 3 CTCs are shown, together with the patient number from which they were isolated. The pore in the ISET filter is indicated, and the *scale bar* is 8 µm. **b** Patient tumour biopsies. The patient from whom the biopsies were obtained is indicated. The *scale bar* is 50 µm

is consistent with the minority of HCC tumours being EpCAM-positive.

A previous study enumerating CTCs in HCC patients using ISET also reports detection of CTCs in a greater proportion of patients. Vona et al. [21] identified ≥1 CTCs/7.5 ml blood in 23 of 44 (52 %) patients, with a range of 3–33 CTCs/7.5 ml blood. This study defined CTCs using morphological criteria as we have done, but

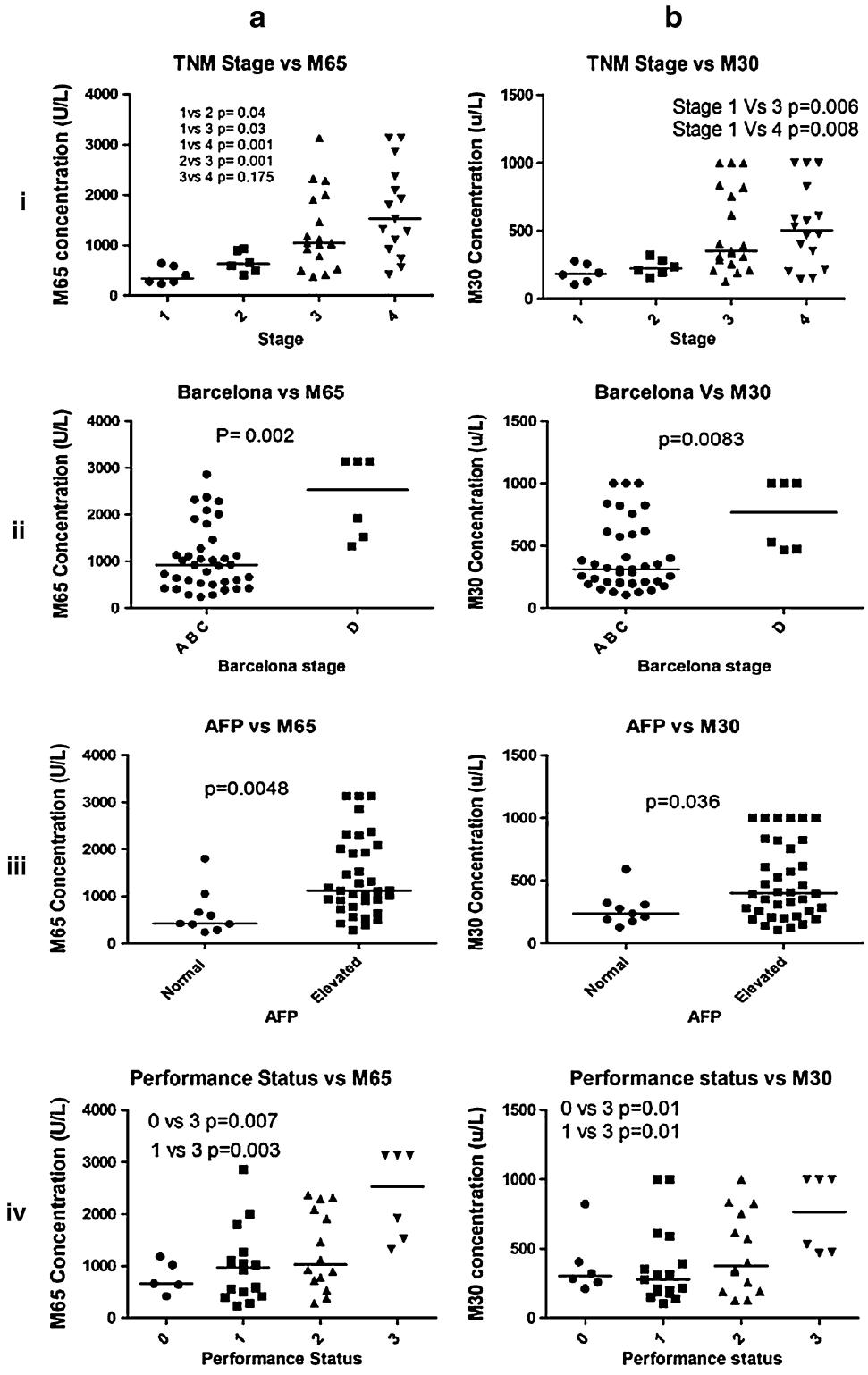

Fig. 3 Association of serum CK18 levels with patient clinical parameters. **a** Full-length CK18 plus caspase-cleaved CK18, measured using M65 ELISA. **b** Caspase-cleaved CK18, measured using M30 ELISA. *i* TMN stage, *ii* Barcelona stage, *iii* Serum AFP level, *iv* Performance status. Statistical significance as determined by the Mann–Whitney *U* test is indicated

with a cell diameter of ≥25 μm. This may account for the lower CTC detection than that reported herein. Ours is the first direct comparison of CTC numbers in HCC using CellSearch and ISET techniques, confirming and extending earlier observations indicating that a minority of HCC patients possess EpCAM-positive CTCs and providing firm evidence that samples apparently lacking CTCs following CellSearch analysis contain numerous CTCs as defined by morphology and size. There are reports in epithelial cancers that down-regulation of EpCAM in CTCs is associated

Table 3 Individual patient clinical data, and corresponding serum levels of full-length CK18 plus caspase-cleaved CK18 (M65), and caspase-cleaved CK18 (M30)

Patient number	TNM stage	Child–Pugh score	PST	AFP (IU/ml)	Serum M65 concentration (Units/L)	Serum M30 concentration (Units/L)
301	3	B	2	2,594	780	257
302	4	A	3	80,636	1,923	467
303	2	A	2	8	900	195
304	4	B	3	26	1,317	531
306	3	A	2	66,542	2,322	837
307	3	A	3	2,042	3,134.1[b]	1,000[b]
308	4	A	2	9	728	400
310	4	A	1	410	422	201
311	4	A	1	10	84.5[a]	152
312	3	A	1	96	2,008	1,000[b]
313	3	A	0	11	89.5[a]	822
314	2	A	0	5	663	323
316	3	A	2	179	1,121	333
317	3	A	0	7	1,185	404
318	1	A	1	4	404	278
319	1	A	1	253	280	105
320	1	A	2	2	286	129
321	3	B	2	19,000	931	352
322	3	A	1	60	501	208
323	3	A	1	1,084	1,025	312
324	3	A	2	338	2,288	617
325	1	A	1	5	238	192
326	2	A	N/A	3,093	938	282
327	2	B	N/A	N/A	509	159
328	1	A	0	13	643	258
329	3	A	0	5	421	211
330	4	A	3	359,600	3,134.1[b]	1,000[b]
331	3	A	0	9,058	1,021	285
332	4	A	1	5,058	922	216
334	3	A	1	5	1,055	310
335	3	B	2	16	1,466	1,000[b]
336	4	B	3	203	3,134.1[b]	1,000[b]
337	4	A	1	96	560	142
338	3	A	1	56	1,049	393
340	4	A	2	3,900	2,373	826
342	3	B	2	11	1,908	756
343	1	A	1	4	593	177
344	2	A	N/A	N/A	599	210
345	3	A	2	339	533	192
346	4	A	1	27	1,111	352
347	4	A	1	44	1,275	612
348	2	B	N/A	2	413	237
349	4	A	3	3,471	1,525	475
350	4	A	1	5	1,800	590
351	3	B	2	79	1,130	409
352	4	A	1	8	2,861.6	1,000
353	3	A	2	8	385	128
354	4	A	2	11	2,091	573

N/A result not available

[a] Values below lower limit of detection of the assay results not included in further analysis

[b] Upper limit of detection of the assay but included in further analysis

with the acquisition of the mesenchymal phenotype and thus a greater disposition towards malignant metastasis [22, 23]. Further characterisation of the CTCs in HCC is warranted to address this issue.

We further characterised the CTC populations in patients by immunostaining the ISET filters for GPC3, a candidate diagnostic and prognostic marker for HCC. In our study, 10 of 12 (83 %) available patient tumour biopsies stained positively for GPC3, and in 18 of 19 (95 %) patients, GPC-positive CTCs could be detected using ISET. GPC3-positive CTCs could be detected in all 5 samples for which matched tumours were available. Therefore, our small sample set indicates a good correlation between GPC3 staining and patient HCC status, although a larger cohort would be necessary to confirm this apparent association between GPC3 expression in primary tumour tissue and in CTCs.

We also examined the relationship between the patient clinical parameters and levels of circulating CK18 fragments. The level of caspase-cleaved CK18 is proposed as a specific indicator of apoptosis, while levels of the full-length CK18 theoretically measure both caspase cleavage of CK18 (apoptosis) and cellular release of intact CK18 (assumed via necrosis) [24]. In addition, higher levels of CK18 (by M65) can report increased epithelial tumour burden, at least in colorectal cancer where it is also an indicator of poor patient prognosis [25]. Levels of cleaved and full-length CK18 have also been reported to indicate poor prognosis in gastric cancer [26] and in renal cell carcinoma [27].

A recent report has indicated an association of high serum levels of CK18 (as measured by the M65 assay) with impaired prognosis [28], and there are two reports of increased plasma levels of caspase-cleaved and full-length CK18 in patients with non-alcoholic steatohepatitis [29, 30]. Here we report statistically significant associations of HCC clinical parameters with both serum M30 and M65 data. Figure 3a, b and Table 3 suggest that serum M65 data are the most significant in terms of clinical parameter associations, and this is borne out by multivariate Cox Regression Analysis (Supplementary Table 1).

In summary, this study identifies for the first time the presence of GPC3-expressing CTCs in HCC, offering opportunity for a minimally invasive biomarker in the development of GPC3 targeted therapies. Also, for the first time in HCC, we present a comparison of CTC enumeration using the Veridex CellSearch and ISET methods, identifying greater numbers of CTCs using ISET. Finally, and in keeping with reports in colorectal, renal and gastric cancers, we show that circulating caspase-cleaved and total CK18 significantly associate with TNM stage, BCLC stage, serum AFP levels and Performance Status in HCC. These advances provide further evidence of the utility of circulating biomarkers in HCC.

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