Research Article

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# Imagestream detection and characterisation of circulating tumour cells – A liquid biopsy for hepatocellular carcinoma?

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**Background & Aims**: The lack of progress in developing and delivering new therapies for hepatocellular carcinoma (HCC) is in part attributed to the risk related avoidance of tumour biopsy at diagnosis. Circulating tumour cells (CTCs) are a potential source of tumour tissue that could aid biological or biomarker research, treatment stratification and monitoring.

**Methods**: An imaging flow cytometry method, using immunofluorescence of cytokeratin, EpCAM, AFP, glypican-3 and DNA-PK together with analysis of size, morphology and DNA content, for detection of HCC CTCs was developed and applied to 69 patient and 31 control samples. The presence of CTCs as a prognostic indicator was assessed in multivariate analyses encompassing recognised prognostic parameters.

**Results**: Between 1 and 1642 CTCs were detected in blood samples from 45/69 HCC patients compared to 0/31 controls. CTCs positive for the epithelial markers cytokeratin and EpCAM were detected in 29% and 18% of patients respectively, while an additional 28% of patients had CTCs negative for all markers other than size and evidence of hyperploidy. CTC number correlated significantly with tumour size and portal vein thrombosis (PVT). The median survival of patients with >1 CTC was 7.5 months versus >34 months for patients with <1 CTC ($p$ <0.001, log-rank), with significance retained in a multivariate analysis (HR 2.34, 95% CI 1.005–5.425, $p$ = 0.049) including tumour size and PVT.

**Conclusions**: The use of multiple parameters enhanced HCC CTC detection sensitivity, revealing biological associations and predictive biomarker potential that may be able to guide stratified medicine decisions and future research.

**Lay summary**: Characteristics of tumour tissues can be used to predict outcomes for individual patients with cancer, as well as help to choose their best treatment. Biopsy of liver cancers carries risks, however, and is usually avoided. Some cancer cells enter the blood, and although they are very rare, we have developed a method of finding and characterising them in patients with liver cancer, which we hope will provide a low risk means of guiding treatment.



Keywords: Circulating tumour cells; Hepatocellular cancer; ImageStream; Biomarkers; Liquid biopsy.

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*Abbreviations:* HCC, hepatocellular cancer; CLD, chronic liver disease; PVT, portal vein thrombosis; EHD, extra-hepatic disease; ECOG, Eastern cooperative oncology group; PST, performance status; CTCs, circulating tumour cells; RBCs, red blood cells; WBC, white blood cells; EpCAM, epithelial cell adhesion molecule; CK, cytokeratin; AFP, alphafetoprotein; GPC-3, glypican-3; DNA-PK, DNA-dependent kinase; ASGPR, asiaglycoprotein receptor; ISET, isolation by size of epithelial cell; HR, hazard ratio; BMI, body mass index; T2DM, type 2 diabetes mellitus; BCLC, Barcelona clinic liver cancer; TTP, time to progression; ARLD, alcohol related liver disease; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; DEB-TACE, drug eluting bead-transarterial chemoembolization; SIRT, selective internal radiation therapy; EASL, European association for the study of the liver; EORTC, European organisation for research and treatment of cancer.

## Introduction

Hepatocellular carcinoma (HCC) complicates chronic liver diseases (CLD) and, owing to a combination of incidence, late stage presentation and lack of therapeutic options, is the second commonest cause of cancer related death globally [1]. The predominant causes of CLD include hepatitis B and hepatitis C, although the prevalence of obesity and alcohol excess are having a major impact, with mortality continuing to rise despite advances in antiviral therapy [2]. The presence of associated CLD severely limits the role of traditional anti-cancer cytotoxic agents in patients with HCC. While 'targeted' medical therapies have emerged for other cancer types in recent years, advances for patients with HCC have been hampered by both a failure to identify or successfully 'drug' the key drivers of hepatocarcinogenesis, as well as an inherent lack of biological markers to support stratification of both traditional and emerging therapies. This handicap is attributed to the lack of tissue biopsy as standard diagnostic practice for patients with HCC. In the majority the diagnosis can be confidently made using imaging criteria, avoiding biopsy and the associated risks of haemorrhage and tumour seeding [3]. There is a need, therefore, for HCC biomarkers other than those acquired by standard staging or tissue biopsy, that can be used to assist treatment stratification and to determine prognosis. The development of a 'liquid biopsy' enabling evaluation at multiple time points would be a major advance.

Cancer

First described by Thomas Ashworth in 1869, circulating tumour cells (CTCs) are those cells that have detached from a primary or secondary tumour and can be detected in the peripheral circulation [4]. Although the potential significance of these cells has long been realised, clinical implementation has been slow owing to a lack of cancer-specific biomarkers and difficulties detecting these rare cells (estimated frequency $\leqslant$1 CTC/ml of blood) against a high background of haematopoietic cells and blood components. Recent technological advances have started to have an impact in metastatic breast, prostate and colorectal cancers, where CTCs positive for epithelial biomarkers such as cytokeratin (CK) and epithelial cell adhesion molecule (EpCAM) have been detected using immunogenic capture with the CellSearch® system (Janssen Diagnostics) and enumeration of CTCs shown to be a useful predictor of prognosis in terms of median overall survival and TTP [5–7].

Currently, there are limited studies detecting and characterising CTCs in HCC, with no standardisation of methods and techniques. CTC detection of $\geqslant$1 or 2 CTC using the CellSearch system is reportedly in the region of 30-35%, although positive cells have occasionally been detected in controls [8–10]. An alternative size based filtration technique (Isolation by Size of Tumour cells or ISET®) identified cells presumed to be CTCs during tumour resection in a study published in 2000, but with a sensitivity that could only detect these cells as 'microemboli' prior to surgery (3 of 7 patients), rather than as single cells [11]. More recently, Morris and colleagues evaluated the ISET and CellSearch methods in patients with HCC, confirming a CellSearch sensitivity similar to that previously reported (15/50; 28%) compared to 100% (19 of 19 patients) for ISET [9]. While ISET sensitivity was promising, specificity was dependent on size only, with no associations in the small number of cases studied with tumour stage or outcome [9]. Other researchers have explored the use of positive immunomagnetic selection with mesenchymal markers in combination with alternative liver specific markers such as asiaglycoprotein receptors (ASPGR), or in-situ hybridisation to detect molecular aberrations (TP53 deletion, HER-2 amplification) [12,13]. As with the other methods, the clinical relevance of detection of these populations of cells in patients with HCC remains to be determined.

We have previously outlined a method for the detection and characterisation of CTCs using the ImageStream (Amnis®, EMD Millipore) imaging flow cytometer [14]. Here we report our subsequent study in patients with HCC, using a combination of image, size and fluorescently detected biomarkers. These included the epithelial biomarkers EpCAM and cytokeratin, HCC specific biomarkers AFP and glypican-3 (GPC3), as well as DNA-PK – a candidate biomarker for treatment stratification in HCC [15]. We have detected at least 1 CTC in 65% (45/69) of patients, showing significant correlations between CTC number and adverse tumour characteristics. Furthermore, the presence of CTCs was an independent predictor of poorer survival.

## Patients and methods

### *Cell culture*

A panel of 6 authenticated HCC cell lines (LGC standards) were assessed for cell area. HepG2, Hep3B, Huh-7 and PLC/PRF/5 were cultured in Dulbecco's modified eagle's medium with 15 mM HEPEs, pyridoxine and $NaHCO_3$ (Sigma-Alrich, UK) supplemented with 10% foetal bovine serum (FBS), (Gibco, UK) and 2.5% 200 mM L-Glutamine solution (Sigma-Alrich, UK). SNU182 and SNU475 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with 10% FBS. Cells were maintained in the growth phase and passaged when ~70% confluent. Regular mycoplasma testing was performed using the MycoAlert mycoplasma testing kit, Lonza, USA.

### *Assessment of biomarker expression in HCC cell lines*

HCC cell lines were assessed for the expression of biomarkers using the ImageStream. The SJSA1 human osteosarcoma bone fibroblast cell line was used as a negative control when assessing the expression of epithelial biomarkers. A population of round single cells was gated by using a scatterplot of brightfield aspect ratio against area. From this population in focus cells were selected by creating a histogram of the root mean square feature which detects differences in intensity between adjacent pixels. A gate of in focus cells was created and assessed for the mean pixel intensity of biomarkers of interest. Numerical data on different cell populations was exported to GraphPad Prism Software, version 6 (GraphPad Prism Software Inc, California, USA).

### *Patient blood samples*

As part of a pilot project exploring the utility of the ImageStream in patients with HCC, blood samples were collected from patients attending the Newcastle-upon-Tyne Hospitals NHS Foundation Trust between November 2012 and January 2015. With the initial aims of optimising enrichment and analysis methodologies, as well as assessing a panel of CTC biomarkers, there was no patient selection criteria other than an established diagnosis of HCC, based on EASL-EORTC guidelines [3], and patient consent, aiming to collect 1 sample per week. Patients with an active malignancy at another site were excluded. Control samples were from 15 healthy volunteers and 16 patients with cirrhosis without cancer. Ethical approval was obtained through the Newcastle hepatopancreatobiliary and gastroenterology research tissue biobank. The patient dataset included age, sex, body mass index (BMI), blood count and biochemistry, as well as tumour staging on cross-sectional imaging – tumour size, portal vein thrombosis (PVT), presence or absence of extra-hepatic disease (EHD). Eastern cooperative oncology group (ECOG) performance status score, Child-Pugh score and Barcelona Clinic for Liver Cancer (BCLC) stages were recorded, as were treatment regimens. Follow-up was until 15th September 2015, recording whenever possible the time to clinical progression and radiological progression using modified response evaluation criteria in solid tumours (mRECIST) criteria [3], as well as date of death, from the date the blood sample for CTC analysis was taken.

### *Patient sample processing*

Patient blood samples were collected in either EDTA (BD Vacutainer) or CellSave preservative tubes (Janssen Diagnostics). EDTA tubes were transported on ice to the laboratory where they were processed immediately. CellSave tubes were transported at ambient temperature and cells were left to fix for at least 1 h. A total of 4 ml blood was processed from EDTA samples and 8 ml from CellSave tubes and cell number for each sample expressed as number of cells per 4 ml whole blood. Patient samples were processed according to a previously developed method which reported recovery of 57.3, 49.2 and 59.0% of cells spiked into whole blood at densities of 500, 50 and 5 per ml respectively [14]. Unfortunately, it was not possible to assess in-study sample reproducibility. Briefly, samples were blocked using bovine serum albumin (BSA) solution and FcR block (MACS®, Miltenyi) to prevent unspecific antigen reactions. The samples were depleted of red blood cells (RBCs) by incubation with BD Phosflow™ lyse/fix red cell lysis buffer (BD Biosciences). The sample was then immunomagnetically depleted of white blood cells (WBCs) using a CD45 depletion kit and BigEasy magnet (StemCell Technologies). The remaining cell suspension was then stained with a series of immunofluorescent antibodies including: pan-CK (CK -4, -5, -6, -8, -10, -13 and -18), EpCAM, AFP, GPC3, DNA-PK and CD45 (Supplementary Table 1). Nuclei were stained with either DRAQ5 or 4′,6-diamidino-2-phenylindole (DAPI). Following staining, cells were washed and re-suspended in PBS prior to processing through the ImageStream. Analysis was performed using the IDEAS software (Amnis, Seattle) and CTCs were identified on the basis of brightfield morphology, size, antigen expression, nuclear signal and the absence of CD45 expression. Objects that did not meet these criteria but had a consistent cell morphology and lack of CD45 expression were also identified. Cell area was calculated by creating a custom brightfield mask based on the standard brightfield mask eroded by 3 pixels to allow closer fit to the brightfield image. A new area feature of this mask was created and area values calculated in $\mu m^2$.