Research Article

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Cancer-associated circulating large extracellular vesicles in cholangiocarcinoma and hepatocellular carcinoma

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Background & Aims: Large extracellular vesicles, specifically AnnexinV$^+$ EpCAM$^+$ CD147$^+$ tumour-associated microparticles (taMPs), facilitate the detection of colorectal carcinoma (CRC), non-small cell lung carcinoma (NSCLC) as well as pancreas carcinoma (PaCa). Here we assess the diagnostic value of taMPs for detection and monitoring of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). Specifically, the aim of this study was to differentiate liver taMPs from other cancer taMPs, such as CRC and NSCLC.

Methods: Fluorescence-activated cell scanning (FACS) was applied to detect various taMP populations in patients' sera that were associated with the presence of a tumour (AnnexinV$^+$ EpCAM$^+$ CD147$^+$ taMPs) or could discriminate between cirrhosis (due to HCV or HBV) and liver cancers (AnnexinV$^+$ EpCAM$^+$ ASGPR1$^+$ taMPs). In total 172 patients with liver cancer (HCC or CCA), 54 with cirrhosis and no liver neoplasia, and 202 control subjects were enrolled.

Results: The results indicate that AnnexinV$^+$ EpCAM$^+$ CD147$^+$ taMPs were elevated in HCC and CCA. Furthermore, AnnexinV$^+$ EpCAM$^+$ ASGPR1$^+$ CD133$^+$ taMPs allowed the distinction of liver malignancies (HCC or CCA) and cirrhosis from tumour-free individuals and, more importantly, from patients carrying other non-liver cancers. In addition, AnnexinV$^+$ EpCAM$^+$ ASGPR1$^+$ taMPs were increased in liver cancer-bearing patients compared to patients with cirrhosis that lacked any detectable liver malignancy. The smallest sizes of successfully detected cancers were ranging between 11–15 mm. AnnexinV$^+$ EpCAM$^+$ ASGPR1$^+$ taMPs decreased at 7 days after curative R0 tumour resection suggesting close correlations with tumour presence. ROC values, sensitivity/specificity scores and positive/negative predictive values (>78%) indicated a potent diagnostic accuracy of AnnexinV$^+$ EpCAM$^+$ ASGPR1$^+$ taMPs.

Conclusion: These data provide strong evidence that AnnexinV$^+$ EpCAM$^+$ ASGPR1$^+$ taMPs are a novel biomarker of HCC and CCA liquid biopsy that permit a non-invasive assessment of the presence and possible extent of these cancers in patients with advanced liver diseases.

Lay summary: Microparticles (MPs) are small vesicles that bleb from the membrane of every cell, including cancer cells, and are released to circulate in the bloodstream. Since their surface composition is similar to the surface of their underlying parental cell, MPs from the bloodstream can be isolated and by screening their surface components, the presence of their parental cells can be identified. This way, it was possible to detect and discriminate between patients bearing liver cancer and chronic liver cirrhosis.

Keywords: ASGPR1; Biomarker; Cancer; CCA; CD147; CD326; EMMPRIN; EpCAM; Extracellular vesicles; HCC; Microparticles; Microvesicles.

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Cancer

Introduction

A decade ago extracellular vesicles (EVs) raised little attention in the scientific communities around the globe. Nowadays, EV research has become intense and acquired more attention, including within the liver research community.[1,2] Over the years, researchers explored the likely role of EVs, including small EVs as exosomes (50–100 nm in diameter)[3] and large EVs, *e.g.* microvesicles/microparticles (MVs/MPs; 100–1000 nm in diameter).[4] Rarely, MVs/MPs were referred to as 'ectosomes'.[5] However, the underlying biogenesis of exosomes and MVs/MPs is different and unique.[6] It was reported that EVs play a role in the horizontal communication between cells.[7] In fact, it was also shown that tumours prepare their own tumour niches via the release of EVs,[8] including a possible suppression of the immune system and the activation of tumour neo-angiogenesis.[9,10] Additionally, EVs are present in all body fluids such as urine,[11] blood serum/plasma,[12,13] and bile.[14] These facts made the isolation, quantification and characterization of circulating EVs a very promising and attractive potential clinical tool,[15] and several methodologies have been established for these purposes.[16] We demonstrated in the past that fluorescence-activated cell scanning (FACS) is accurate and reliable to detect MPs isolated from human serum and plasma.[12,13,17] Additionally, we reported that MP profiling for distinct MP populations that are associated with chronic liver diseases robustly discriminates between non-alcoholic fatty liver disease and chronic hepatitis C virus (HCV) infection,[12] thus providing a novel liquid biopsy tool based on serum analyses. Since almost every cell can release EVs upon stimuli, it is likely that tumour cells release EVs that reach the peripheral blood flow or other body fluids and that these particles might reveal the presence of a tumour.[15,18] We took advantage of this hypothesis and showed that tumour-released MPs carry one, two or multiple tumour-associated antigens simultaneously and could indeed indicate the presence of tumours.[17] Furthermore, we showed that $EpCAM^+$ and $CD147^+$ tumour-associated MPs (taMPs) accurately detected colorectal, non-small cell lung, and pancreatic cancers.[17] Additionally, we reported that cell-derived taMP release was independent of a metastatic phenotype.[17] $Glypican1^+$ exosomes have been used for the detection of pancreatic and breast cancer, but have failed to discriminate between these two entities.[19] Nevertheless, few publications are available showing the diagnostic potential of EVs for cancer detection by exploring surface antigens on EVs. Importantly, based on our multiplex surface staining strategy, we successfully differentiated hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) from chronic diseases without liver tumours.

Material and methods

Cell culture

All tissue culture work was performed under sterile conditions in a laminar flow hood. Cells were invariably incubated at 37°C and 5% CO_2 in a CO_2 incubator. The culture medium used throughout all tissue culture work consisted of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco by Life Technologies, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco by Life Technologies, Paisley, UK) and 1% (v/v) penicillin-streptomycin (P/S; 10,000 U/ml, Gibco by Life Technologies, Paisley, UK) dissolved in phosphate buffered saline (PBS, Sigma Aldrich, Steinheim, Germany) containing 4 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen by Life Technologies, Paisley, UK).

Human cancer cell lines

The human HCC cell lines HuH7 (CLS Cell Lines Service GmbH, Eppelheim, Germany, #300156) and HepG2 (CLS Cell Lines Service GmbH, Eppelheim, Germany, #300198) and the human hepatic adenocarcinoma cell line SK-HEP-1 (CLS Cell Lines Service GmbH, Eppelheim, Germany, #300334) were used as an *in vitro* liver cancer model for surface antigen validation. Additionally, the human pancreas ductal adenocarcinoma cell lines Panc-1 (CLS Cell Lines Service GmbH, Eppelheim, Germany, #300228) and the human pancreatic adenocarcinoma cell lines Capan-1 (CLS Cell Lines Service GmbH, Eppelheim, Germany, #300143) and Capan-2 (CLS Cell Lines Service GmbH, Eppelheim, Germany, #300144) were utilized as an *in vitro* negative control.

Surface staining of human cancer cell lines

For the surface staining of human cancer cells 10^5 freshly harvested cells were used for each staining. After determining the cell count the corresponding volume of the cell suspension was transferred to a fresh 1.5 ml reaction tube and the cells were centrifuged at 300 g for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in 50 µl Fc Block Mix containing 47.5 µl FACS buffer and 2.5 µl Fc Block (unfiltered) for each staining. Samples were then incubated for 5 min on ice. After the incubation, 50 µl of the Multi Antibody Mix 1 (EpCAM/CD147) or Multi Antibody Mix 2 (EpCAM/ASGPR1/CD133) containing 50 µl FACS buffer and 1 µl (pre-dilution necessary) of the corresponding antibodies according to Table S1 were added to each staining and the samples were incubated for 15 min on ice in the dark. After the incubation 400 µl of FACS buffer was added to each staining and the samples were centrifuged at 300 g for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 400 µl FACS buffer. For FACS measurement 4 µl of 1:10 PI was added to each sample and 150 µl was measured using the MACSQuant® Analyzer 10.

Human study cohort

The Ethics Commissions of: (i) the State Chambers of Medicine in Rhineland-Palatinate; (ii) Saarland; (iii) San Sebastian, Spain; as well as (iv) Warsaw approved the current study (approval numbers: 837.151.13 (8836-F), 167/11, PI2014187, KB/41/A/2016 and AKB/145/2014, respectively) and patients gave their informed consent. Patients with a major second or third known comorbidity that could affect immune cell activation, such as acute inflammation, chronic inflammation, autoimmune diseases or viral infections besides HBV/HCV and liver cirrhosis, were excluded. Additionally, patients who underwent chemotherapy or were receiving chemotherapy or were subjected to any other anti-tumour therapy during the time blood samples were taken were excluded as well, except for patients who participated in the R0 resection study section. The characteristics of the patients are summarized in Tables S2 and S3.

Isolation of cell derived microparticles from human serum

Blood was collected in standard S-Monovette® 7.5 ml, Serum Gel with Clotting Activator (Sarstedt AG&Co., Nümbrecht, Germany) and left for 30 min at 37°C to allow for clot formation followed by centrifugation at 4,000 rpm for 20 min at 4°C. Clots were carefully separated and supernatants were stored at −80°C for further MP isolation. MPs from serum samples were isolated by differential centrifugation between 2,000 and 20,000 g as described by others and us.[10,11,14] MPs sedimenting at 20,000 g were characterized by FACS using staining for the corresponding antibodies according to Table S4 referred to Multi Antibody Mix 1 or 2 (1: AnnexinV/EpCAM/CD147; 2: AnnexinV/EpCAM/ASGPR1/CD133). Multi Antibody Mix 2 was optimized for a better detection of liver tumours. All antibodies were titrated against the matching isotype control on patient's samples prior to use. MP preparations were characterized on a MACSQuant 10 Analyser (Miltenyi Biotec, Bergisch Gladbach, Germany) and FACS raw-data was analysed with FlowJo X software for MAC OSX (Tree Star, Inc., Ashland, Oregon). To avoid non-specific antibody binding, Fc receptors on MPs and target cells were blocked with FcR Blocking Reagent (eBioscience™, San Diego, CA, USA) and 0.05% BSA. Used BSA blocking solution was centrifuged at 20,000 g prior to FACS to avoid artefacts due to aggregation.[17] All solutions except antibody containing solutions were centrifuged or filtered (0.2 µm) prior to their use to remove contaminations such as possible protein aggregates or particles with similar size as larger EVs.