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Isolation of Circulating Plasma Cells from Blood of Patients Diagnosed with Clonal Plasma Cell Disorders using Cell Selection Microfluidics

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Abstract

Blood samples from patients with plasma cell disorders were analysed for the presence of circulating plasma cells (CPCs) using a microfluidic device modified with monoclonal anti-CD138 antibodies. CPCs were immunophenotyped using a CD38/CD56/CD45 panel and identified in 78% of patients with monoclonal gammopathy of undetermined significance (MGUS), all patients with smouldering and symptomatic multiple myeloma (MM), and none in the controls. The burden of CPCs was higher in patients with symptomatic MM compared with MGUS and smouldering MM ($p < 0.05$). FISH analysis revealed the presence of chromosome 13 deletions in CPCs that correlated with bone marrow results. Point mutations in *KRAS* were identified, including different mutations from sub-clones derived from the same patient. The microfluidic assay represents a

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].

Conflicts of interest

SAS holds equity shares in BioFluidica, Inc., a company that holds commercialization rights to CTC isolation technology described herein. MAW declares COI as a spouse of a BioFluidica, Inc employee.

highly sensitive method for enumerating CPCs and allows for the cytogenetic and molecular characterization of CPCs.

Introduction

Plasma cell disorders (PCDs) are a diverse group of maladies that include monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (SMM), and multiple myeloma (MM). These diseases are characterized by the monoclonal expansion of plasma cells and production of a monoclonal M-protein in the serum or urine. Clinical outcomes for patients with symptomatic MM continue to improve owing to new therapeutic agents, however, the majority of patients still suffer multiple relapses and ultimately succumb to refractory disease.¹

The International Staging System (ISS) and the presence of high risk cytogenetic abnormalities are commonly used for prognosis and to guide therapy decisions for MM.² M-protein isotype and concentration, the serum free light chain ratio, immunoparesis, presence of occult bone lesions, and cytogenetics can in some cases identify patients at higher risk of disease progression, however, distinct differences in outcomes still persist even in well-defined risk groups.³ It has become increasingly clear that MM is a heterogeneous disease and as such, further advances in understanding the pathogenesis and prognostication of MM are critical to improve patient outcome. Recent evidence has demonstrated that virtually all cases of symptomatic MM evolve from an antecedent diagnosis of MGUS or SMM.^{4, 5} Therefore, additional markers to better risk stratify MGUS and SMM patients are needed.

Circulating plasma cells (CPCs) in peripheral blood have emerged as an important prognostic marker in patients with PCDs, even though CPC burden in peripheral blood is reported to be >100-fold lower than in bone marrow.^{6, 7} The presence of CPCs in peripheral blood as assessed by immunofluorescence microscopy (IM) in patients with MGUS was associated with a shorter time to development of SMM or symptomatic MM as well as inferior overall survival (OS).⁸ Similarly, based on CPC isolated from peripheral blood, 71% of patients with SMM with $>5 \times 10^6$ CPCs/l or >5 CPCs per 100 cytoplasmic immunoglobulin (Ig)-positive mononuclear cells progressed to symptomatic disease in 2 years, in contrast to 24% of those who did not meet either criteria. The median OS of SMM patients with high levels of CPCs was 49 months versus 148 months for those with lower levels of CPCs.⁹ A more recent study evaluating CPCs isolated from peripheral blood in SMM patients via multi-parameter flow cytometry (MFC) also demonstrated shorter time to progression to symptomatic MM and inferior OS for those with >150 CPCs per 150,000 cell events.¹⁰ Lastly, increased levels of CPCs in patients with relapsed and newly-diagnosed symptomatic MM were associated with inferior progression-free survival (PFS) and OS.^{11, 12} The presence of high levels of CPCs in peripheral blood retained prognostic significance in multivariate analyses incorporating commonly utilized prognostic markers and in patients treated with modern therapy.¹³

CPCs may constitute $<<0.1\%$ of total blood components,¹⁴ and the frequency of these cells depends on the nature of the PCD. CPC detection has relied primarily on slide-based IM,¹⁵⁻¹⁷ MFC,¹⁸⁻²⁰ or molecular methods for detecting clonal Ig gene rearrangements.¹⁷ IM is

challenging for MGUS or SMM staging due to the low frequency of CPCs in peripheral blood. Though MFC has been shown to be more sensitive compared with morphological-based methods (1 CPC per 10,000 peripheral blood cells), MFC requires a large sample volume and involves elaborate processing steps, such as red blood cell lysis, centrifugation and washings between cell staining steps, all of which may lead to CPC loss, which can affect clinical results.

CellSearch®, which uses a ferrofluid consisting of magnetic nanoparticles surface decorated with the appropriate antibody, has been used for the enumeration of CPCs in peripheral blood.²¹ CPCs were selected using anti-CD138 monoclonal antibodies and enumerated via the following panel: CD38+/CD45-/CD19-. Using spike in experiments of H929 cells over a range of 0-2,000 cells per 4 ml of normal blood, the recovery was 50%. From a pilot clinical study and using CD138 as the selection antigen, 33% of non-diseased subjects had low levels of CPCs detected. For patients with active MM, >1 CPC was detected in 91% of the sample cohort. For MGUS/SMM patients (each group was not reported individually), >1 CPC was detected in 60% of the patients tested. FISH results for CPCs enriched from blood agreed with results from bone marrow in symptomatic MM patients.^{21, 22}

Microfluidic devices utilizing positive selection with antibodies to isolate cells of interest have been shown to provide high sensitivity and specificity for the detection of low abundant cells in peripheral blood, such as circulating tumour cells (CTCs), with a frequency of occurrence on the order of 1:10⁹ peripheral blood cells²³⁻²⁷ or detection of circulating leukemic cells (CLC) from peripheral blood of patients with acute myeloid leukaemia.²⁸ A number of studies using microfluidic enrichment of CTCs or CLCs have demonstrated superior performance in terms of specificity, recovery, and throughput compared to IM or MFC.^{24, 29}

Recently, a herringbone type microfluidic device was used to enrich CD138 expressing CPCs from whole blood;³⁰ the recovery of this microfluidic device was determined from a model cell line spike-in experiment (0–100 cells/ml) into EDTA preserved blood samples from healthy controls and found to be 45%. The analysis of active MM patients indicated that the number of captured CPCs was 20 – 184/ml with a purity of 1 – 5%. The analysis of CPCs in patients with MGUS or SMM was not reported.

We have reported a microfluidic device that uses sinusoidally-shaped microchannels providing the ability to efficiently capture rare epithelial cells (recovery of 97% for EpCAM expressing MCF7 cells; purity >85%) using surface-immobilized antibodies.^{26, 27, 31, 32} The microfluidic device can process whole blood directly without pre-processing, minimizing potential sample loss and/or contamination. The antibodies could be tethered to the walls of the microfluidic device using a single-stranded oligonucleotide bifunctional linker, which can be cleaved enzymatically to release the captured cells while maintaining their viability.³³ The sinusoidal microfluidic device offers high sensitivity with a simple workflow for capturing, enumerating, and characterizing rare cells from blood samples.

Herein, we evaluated the ability of the sinusoidal microfluidic device to affinity-select CPCs for different PCDs (*i.e.*, MGUS, SMM and symptomatic MM), and also demonstrate the

ability to release CPCs and perform cytogenetic and molecular analysis on the enriched cells. Blood of patients diagnosed with different PCDs and healthy controls were analysed and the CPCs affinity selected using anti-human CD138 monoclonal antibodies (mAb) attached to the surface of the microfluidic channels. We enriched the CPCs using CD138, which has been found to be expressed in both normal and diseased CPCs, but with a higher expression in diseased CPCs;^{14, 34} 100% CPCs in MM patients were found to express CD138 when cells were sampled from bone marrow.³⁵

Identification of diseased CPCs was performed via immuno-phenotyping with fluorescently-labelled anti-CD38, and anti-CD45 mAbs. A cell displaying a CD138+/CD38+/CD45– phenotype was classified as a CPC. We also assessed the clonality with anti-CD56^{36, 37} and performed cytoplasmic immunoglobulin (cIg) staining for κ and λ Ig light chains. Isolated cells were assessed for mutations in *KRAS* using a highly sensitive polymerase chain reaction / ligase detection reaction / capillary gel electrophoresis (PCR/LDR/CGE) assay and chromosomal abnormalities detected via FISH. Our microfluidic device (coined hereafter as a CPC selection device) demonstrated high sensitivity for isolating CPCs directly from the peripheral blood of PCD patients, even those with MGUS. The burden of CPCs was higher in patients with symptomatic MM compared to those with SMM and MGUS. We also identified the presence of chromosome 13q deletions and *KRAS* mutations in anti-CD138 selected CPCs.

Experimental

CPCs selection device fabrication and assembly

The CPC selection device was made from the plastic cyclic olefin copolymer (COC) owing to its ability to be replicated with the prerequisite microstructures, its high optical clarity, propensity to be UV/O₃ activated and subsequently modified with selection mAbs with high efficiency and minimal amounts of non-specific adsorption of contaminating cells to its surface.³⁸ The CPC selection device consisted of an array of 50 sinusoidal microchannels with a nominal width of 25 μ m and depth of 150 μ m serving as the selection bed (Fig. 1). The selection bed was addressed using a single inlet and outlet microchannel and arranged in a unique Z-configuration.²⁷ The microfluidic chip fabrication procedure is discussed in the supplementary data.

Monoclonal antibody attachment to the COC surface

After chip assembly, which involved sealing a cover plate to the fluidic network embossed into the COC substrate, the attachment of CD138 mAbs to the UV/O₃ activated COC surfaces of the CPC selection device was undertaken. The procedure followed a method reported by our group and used a bifunctional linker (single-stranded oligonucleotide) that contained a uracil residue that could be cleaved using the USERTM enzyme system to allow for release and collection of the selected CPCs from the microfluidic device.³³ Briefly, mAbs were reacted with a sulfo-NHS ester of succinimidyl trans-4 (maleimidylmethyl) cyclohexane-1-carboxylate (SMCC), yielding a maleimide-labelled mAb (SMCC-mAb). Once purified, the SMCC-mAb was covalently attached to the reduced 3'-disulfide group (sulfhydryl) of a single-stranded oligonucleotide bifunctional linker that was immobilized to

the UV/O₃ activated COC surface. Oligonucleotides were attached to the COC surface-confined carboxylate groups using 20 mg/ml EDC and 2 mg/ml NHS via a 5'-amino group. The optimum concentration of the mAb was chosen based on studies to optimize CTC recovery as reported elsewhere.²⁷

Clinical samples

Patients with different PCDs (MGUS, SMM and symptomatic MM) were consented according to a protocol approved by the University of North Carolina's Institutional Review Board (IRB). Lab personnel were blinded to the nature of the sample. A total of 47 PCD patients were analysed for CPCs with an additional 5 healthy donor samples obtained from UNC's blood donation centre, which served as negative controls. All specimens were collected into BD Vacutainer® (Becton-Dickinson, Franklin Lakes, NJ) tubes containing EDTA. Tubes with blood were placed on a nutator until processed within 6 h of the blood draw.

CPC selection device operation

Prior to blood sample infusion, the CPC selection device was washed with 0.5% BSA/PBS pH 7.4 at a volumetric flow rate of 40 µl/min for at least 10 min to remove unbound mAb from the microchannel walls. Between 0.5 and 2 ml of patient blood was transferred into a disposable 3 ml Luerlock™ syringe (BD Biosciences, Franklin Lakes, NJ) using a BD vacutainer female luer transfer adapter. Immediately after transfer, blood samples were processed through the CPC selection device. A PHD2000 syringe pump (Harvard Apparatus, Holliston, MA) was used to hydrodynamically drive the blood through the CPC selection device at the appropriate volumetric flow rate (0.9 ml/h) to attain an average linear velocity of sample through each sinusoidal microchannel of 1.1 mm/s that maximized recovery of the CPCs. During the course of blood sample introduction, the syringe containing the peripheral blood sample was rotated by 180° orthogonal to its longitudinal axis to prevent sedimentation of the blood components inside the syringe. Finally, the CPC selection device was flushed with 2.0 ml of 0.5% BSA/PBS pH7.4 at a linear velocity of 4 mm/s (3.3 ml/h) to remove non-specifically bound cells. At this point, the device was either submitted for in situ on-chip immunofluorescence staining for identification/enumeration of the CPCs or for release³³ and mutation detection or fluorescence in situ hybridization (FISH) of the released CPCs. For CPC release the devices were filled with 10 µl of USER™ enzyme (4 U per 10 µl CutSmart buffer) and incubated 45 min at 37°C to cleave the oligonucleotide linker. Cells were then washed from the chip with PBS. The volume in which CPCs were eluted in was determined by the volume of the isolation bed which was 10 µl, therefore providing a 100× volume reduction of the CPCs when 1 ml of blood was processed.

Immunostaining

CPCs were analysed for surface antigens via immunostaining by; (i) treating with Fc blocker (IgG) for 15 min; (ii) incubation with anti-human CD45-FITC mAbs, anti-human CD38-APC, and anti-human CD56-Texas Red for 30 min; (iii) cell fixation with 2% PFA for 10 min; (iv) permeabilization with 0.1% Triton-X100 for 10 min; and (v) 2 min incubation with a nuclear dye, DAPI. For the purposes of our analyses, CPCs selected via anti-CD138 mAbs were defined as DAPI+/CD38+/CD45-. For clonal population staining, selected cells were:

(i) fixed with 2% PFA for 10 min; (ii) permeabilized with 0.1% Triton-X100 for 10 min; and (iii) incubated for 30 min with anti-human-Ig kappa light chain-Texas Red, anti-human Ig lambda light chain-APC and anti-human CD45-FITC and DAPI.

The stained cells were imaged using an inverted Olympus IX71 microscope (Center Valley, PA) using 10 $\times$, 20 $\times$, 40 $\times$, and 63 $\times$ dry objectives equipped with a high resolution (1344 $\times$ 1024) CCD camera (Hamamatsu ORCA-03G) and a mercury arc lamp as an illumination source. Images were collected and analysed using Metamorph imaging software (Olympus).

KRAS mutational analysis

PCR/LDR³⁹ was used to detect point mutations in *KRAS* from the selected CPCs. Discriminating and common primers used for the LDR were designed to produce ligation products with different sizes (see supplementary data Table S1) with the ligated products separated by capillary gel electrophoresis and the mutation identified based on the size of the LDR product. PCR was performed on HT-29 (wild-type), RPMI-8226, and CPCs genomic (g) DNA using primers targeting a 290 bp fragment of the *KRAS* gene. The presence of amplicons was confirmed by agarose gel electrophoresis. Detailed procedures can be found in the supplementary data.

FISH analysis

FISH analysis was performed at the University of North Carolina Cytogenetics Laboratory. Analysis was performed on both RPMI-8226 cells as a control and on CPCs selected from patient blood. The DLEU 13q14 Kretech probe was used. Detailed FISH procedures are provided in the supplementary data.

Results and discussion

Microfluidic Device

The CPC selection device was designed to select CPCs based on positive affinity selection using a design optimized to provide high recovery, even for low-expressing target cells, with high purity and the ability to process 1 ml of whole blood in ~1 h.^{26–28, 31} The affinity bed of the CPC selection device consisted of 50 sinusoidal channels with dimensions of 25 μ m (width) and 150 μ m (depth) arranged in a Z-configuration (Fig. 1A, B). The channel width was designed to be as close as possible to the CPCs' average diameter to improve recovery, while a depth of 150 μ m was selected to allow processing of relatively large volumes of blood in short time periods.³¹ As noted previously, COC was selected as the substrate material for the microfluidic due to its ability to be efficiently UV/O₃ activated to generate high loads of surface-bound antibodies and minimal amounts of non-specific adsorption of blood components.³⁸

Plasma cells have been found to possess a round shape with diameters ranging from 9–20 μ m; the average cell diameter for the model cell line used here, RPMI-8226, was 14 $\pm$ 2 μ m as determined by using optical analysis. The selected channel width of 25 μ m provided high recovery of CPCs while at the same time minimizing channel blockage by hematopoietic cells (Fig. 1C).³¹

Optimal capture conditions for cells travelling through sinusoidal channels were based on the Chang/Hammer model, which describes the dependence of the translational velocity of cells containing antigen-bearing targets on the encounter rate and probability of reaction between solution-borne cells and surface tethered antibodies.⁴⁰ We investigated the optimal linear velocity for the maximum recovery for CPCs using RPMI-8226 as a model and anti-CD138 mAbs as the affinity agent. Based on our findings (see Fig. 2A), the maximum cell capture efficiency occurred at a cell translational velocity of 1.1 mm/s.

A plot of different RPMI-8226 cell numbers seeded into healthy donor blood versus the number of isolated cells generated a recovery of 69% ($R^2 = 0.999$), which was determined from the slope of the plot (see Fig. 2B). From our previous work demonstrating the isolation of circulating leukemia cells, we were able to show that the same device could isolate >90,000 cells and still showed no signs of saturation;²⁸ we are operating well below saturation for the clinical results shown herein (see Fig. 5).

The purity of the RPMI-8226 cells determined from these experiments was 11%, 54%, 63%, and 79% when 12, 110, 153, and 350 RPMI-8226 cells were isolated, respectively. On average we observed 90 nucleated cells co-isolated with RPMI-8226 when 1 mL of healthy blood was processed.

The size of the cell selection microchannel with respect to the size of the cell of interest can have an influence on cell recovery. The average diameter of RPMI-8226 cells is smaller compared to MCF7 cells that were used in earlier studies and generated higher recoveries.²⁷ Other factors affecting the recovery results for the RPMI-8226 cells included decreased centrifugal forces due to the smaller size of the RPMI-8226 cells, and lower selection antigen expression. In all cases, the efficiency of cell capture is affected by encounter duration and strength of adhesion between a cell-bound receptor and tethered ligand, which can be controlled by the flow velocity as stipulated by the Chang/Hammer Model.⁴⁰

The CPC selection technology presented herein uses a microfluidic device that has some attractive characteristics for the enrichment of CPCs directly from whole blood, such as high recovery (69% for CPCs expressing CD138) and purity, defined as $[CPC/(CPC + leukocytes)]$ (average of 68%, ranging between 1.8% and 97.0%, see supplementary data) and can process 1 ml of whole blood in 1.1 h (Fig. 1). These operational metrics are important because it provides the ability to search for rare target cells, such as those found in MGUS and SMM, and perform molecular analysis on the enriched cells without requiring single cell picking due to the favorable purity of the selected cells.

The surfaces of the microchannels for our CPC selection device were modified with covalently attached mAbs for selecting CPCs that expressed CD138. Interactions between the cells and the surface immobilized mAbs are promoted by the narrow channel width and the sinusoids' radius of curvature that induced centrifugal forces and propels cells out of the laminar streamlines and toward the channel walls that provided high recovery.⁴¹ For example, the model RPMI-8226 cell line seeded into healthy donor blood provided a recovery of 69% using the sinusoidal device, while the recovery for a herringbone device for the same cell line was 45%.³⁰ As a reference, the CellSearch® platform, which uses

immuno-magnetic beads and anti-CD138 monoclonal antibody enrichment, produced a recovery for CPCs of ~50%.²¹ The design of the sinusoidal channel enables uninterrupted cell rolling along the affinity surface improving the probability of antigen/mAb binding.⁴¹ Thus, the CPC selection device represents a promising platform for high CPC recovery that can circumvent the sensitivity limitations associated with other methods (IM, MFC) to make it appropriate for selecting CPCs from the peripheral blood of MGUS and SMM patients as well as symptomatic MM patients.

Phenotype and clonal identification of CPCs from clinical samples

Fluorescence staining conditions were optimized using the RPMI-8226 cell line and results were confirmed using flow cytometry (see supplementary data Fig. S1). Results of immunophenotyping of this model cell line are presented in Fig. 3A.

Using the optimized CPC selection conditions and staining, patients and normal controls were analysed for the presence of CPCs (see Tables S2, S3 in supplementary data for patient details). Cells were identified as CPCs when showing positive staining for DAPI and CD38, while staining negative for CD45 (see Fig. 3B and supplementary data Fig. S2). The clonality was also assessed by evaluating for the expression of CD56 that is expressed by neoplastic plasma cells. In the case of CPCs, the loss of CD56 expression may be associated with more aggressive disease.^{36, 37} Of the CD138+/CD38+/CD45– cells, 57%, 80%, 64% and 65% were negative for CD56 in MGUS, SMM, symptomatic, and treated symptomatic MM patients, respectively (supplementary data Table S4).

To further identify clonal populations of isolated CPCs, we used fluorescently-labelled anti-cytoplasmic Ig (κ) and anti-human Ig (λ) along with anti-CD45 to exclude leukocytes. We established conditions for cytoplasmic light chain staining using the RPMI-8226 cell line. The RPMI-8226 cell line demonstrated restricted lambda light chain expression (see Fig. 4A), which is in agreement with the literature.⁴²

Six symptomatic MM patients were evaluated for CPC isotypic restriction (*i.e.*, homogeneous expression of either kappa or lambda light chains) resulting from clonal proliferation of neoplastic plasma cells. Fig. 4B presents κ + light chain restricted CPCs isolated from patient #20. Two other symptomatic MM patients (#26 and #63) were also identified as having CPC with λ + free light chain while the remaining CPCs presented both κ + and λ +. Fig. 4C shows a λ + light chain restricted CPC from symptomatic MM patient #64. In two MM patients (#19 and 31) CPCs did not show isotypic restriction (see Table S6).

The typical serum free chain ratio for κ + to λ + is between 0.26 and 1.65,⁴³ while a ratio value outside this range correlates with elevated M-protein expression. This ratio can be associated with the number of CPCs expressing either cytoplasmic Ig (κ) or anti-human Ig (λ). Interestingly, Qasaimeh *et al.*³⁰ observed a positive correlation between the number of isolated CPCs and the corresponding serum M-protein levels.

Enumeration of CPCs in PCD patients

We initiated a pilot study for the isolation and enumeration of CPCs in patients with MGUS, SMM, and symptomatic MM using our CPC selection device and enumeration assay, which

consisted of staining the selected cells using the DAPI/CD38/CD56/CD45 panel (Fig. 5). A total of 52 blood samples were analysed, including 5 negative controls, 9 MGUS, 11 SMM, 19 symptomatic MM, and 8 symptomatic MM patients on active treatment (supplementary data Tables S2 and S3). CPC counts were all normalized to 1 ml of blood. For patients diagnosed with MGUS, the mean CPC number was 64/ml (range = 0 – 228/ml) and for SMM patients it was 69/ml (range = 5 – 192/ml). In symptomatic MM patients, we detected a mean CPC number of 818/ml (range = 5 – 5,820/ml) and lower CPC counts of 169/ml (range of 4 – 715/ml) for symptomatic MM patients undergoing treatment. Importantly, CPCs were rarely detected in healthy donors (mean = 0.3/ml, range = 0 – 1, n = 5); CD138(+) and CD38(+) cells are rarely found in healthy controls, as CD138 expression is absent on highly proliferative normal plasma blasts and earlier B-cell stages.^{44, 45} All data are summarized in supplementary data Table S4. CPCs were detected in 78% of MGUS patients and 100% of patients with SMM and symptomatic MM. Data obtained for SMM, symptomatic MM, and symptomatic MM patients undergoing treatment were statistically different from the counts observed for healthy controls ($p < 0.05$). Additionally, symptomatic MM patients had higher levels of CPCs than the other PCD groups tested (Fig. 5). The average purity of isolated CPCs from symptomatic MM was 68% (supplementary data Tables S2, S3).

High levels of CPCs in patients with PCDs have been shown to be predictive of PFS and OS not only in symptomatic MM, but MGUS and SMM as well.⁴⁶ Importantly, CPCs retained significance in multivariate analyses incorporating commonly utilized prognostic markers, including $\beta 2$ -microglobulin, lactate dehydrogenase, ISS stage, and the presence of high risk cytogenetic abnormalities.⁴⁷ CPC enumeration represents a promising tool for residual disease determination, prognostication, and potentially refinement of therapy. CPCs enumeration to date has been performed via IM, MFC or sequencing primarily using bone marrow biopsy samples.^{13, 48} Application of IM for CPC enumeration from peripheral blood has been limited due to the lack of fluorescence sensitivity and the technically challenging methodology required for this assay. MFC lacks in situations where the CPC burden is low, especially in MGUS and SMM patients. To overcome these issues, IM or MFC require the use of bone marrow aspirates, which can be a painful and invasive procedure, thus limiting the frequency of sampling. Therefore, a compelling need exists for a highly sensitive method for CPC isolation and enumeration from blood (minimally invasive procedure), and an assay that allows molecular characterization of the CPCs as well.

Our pilot data indicated that the CPC analysis of blood from patients with PCDs yielded test positivity of 78% in MGUS and 100% in patients with SMM and symptomatic MM, which is higher than what IM and MFC can offer. The MFC is capable of detecting 1 CPC among 10^4 WBC while our assay provided the ability to detect CPCs from blood containing of 10^7 WBCs and 10^9 RBCs.^{27, 28}

The advantage of the sinusoid-based geometry used in the CPC selection device is that centrifugal forces induce extended rolling of cells along the mAb-decorated channel walls, and thus low-expressing targets can be captured providing the ability to efficiently capture CPCs with ~700 antigens per cell.⁴¹ However, CPCs that show CD138 expression $\ll 700$ per cell will require the use of additional selection antigens.^{49, 50, 51}

Although we identified CD138(+) CPCs that expressed CD38 and CD56 in a subset of PCD samples, and demonstrated Ig light chain restriction, we did not rigorously assess the clonality of CPCs. Whether there is additional value to better characterizing the CPCs as clonal or not will require prospective, longitudinal studies with more comprehensive immuno-phenotyping. Such studies can determine if there is a difference in clinical outcome when total CPCs are assessed versus clonal CPCs.

***KRAS* mutational analysis of isolated CPCs using PCR/LDR/CGE**

We performed *KRAS* mutational analysis from gDNA extracted from both RPMI-8226 cells and CPCs from 3 clinical samples (see supplementary data Table S5). gDNA from cells was harvested and subjected to PCR with primers spanning codons 12 and 13 for the detection of possible *KRAS* mutations. gDNA extracted from the HT-29 and RPMI-8226 cell lines was used as a negative and positive controls, respectively, as it is documented that HT-29 bears no *KRAS* mutations, while the RPMI-8226 cell line is heterozygous bearing both the wild type allele and a *KRAS* G12A mutant allele.

PCR amplicons were used for LDR and the LDR products were analysed using CGE. Results from CGE (Fig. 6A-E) indicated the presence of a 47 nt LDR product in the RPMI-8226 electropherogram denoting the G12A mutation (Fig. 6A) while no LDR products were seen for the HT-29 cells (Fig. 6B) for all LDR primer sets tested.

A summary of the LDR analyses for patient CPCs are presented in supplementary data Table S5. Two patients with symptomatic MM (#35, #38) and one with SMM (#36) showed different/multiple *KRAS* mutations indicating sub-clones among the enriched CPCs.⁵² PCR/LDR outperformed Sanger sequencing in terms of the ability to detect rare mutated DNA. As shown in Fig. 6F, for patient #36, Sanger sequencing detected the presence of only a G13S mutation, while an additional 3 were detected in PCR/LDR/CGE analyses (see supplementary data Table S5).

An advantage of the CPC selection device is the ability to release affinity-selected cells and perform molecular analysis on the cargo of the CPCs. *KRAS* and *NRAS* mutations are of oncogenic significance in MM^{53, 54} and have been documented with a frequency of 25-39% in newly diagnosed MM patients. *NRAS* mutations have been shown to be predictive of resistance to bortezomib monotherapy in relapsed MM patients.⁵⁵ Given the prevalence of *RAS* mutations in MM, there is an interest to develop *RAS*-targeted therapies for these patients. Indeed, the ability to test for the presence of these mutations in a non-invasive manner is clinically valuable; our assay can be used as a companion diagnostic for discovering new *RAS*-targeted therapies.

LDR coupled with PCR has been shown to be able to detect mutated DNA in an excess of wild-type DNA with a sensitivity approaching 1 mutant copy in 4,000 wild type copies.⁵⁶ Utilizing PCR/LDR/CGE, *KRAS* mutations in CPCs isolated from blood samples were identified and interestingly we observed multiple *KRAS* mutations within the same patient sample, in line with existing data demonstrating the presence of multiple sub-clones in MM patients.^{52, 53, 57}

FISH analysis

MM is associated with many chromosomal abnormalities, and FISH has been used extensively to identify and characterize MM cells. Chromosome 13 deletions (usually consist of large deletions in the q arm) are especially prevalent in MM, and have been found in 42–54% of patients.^{58, 59} FISH is typically performed on MM cells obtained via bone marrow aspiration, the invasive nature of which limits frequent assessment of cytogenetic evolution in patients. Additionally, collection of representative samples from bone marrow aspirates can be confounded by the intra-patient biologic heterogeneity of MM cells from one focus of marrow disease to the next.

Thus, an assay that utilizes peripheral blood for FISH analysis would be valuable in order to negate the need for bone marrow biopsies and account for tumour heterogeneity.

Both RPMI-8226 cells (FISH negative control) and CPCs secured from a patient using the CPC selection device and released from the capture surface were transferred onto microscope slides. The DLEU 13q14 Kreatech probe with 13qter control probe was used for this analysis. The control probe, which is labelled with a green fluorescent probe, locates chromosome 13 while the red probe is used to detect the DLEU gene region at 13q14. The 13q14 probe will bind to chromosome 13 not bearing the deletion; if a deletion is present, there is a loss of a red signal within the cell. In all control RPMI-8226 cells, chromosome 13q deletion was not observed (Fig. 7A) which is in agreement with the karyotype of this cell line. However, a polyploid character of some cells was demonstrated by multiple copies of chromosome 13. In MM patients' blood, CPCs had both non-deleted (Fig. 7B) and 13q deletions (Fig. 7C). This data was in agreement with FISH results from paired bone marrow aspirates.

Conclusions

We have demonstrated the utility of a microfluidic device to isolate CPCs via positive affinity selection directly from clinical samples – the blood of patients diagnosed with PCDs. This device was able to isolate CPCs using CD138 mAbs as the affinity agent. The CPC selection device did not require sample pre-processing, such as the lysis of RBCs, therefore eliminating steps that could lead to CPC loss and simplifying the workflow making it particularly attractive for clinical implementation. The recovery of CPCs was high, as demonstrated by the detection of CPCs in MGUS patients and all SMM and symptomatic MM patients. The feasibility of performing immunophenotyping, clonality testing, FISH analysis and *KRAS* mutation testing was demonstrated. Prospective studies are needed to assess the prognostic significance of CPC detection in PCDs using our CPC selection device.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Insight, innovation, integration

High levels of CPCs in MM patients' blood are associated with more aggressive disease and worse outcomes. To date, CPC analyses have relied on methods such as slide-based immunofluorescence or flow cytometry that are cumbersome and/or lack sensitivity for detecting low levels of CPCs in blood. Herein, we demonstrate the use of a highly sensitive microfluidic assay as a clinically viable tool for the quantification and characterization of CPCs directly from the peripheral blood of patients with clonal plasma cell disorders (PCDs). We also demonstrate the ability to perform fluorescent in situ hybridization (FISH) and mutational analyses on the affinity selected CPCs. Our assay represents a novel and potentially powerful method for the enumeration and analysis of CPCs in patients with PCDs.

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Figure 1.

CPC selection device and assay format. (A) CAD drawing of the CPC selection device, which consisted of 50 sinusoidal channels that made up the selection bed with a footprint of $\sim 3.4 \text{ cm}^2$. The fluid flow followed a Z-configuration through the microchannel network as indicated by the red arrows. (B) SEM image of the sinusoidal channels fabricated in a COC polymer using hot embossing. (C) An image of DAPI stained RPMI-8226 cells affinity-selected within the device. (D) Schematic representation of the selection process of CPCs using the CPC selection device. A clinical blood sample obtained from a patient was processed using the optimized flow parameters through the microdevice containing surface decorated anti-CD138 antibodies. Anti-CD138 antibodies were attached via an oligonucleotide linker with a uracil (red dot) residue that could be enzymatically cleaved, thereby releasing intact cells from the selection surface.

Figure 2.

(A) RPMI-8226 cell recovery versus cell translational velocity. In these experiments, ~500 RPMI-8226 cells were seeded into whole blood from healthy donors (1 ml). The cells were pre-stained with a Hoechst 33342 dye, spiked in blood and introduced in the CPC selection device at linear velocities ranging from 0.4 – 2.0 mm/s (3 measurements for each velocity were performed). Capture efficiency was determined by the ratio of RPMI-8226 cells captured by the CPC selection device to the total number of RPMI-8226 cells seeded into the blood sample. The number of cells captured was determined by fluorescence microscopy. (B) Calibration plot of RPMI-8226 cells seeded (20-500 cells, n=3) of healthy donor blood and processed through the selection device at the optimized linear velocity of 1.1 mm/s. Recovery of the RPMI-8226 cells was given by the slope of this plot.

Figure 3.

Immuno-phenotyping of CPCs. (A) RPMI-8226 cells and (B) CPCs from a symptomatic MM patient captured in the CPC selection device via affinity selection using anti-human CD138 mAbs immobilized to the channel wall, (C) co-isolated leukocyte. CPCs were identified based on staining with (i) DAPI, and phenotyping: (ii) CD56-TR, (iii) CD38-APC, (iv) leukocyte specific antigen, CD45-FITC. Cells positive for DAPI, CD38 and/or CD56 and negative for CD45 were considered CPCs. (v) Composite image of (i) through (iv).

Figure 4.

Identification of CPCs using Kappa (Ig- κ) and Lambda (Ig- λ) Ig light chain markers. Panel of fluorescent images from (A) the lambda light chain expressing MM cell line RPMI-8226, (B) from kappa light chain expressing CPCs isolated from a symptomatic MM clinical sample. (C) lambda light chain expressing CPCs from a symptomatic MM patient, (D) kappa light chain and lambda light chain expressing CPCs from asymptomatic MM patient, (E) kappa light chain and lambda light chain expressing blasts stained with: (i) DAPI for nucleus identification; (ii) anti-human Ig- κ -TR; (iii) anti-human Ig- λ -APC; and (iv) anti-CD45 -FITC, (v) merged image.

Figure 5.

CPC recovery from clinical PCD samples. Pilot clinical data secured from patients with various PCDs including MGUS, smoldering MM, symptomatic MM, and symptomatic MM patients undergoing treatment (n indicates number of patients). Between 0.5–2 ml of whole blood was analysed and results were normalized to 1 ml of blood. The p-values were calculated using the Mann-Whitney test. The dotted line in the box plots indicates the mean and the solid line indicates the median.

Figure 6.

KRAS molecular profiling of CPCs. Capillary gel electropherograms (CGE) showing the separation of LDR products secured from RPMI-8226 cells (A), HT-29 cells (B), and CPCs isolated from clinical samples (C-E). LDR product peaks are represented with an asterisk. (A) 47 nt LDR product denoting the G12A (G35C) mutation found in RPMI-8226; (B) no LDR product at the G12A locus for HT-29; (C) 52 nt LDR product denoting the G12S (G34A) mutation for patient 35 (symptomatic MM); (D) no LDR products at the G12S locus for patient 36 (SMM); and (E) 43 nt LDR product denoting the G13S (G37A) mutation for patient 36 (SMM). DNA size markers of 20 and 80 nt were co-electrophoresed with the LDR products. (F) Sanger sequencing trace for gDNA secured from patient 36 over codons 12 and 13 with an additional peak at c13.1A denoting the presence of mutant copies bearing the G13S mutation. LDR consisted of 20 cycles; initial denaturation was performed at 95°C for 2 min and each cycle consisted of: 95°C (30 s); 60°C (2 min); and 4°C as a final hold. LDR products were separated at a capillary temperature of 60°C with an initial denaturation step prior to injection at 90°C for 3 min. CGE (column length = 30 cm) injection was performed at 2.0 kV for 30 s and separation was done at 6.0 kV.

Figure 7.

Chromosome 13 deletion detection using FISH. FISH probe del13q14 was used in (A) RPMI-8226 cells, and in (B) and (C) cells that were selected from MM patient blood using anti-CD138 mAbs. The green fluorescent signal indicates the presence of chromosome 13, and the red fluorescent signal indicates an absence of a chromosome 13 deletion. The MM cell line RPMI-8226 displayed the absence of the deletion as both green and red dots were observed, which is in agreement with the karyotype of this cell line. Cells from PCD patient samples revealed cells without the 13q deletion (B) and with the deletion (C).