Detection and Characterization of Circulating Tumour Cells in Multiple Myeloma

Original Research Article

Liangxuan Zhang¹*, Sharon Beasley², Natalie L. Prigozhina², Renee Higgins², Shoji Ikeda¹, Florence Y. Lee², Dena Marrinucci² and Shidong Jia¹,³*

¹ Departments of Oncology Biomarker Development, Genentech Inc, South San Francisco, CA, USA
² Epic Sciences Inc., San Diego, CA, USA
³ Predicine Inc, Hayward, CA, USA
*Corresponding author(s) E-mail: zhangl60@gene.com; sjia@predicine.com

Received 16 March 2016; Accepted 06 May 2016
DOI: 10.5772/64124
© 2016 Author(s). Licensee InTech. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Multiple myeloma (MM) remains an incurable disease despite recent therapeutic improvements. The ability to detect and characterize MM circulating tumour cells (CTCs) in peripheral blood provides an alternative to replace or augment invasive bone marrow (BM) biopsies with a simple blood draw, providing real-time, clinically relevant information leading to improved disease management and therapy selection. Here we have developed and qualified an enrichment-free, cell-based immunofluorescence MM CTC assay that utilizes an automated digital pathology algorithm to distinguish MM CTCs from white blood cells (WBCs) on the basis of CD138 and CD45 expression levels, as well as a number of morphological parameters. These MM CTCs were further characterized for expression of phospho-ribosomal protein S6 (pS6) as a readout for PI3K/AKT pathway activation. Clinical feasibility of the assay was established by testing blood samples from a small cohort of patients, where we detected populations of both CD138⁺ and CD138⁻ MM CTCs. In this study, we developed an immunofluorescent cell-based assay to detect and characterize CTCs in MM.

Keywords Circulating Tumour Cells, Rare Cells, Liquid Biopsy, Multiple Myeloma, Biomarkers, Peripheral Blood, Drug Development

1. Introduction

Multiple myeloma (MM) is a neoplasm of plasma cells and is the second most common blood malignancy worldwide [1], accounting for 1% of all cancers, 13% of hematologic malignancies [2], and causing approximately 20% of hematologic malignancy-related deaths [3]. Though MM is still incurable, in the last decade patients with MM have experienced an increased overall survival (up to eight years) due to considerable improvements in disease management, disease monitoring, and the introduction of several new therapeutics, including bortezomib, lenalidomide and thalidomide [1, 2, 4-9].

A number of signalling pathways are known to be dysregulated in MM, which contributes to disease progression. In particular, aberrant activation of growth factor pathways...
results in downstream activation of the PI3K/AKT signalling cascade, promoting cell proliferation, survival and tumour growth [10, 11]. Targeting the PI3K/AKT pathway in MM has been shown to induce cell-cycle arrest and apoptosis in MM cell lines and patient myeloma cells [12-14]. Multiple clinical trials involving the use of PI3K/AKT pathway inhibitors to treat relapsed or refractory MM are underway ([10, 15, clinicaltrials.org]).

Further improvements in MM treatment will require development of novel targeted therapies in combination with diagnostic tests, as well as sensitive technologies to monitor residual disease in patients during remission. Since cancer is a heterogeneous and dynamic disease as demonstrated by inter-patient variability [16, 17] and intra-patient evolution over time [17-22], the management of patients on targeted therapies requires detailed and up-to-date molecular roadmaps, for which repeated and non-invasive sampling will be crucial [23, 24]. Current standard of care requires invasive and painful bone marrow (BM) biopsies, which are not ideal for routine observation of patients’ progress in real time. Thus, the development of a liquid (blood) biopsy for detection and molecular characterization of MM tumour cells in peripheral blood could have broad clinical utility and improve patients’ quality of life.

While originating in the BM, MM tumour cells are able to migrate into the peripheral blood stream, from where they can be isolated and characterized [25] and references therein). These MM circulating tumour cells (CTCs) have been used as biomarkers to indicate active disease [26], to assess disease stage [27], to stratify MM patients for autologous stem-cells transplantation [28, 29], to predict survival [30] and to monitor response to therapy [31]. While flow cytometry is the most commonly used method to analyse MM CTCs, the sensitivity of this technology remains relatively low. Different groups using flow cytometry report varying numbers of patients with detectable CTCs ranging from 30-75% [27, 29, 32-34]. In addition, for patients with MM CTCs, the numbers of MM CTCs identified in peripheral blood were generally low [27, 33]. As an alternative to flow cytometry methods, CellSearch® technology, which utilizes magnetic particles coated with anti-CD138 (syndecan-1) antibodies to enrich MM CTCs from the blood, has been explored for its ability to detect MM CTCs. Using this technology, MM CTCs were isolated from 68% of MM patients [35-37]. However, this enrichment-based approach would miss MM CTCs that are CD138\textsuperscript{neg}, which have been shown to possess stem cell-like qualities and a higher clonogenic potential than their CD138\textsuperscript{pos} counterparts [38-41].

To overcome the limitations of BM biopsies and enrichment-based CTC detection methods, we aimed to develop an assay that can detect all MM CTC subtypes, is flexible for downstream molecular characterization and, at the same time, can provide high-content morphological information. Here we describe an enrichment-free MM specific CTC assay based on the Epic Platform, which has recently been analytically validated for CTCs of epithelial origin [42]. This platform has demonstrated increased sensitivity over the CellSearch® method [43-45], and has been tested in a variety of solid tumour indications including non-small cell lung, prostate, bladder, pancreatic, ovarian and breast cancers [45-47].

The MM CTCs assay used a combination of CD138 and CD45 antibodies as well as a DAPI nuclear stain and morphology. Although CD138 has been shown to be highly expressed on most MM CTCs and is required for myeloma cell adhesion [48, 49], a clinically significant population of MM CTCs that express little to no CD138 has been described [38-41]. To increase sensitivity and ensure identification of the greater population of MM CTCs, including CD138\textsuperscript{neg} CTCs, we combined CD138 expression with morphology characteristics and a secondary biomarker. Due to the importance of the PI3K/AKT pathway in MM, we elected to multiplex the MM CTC assay with a phospho-ribosomal protein S6 (pS6), a common downstream readout in PI3K/AKT signalling pathway studies [50]. This approach provides a platform for enumerating and characterizing CTC in MM patients.

2. Methods

2.1 Sample receipt, processing, and CTC detection using the epic platform

Blood samples were collected in 10 mL cell-free DNA preservative blood tubes (Streck, Omaha, NE) and shipped to Epic Sciences for processing. Sample processing and slide preparation procedures were described previously [42, 44] and also summarized in Figure 1. Briefly, red blood cell (RBC) lysis was performed using an ammonium chloride-based buffer. Following centrifugation, all nucleated cells were plated on up to 12 glass slides at a concentration of three million nucleated cells per slide. Slides were then frozen at -80 °C until CTC analysis. On testing, two slides were thawed and immunofluorescently stained with an antibody cocktail targeting CD138 (BD Pharmingen, CA), CD45, pS6; nuclei were visualized with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, ThermoFisher). Slides were scanned using a high-speed fluorescent imaging system. A proprietary digital pathol-
• Apoptotic CTCs: CD138\textsuperscript{pos}/CD45\textsuperscript{neg} cells with DAPI pattern of chromosomal condensation/fragmentation and/or membrane blebbing.

The number of CTCs detected was reported as either CTC/slide or CTC/ml of blood. Data analysis and graphing were performed using Excel (Microsoft) and Prism software (GraphPad).

2.3 Patient feasibility of CTC detection in MM patients

Blood samples from three Stage III MM patients (on active treatment) were sourced from Conversant Bio (Huntsville, AL), collected in EDTA (BD Vacutainer\textregistered) tubes and shipped overnight to Epic Sciences for processing. Standard sample processing procedures were followed except blood was lysed using BD Phosflow\texttrademark Lyse/Fix Buffer 5X (BD Biosciences, San Diego, CA), and two slides from each patient were immunofluorescently labelled with an antibody cocktail against CD138, CD45, pS6 and stained with DAPI for MM CTC analysis. Following staining, CTCs were identified using Epic’s proprietary algorithm as described above, and expression levels of CD138 and pS6 were measured.

3. Results

3.1 CD138 MM CTC assay development

The anti-CD138 antibody was titrated on samples prepared from spiking a MM cell line, MM.1S, into HD WBCs, and the optimal antibody concentration providing the highest signal levels for the MM CTC assay was determined to be 1 μg/mL (Figure 2A). Although MM.1S cells represented a clonal population, a wide range of CD138 expression was observed (Figure 2B), which likely reflects the expected CD138 expression range in patient cell populations. To assess the specificity of the assay, we used no primary antibody control, where the anti-CD138 antibody was omitted, and an isotype control, where the anti-CD138 antibody was substituted with an appropriate isotype control antibody. As expected, neither of the controls detected any CD138\textsuperscript{pos}/CD45\textsuperscript{neg} cells (Figures 2A, 2B, and Figure 2C), indicating the anti-CD138 antibody was specific. Representative images of an MM.1S cell with an irregularly shaped nucleus and small cytoplasm stained with the MM CTC assay (Figure 2D), showed strong and specific membrane-localized CD138 signals and absence of CD45 signals on the MM.1S cell, while the surrounding WBCs were CD45\textsuperscript{pos}/CD138\textsuperscript{neg}.

3.2 CD138 MM CTC assay qualification: Specificity, sensitivity, linearity, accuracy, reproducibility and reproducibility

To qualify the MM CTC assay for patient sample analysis, assay specificity, sensitivity, linearity, accuracy, reproducibility and repeatability were evaluated. A schematic of the assay qualification setup is shown in Figure 3A. To further confirm the specificity of the MM CTC assay (in...
addition to the no primary and isotype controls described above, the assay was tested on WBCs isolated from five individual healthy donors and no CD138 positive cells were detected (data not shown).

| Run | Expected | Mean | St dev | % CV |
|-----|----------|------|--------|------|
| 1   | 1        | 1    | 0.6    | 43.3 |
|     | 10       | 9    | 1.5    | 17.6 |
|     | 100      | 62   | 9.1    | 14.7 |
|     | 1000     | 994  | 26.1   | 2.6  |
| 2   | 1        | 1    | 1.2    | 173.2|
|     | 10       | 5    | 0.6    | 10.8 |
|     | 100      | 57   | 5.7    | 10.0 |
|     | 1000     | 948  | 46.2   | 4.9  |
| 3   | 1        | 0    | 0.6    | 173.2|
|     | 10       | 7    | 3.5    | 49.5 |
|     | 100      | 59   | 8.7    | 14.9 |
|     | 1000     | 951  | 23.3   | 2.5  |

Table 1. The MM CTC assay is repeatable across titration points. Intra-assay variability was calculated for each titration point from triplicates stained on three separate days. High consistency within the assay (low intra-assay variability, % CV<20) was demonstrated for most conditions. High % CV for n = 1 and 10 was expected due to high variability for detecting low number of cells.

Assay sensitivity was measured by the ability of the MM CTC assay to detect down to a single MM.1S cell spiked-in to three million HD WBCs on a single slide. The results confirmed the assay is highly sensitive and validated its cut-off value. On average, one MM.1S cell per slide was detected in slides at the lowest titration point, with a range of 0 to 2 MM.1S cells detected per slide (Table 2). Further-

more, the assay showed excellent linear correlation (R²=0.9986) and an accuracy of 97.5% between number of expected cells and number of recovered cells ranging from 1 to 1000 cells (Figure 3B). These results also suggest the assay is unbiased since the CD138 expression profile for the CLC population remained consistent regardless of the total number of cells detected, and similar to that observed previously (Figure 3C).

Repeatability (intra-assay variability) of the MM CTC assay was determined by staining triplicate slides per titration point, while reproducibility (inter-assay variability) of the assay was determined by staining three sets of slides on three separate days. The results showed that the assay is highly repeatable and reproducible. At each titration (except for the lowest titration points, as expected), the intra-assay variability expressed as % CV between the triplicate slides (Table 1), and the inter-assay variability expressed as % CV between three runs (Table 2) were below 20%.

Table 2. The MM CTC assay is reproducible across titration points. Inter-assay variability was calculated from data for each titration point across the three runs. High consistency between assay runs (low inter-assay variability, % CV<20) was demonstrated for most data points. High % CV for n = 1 and 10 was expected due to high variability for detecting low number of cells.

Figure 3. The Epic MM CTC assay is sensitive, specific, linear, accurate, repeatable and reproducible. (A) Experimental scheme for assay characterization is shown. Sample slides were prepared with a 10-fold titration series of 1, 10, 100, and 1000 MM.1S cells spiked into three million (3M) HD WBCs per slide. Triplicate slides from each titration point were stained with the MM CTC assay on three separate days. (B) The MM CTC assay is sensitive (limit of detection down to one cell), linear (R²=0.9986), with an accuracy of 97.5% (derived from the slope of the line). The graph shows mean ± SD numbers of MM.1S cells detected plotted against the number of MM.1S cells expected. (C) Heterogeneity of CD138 signals (mean ± SEM) were observed in MM.1S cells, but profiles of CD138 signals remained consistent regardless of number of MM.1S cells detected. The data were combined from the total of nine replicates (triplicate slides, three runs).
Taken together, these results demonstrated that the MM CTC assay was specific, sensitive, linear, accurate, unbiased, repeatable and reproducible for detecting MM-derived CLCs.

3.3 Patient feasibility studies using CD138 MM CTC assay

To determine the clinical feasibility of the MM CTC assay, we tested blood samples from three Stage III MM patients undergoing active treatment. Since previous work demonstrated the utility of pS6 in cytometry-based multiple myeloma CTC assays [50] and because the addition of an extra MM biomarker could potentially help identify a subpopulation of CD138<sup>pos</sup>/CD45<sup>neg</sup> MM CTCs, we multiplexed the MM CTC assay to include pS6 as a marker for PI3K/AKT pathway activation.

The MM CTC assay identified CD138<sup>pos</sup> MM CTCs in all three patient samples tested (Figures 4A, 4B, Table 3), confirming the assay’s ability to detect MM CTCs in clinical samples. Average CD138<sup>pos</sup> MM CTC counts of 63 CTCs/ml, 499 CTCs/ml and 450 CTCs/ml were identified in samples from patients one, two and three, respectively. Notably, each patient sample displayed a heterogeneous population of CTCs with varying CD138 levels (Figure 4B). In addition to traditional MM CTCs, CD138<sup>pos</sup> CTCs, apoptotic CTCs (characterized by abnormal nuclear morphologies, such as chromosomal condensation and/or nuclear fragmentation) and CTC clusters (defined as two or more touching CTCs) were also detected in these samples (Table 3).

### Table 3. CTC populations in patient samples. The table summarizes the total number of CTCs from each category identified on two slides per patient. The percentage of total CTC count is reported in parentheses.

| CTC category (all CD138<sup>pos</sup>) | Patient 1 | Patient 2 | Patient 3 |
|--------------------------------------|----------|----------|----------|
| Traditional CTCs                    | 157      | 1299     | 841      |
| CTC clusters                         | 0        | 1        | 2        |
| Apoptotic CTCs                       | 3        | 77       | 421      |
| CD138<sup>pos</sup>/pS6<sup>pos</sup> | (1.9%)   | (6.0%)   | (43.8%)  |
| (CD138<sup>pos</sup>/pS6<sup>neg</sup>) | 155     | 1,214    | 423      |
| CD138<sup>neg</sup>/pS6<sup>pos</sup> | (98.1%)  | (93.8%)  | (44.0%)  |
| CD138<sup>neg</sup>/pS6<sup>neg</sup> | 0        | 3        | 118      |
| CD138<sup>pos</sup>                  | (0%)     | (0.2%)   | (12.2%)  |

| Total CTCs (CD138<sup>pos</sup> and CD138<sup>neg</sup>) | 158 | 1,294 | 962 |
|-----------------------------------------------------------|----|------|----|
| (100%)                                                    | (100%) | (100%) |

(59 CD138<sup>pos</sup>/pS6<sup>pos</sup> CTCs out of 286 pS6<sup>pos</sup> CTCs per mL of blood). These CD138<sup>pos</sup>/pS6<sup>pos</sup> cells were classified as ‘putative’ MM CTCs based on CD45 negativity and distinct morphology from surrounding WBCs (Figure 4E), including the presence of eccentric nuclei, which is a common feature of MM cells [51].

4. Discussion

In this study, we developed a slide-based, enrichment-free immunofluorescence assay using the Epic Platform to detect, enumerate and characterize MM CTCs. This assay utilized CD138 and CD45 expression levels as well as morphological parameters to distinguish MM CTCs from normal WBCs. Using a titration series of MM.1S cells spiked into normal blood, we demonstrated the assay to be linear and accurate. The assay can also be multiplexed with one or two additional biomarkers to further characterize MM CTCs (discussed below).

Patient feasibility of the MM CTC assay was established by testing three patient samples, where MM CTCs were
identified in all three patients ranging from 63 CTCs/mL to 498 CTCs/mL and pS6 expression levels were quantitated. Patient MM CTCs exhibited a wide range of CD138 expression levels similar to that observed in MM.1S cells. The intra- and inter-patient heterogeneity of MM CTCs was also exemplified by varying cellular morphologies as well as pS6 signal levels. Importantly, in addition to CD138<sub>pos</sub>/CD45<sub>neg</sub> MM CTCs, a population of CD138<sub>pos</sub>/pS6<sub>pos</sub>/CD45<sub>neg</sub> cells was also identified based on their pS6 expression and distinct MM-like cellular morphology. CD138<sub>pos</sub> ‘putative’ MM CTCs are of particular interest as they have previously been reported to possess increased proliferative potential and correlate with poor prognosis [38-41]. A further investigation is needed to analyse characterization and confirmation of these ‘putative’ CTCs.

Basic understanding of the significance of MM CTCs and how they differ from clonal MM cells in the BM has been hampered by the lack of a sensitive and unbiased method to molecularly characterize these cells. Others have endeavoured to profile MM CTCs by capturing, culturing and comparing these cells to their resident BM counterparts [25]. However, this type of approach could introduce additional complexity due to ex vivo culturing of the MM CTCs. The multiplexing and downstream cell-picking capabilities of the Epic platform allow for biomarker expression and genomic profiling using the MM CTC assay described here with single-cell resolution in their native environment, providing a unique opportunity to understand disease heterogeneity.

The ability to assess and characterize MM CTCs also opens up new avenues for improving patient disease management. The invasiveness of BM biopsies limits their clinical utility typically to time of diagnosis or at disease progression. While they have been invaluable for diagnosis and for stratifying patients into proper treatment groups and clinical studies, they provide just a snapshot of a patient’s disease state and are not feasible for continuous routine observations. Blood tests, on the other hand, are minimally invasive and are already performed at follow-up visits for routine disease monitoring. Adding a blood-based MM CTC assay to the current standard of care could present physicians with a movie rather than a snapshot of patients’ disease evolution in real time, allowing for monitoring of treatment response, emergence of treatment resistance, appearance of potential new molecular targets and informing treatment selection.

Due to the heterogeneous nature of patient CTCs, a method capable of unbiased detection and analysis of all CTCs is required to ensure accurate assessment of a patient’s disease state. The MM CTC assay described here provides an advantage over enrichment-based technologies, which are inherently incapable of detecting the full array of CTCs within each patient. Importantly, in addition to CD138<sub>pos</sub>/CD45<sub>neg</sub> MM CTCs, in two of the three patients a clinically important population of CD138<sub>pos</sub>/pS6<sub>pos</sub>/CD45<sub>neg</sub> cells was also detected, based on their pS6 expression and distinct MM-like cellular morphology. This approach may prove useful clinically for pharmacodynamic testing in new therapeutics development and for monitoring and characterizing an individual MM patient’s disease via liquid biopsies of the blood.

5. Conflict of Interest

Z.L., M.L., and S.J are employed by Genentech and have equity in Roche.SB, NP, RH, PL, DM are employed by Epic Sciences.

6. Abbreviations

Circulating Tumour Cell (CTC), Cell Line Cell (CLC), White Blood Cell (WBC), Multiple Myeloma (MM), Bone Marrow (BM), Healthy Donor (HD), Healthy Donor White Blood Cells (HD WBCs), Phospho-ribosomal protein S6 (pS6)

7. Acknowledgements

We thank Dr. Ryon Graf for his contribution to manuscript preparation.

8. References

[1] Palumbo, A., et al., Role of consolidation/maintenance therapy in multiple myeloma. Clin Lymphoma Myeloma Leuk, 2013. 13 Suppl 2: p. S349-54.

[2] Palumbo, A. and K. Anderson, Multiple myeloma. N Engl J Med, 2011. 364(11): p. 1046-60.

[3] Ye, X., et al., Maintenance therapy with immunomodulatory drugs after autologous stem cell transplantation in patients with multiple myeloma: a meta-analysis of randomized controlled trials. PLoS One, 2013. 8(8): p. e72635.

[4] Pineda-Roman, M., et al., VTD combination therapy with bortezomib–thalidomide–dexamethasone is highly effective in advanced and refractory multiple myeloma. Leukemia, 2008. 22(7): p. 1419-1427.

[5] Nishihori, T., et al., Maintenance therapy with immunomodulatory drugs after autologous stem cell transplantation in patients with multiple myeloma: a meta-analysis of randomized controlled trials. PLoS One, 2013. 8(8): p. e72635.

[6] Kouroukis, T.C., et al., Bortezomib in multiple myeloma: systematic review and clinical considerations. Curr Oncol, 2014. 21(4): p. e573-603.

[7] Jeong, T.D., et al., Simplified flow cytometric immunophenotyping panel for multiple myeloma. CD56(CD19)/CD138(CD38)/CD45<sub>pos</sub>, to differentiate neoplastic myeloma cells from reactive plasma cells. Korean J Hematol, 2015. 47(4): p. 260-6.

[8] Lonial, S. and K.C. Anderson, Association of response endpoints with survival outcomes in multiple myeloma. Leukemia, 2014. 28(2): p. 258-268.

[9] Harousseau, J.-L., et al., Better quality of response to lenalidomide plus dexamethasone is associated with
improved clinical outcomes in patients with relapsed or refractory multiple myeloma. Haematologica, 2010. 95(10): p. 1738-1744.

[10] Fuhler, G.M., et al., Widespread deregulation of phosphorylation-based signaling pathways in multiple myeloma cells: opportunities for therapeutic intervention. Mol Med, 2011. 17(7-8): p. 790-8.

[11] Younes, H., et al., Targeting the phosphatidylinositol 3-kinase pathway in multiple myeloma. Clin Cancer Res, 2007. 13(13): p. 3771-5.

[12] Munugalavadla, V., et al., The PI3K inhibitor GDC-0941 combines with existing clinical regimens for superior activity in multiple myeloma. Oncogene, 2014. 33(3): p. 316-25.

[13] Steinbrunn, T., et al., Combined targeting of MEK/ MAPK and PI3K/Akt signalling in multiple myeloma. Br J Haematol, 2012. 159(4): p. 40-40.

[14] Baumann, P., et al., Simultaneous targeting of PI3K and mTOR with NVP-BGT226 is highly effective in multiple myeloma. Anticancer Drugs, 2012. 23(1): p. 131-8.

[15] Ikeda, H., et al., PI3Kp110(del) is a novel therapeutic target in multiple myeloma. Blood, 2010. 116(9): p. 1460-8.

[16] Kandoth, C., et al., Mutational landscape and significance across 12 major cancer types. Nature, 2013. 502(7471): p. 333-35.

[17] de Bruin, E.C., et al., Spatio-temporal diversity in genomic instability processes defines lung cancer evolution. Science, 2014. 346(6206): p. 251-6.

[18] Burrell, R.A., et al., The causes and consequences of genetic heterogeneity in cancer evolution. Nature, 2013. 501(7471): p. 336-345.

[19] Greaves, M. and C.C. Maley, Clonal evolution in cancer. Nature, 2012. 481(7381): p. 306-13.

[20] Hiley, C., et al., Deciphering intratumor heterogeneity and temporal acquisition of driver events to refine precision medicine. Genome Biol, 2014. 15(8): p. 453.

[21] Barber, L.J., M.N. Davies, and M. Gerlinger, Dissecting cancer evolution at the macro-heterogeneity and micro-heterogeneity scale. Curr Opin Genet Dev, 2014. 30(c): p. 1-6.

[22] Carreira, S., et al., Tumor clone dynamics in lethal prostate cancer. Sci Transl Med, 2014. 6(254): p. 254ra125.

[23] Mateo, J., et al., The promise of circulating tumor cell analysis in cancer management. Genome Biol, 2014. 15(8): p. 448.

[24] Crowley, E., et al., Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol, 2013. 10(8): p. 472-84.

[25] Paiva, B., et al., Detailed characterization of multiple myeloma circulating tumor cells shows unique phenotypic, cytogenetic, functional, and circadian distribution profile. Blood, 2013. 122(22): p. 3591-8.

[26] Witzig, T.E., et al., Quantitation of circulating peripheral blood plasma cells and their relationship to disease activity in patients with multiple myeloma. Cancer, 1993. 72(1): p. 108-13.

[27] Rawstron, A.C., et al., Circulating plasma cells in multiple myeloma: characterization and correlation with disease stage. Br J Haematol, 1997. 97(1): p. 46-55.

[28] Dingli, D., et al., Flow cytometric detection of circulating myeloma cells before transplantation in patients with multiple myeloma: a simple risk stratification system. Blood, 2006. 107(8): p. 3384-8.

[29] Gertz, M.A., et al., Monoclonal plasma cells in the blood stem cell harvest from patients with multiple myeloma are associated with shortened relapse-free survival after transplantation. Bone Marrow Transplant, 1997. 19(4): p. 337-42.

[30] Nowakowski, G.S., et al., Circulating plasma cells detected by flow cytometry as a predictor of survival in 302 patients with newly diagnosed multiple myeloma. Blood, 2005. 106(7): p. 2276-9.

[31] Paiva, B., J.J. van Dongen, and A. Orfao, New criteria for response assessment: role of minimal residual disease in multiple myeloma. Blood, 2015. 125(20): p. 3059-68.

[32] Billadeau, D., et al., Clonal circulating cells are common in plasma cell proliferative disorders: a comparison of monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, and active myeloma. Vol. 88. 1996. 289-296.

[33] Luque, R., et al., Normal and clonal B lineage cells can be distinguished by their differential expression of B cell antigens and adhesion molecules in peripheral blood from multiple myeloma (MM) patients—diagnostic and clinical implications. Clinical and Experimental Immunology, 1998. 112(3): p. 410-418.

[34] Schneider, U., et al., Two subsets of peripheral blood plasma cells defined by differential expression of CD45 antigen. Br J Haematol, 1997. 97(1): p. 56-64.

[35] Gross, S., et al., Assay to capture and detect circulating multiple myeloma cells from blood. 2013, Google Patents.

[36] Gross, S., et al. Automated Enumeration and Characterization of Circulating Multiple Myeloma Cells in Blood. in ASH Annual Meeting Abstracts. 2011.

[37] Weiss, B., et al., Circulating Multiple Myeloma Cells (CMMC): A Novel Method for Detection and Molecular Characterization of Peripheral Blood Plasma Cells in Multiple Myeloma Precursor States. Blood, 2014. 124(21): p. 2031-2031.

[38] Reid, S., et al., Characterisation and relevance of CD138-negative plasma cells in plasma cell myeloma. Int J Lab Hematol, 2010. 32(6 Pt 1): p. e190-6.

[39] Matsui, W., et al., Characterization of clonogenic multiple myeloma cells. Blood, 2004. 103(6): p. 2332-2336.

[40] Jacak, J., et al., Expression analysis of multiple myeloma CD138 negative progenitor cells using single molecule

Liangxuan Zhang, Sharon Beasley, Natalie L. Prigozhina, Renee Higgins, Shoji Ikeda, Florence Y. Lee, Dena Marrinucci and Shidong Jia: Detection and Characterization of Circulating Tumour Cells in Multiple Myeloma
microarray readout. Journal of Biotechnology, 2013. 164(4): p. 525-530.

[41] Kawano, Y., et al., Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. Int J OncoL, 2012. 41(3): p. 876-84.

[42] Werner, S.L., et al., Analytical Validation and Capabilities of the Epic CTC Platform: Enrichment-Free Circulating Tumour Cell Detection and Characterization. J Circ Biomark, 2015. 4(3): p. doi: 10.5772/60725.

[43] Dittamore, R., et al. Molecular characterization of circulating tumor cells (CTC) and CTC subpopulations in baseline and progressive metastatic castration resistant prostate cancer (mCRPC). In ASCO Annual Meeting Proceedings. 2014.

[44] Marrinucci, D., et al., Fluid biopsy in patients with metastatic prostate, pancreatic and breast cancers. Phys Biol, 2012. 9(1): p. 016003.

[45] Nieva, J., et al., High-definition imaging of circulating tumor cells and associated cellular events in non-small cell lung cancer patients: a longitudinal analysis. Phys Biol, 2012. 9(1): p. 016004.

[46] Punnoose, E., et al., PTEN loss in Circulating Tumor Cells (CTCs) correlates with PTEN loss in fresh tumor tissue from Castration-Resistant Prostate Cancer (CRPC) patients. Br J Cancer, 2015(submitted).

[47] Scher, H.I., et al., Predictive biomarkers of sensitivity to androgen receptor signaling (ARS) and taxane-based chemotherapy in circulating tumor cells (CTCs) of patients (pts) with metastatic castration resistant prostate cancer (mCRPC). J Clin Oncol, 2015. 33(suppl 7, abstr 147).

[48] Khotskaya, Y.B., et al., Syndecan-1 Is Required for Robust Growth, Vascularization, and Metastasis of Myeloma Tumors in Vivo. Journal of Biological Chemistry, 2009. 284(38): p. 26085-26095.

[49] Kumar, S., T. Kimlinger, and W. Morice, Immunophenotyping in multiple myeloma and related plasma cell disorders. Best practice & research. Clinical haematology, 2010. 23(3): p. 433-451.

[50] Li, C., et al., Development of a robust flow cytometry-based pharmacodynamic assay to detect phospho-protein signals for phosphatidylinositol 3-kinase inhibitors in multiple myeloma. J Transl Med, 2013. 11: p. 76.

[51] San Miguel, J.F., et al., Conventional diagnostics in multiple myeloma. Eur J Cancer, 2006. 42(11): p. 1510-9.