Clinical significance and molecular characteristics of circulating plasma cells in multiple myeloma

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Research Article

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Abstract

Multiple myeloma (MM) is one of the most common hematological malignancies and remains incurable to date. Clonal plasma cells can be detected in the peripheral blood of most MM patients, and previous studies have shown an association between the quantification of circulating plasma cells (CPC) and prognosis. However, the significance of CPC for response assessment and prognosis prediction as well as the mechanism underlying CPC formation in Chinese population has not been fully elucidated. In this investigation, we used multi-parameter flow cytometry (MFC) for CPC quantification and next-generation sequencing (NGS) technology for mutational landscape mapping to identify the correlation of CPC level with clinical characteristics and the mutations. As a result, we demonstrated that CPC quantification could effectively mirror the tumor load, and CPC≥0.105% at diagnosis or detectable CPC after therapy indicates poor treatment response and adverse outcome, and it is verified that the introduction of CPC level into the R-ISS system enables a more accurate risk stratification. We also noticed an elevated percentage of light-chain MM in patients with high CPC, this is the first time, to the best of our knowledge, to report the subtype pattern regarding CPC. Mutational landscape revealed that TP53 mutations and pathways involving chromatin or chromosome regulation and adhesion may be potential mechanisms accounting for CPC formation. Accordingly, the detection of CPC by MFC may provide a less-invasive, convenient and, more importantly, reliable approach for risk stratification and disease monitoring in Chinese population.

Introduction

Multiple myeloma (MM), one of the most common hematological malignancies, is caused and characterized by clonal proliferation of malignant plasma cells\textsuperscript{[1]}. Clonal plasma cells are predominantly distributed in the bone marrow (BM), mainly due to the adhesion of these cells to the bone marrow microenvironment\textsuperscript{[2, 3]}. Along with the technical improvement, it has become clear that clonal plasma cells not merely reside in the bone marrow but can passage into the circulation and then home to intramedullary or distant tissues, a process that allows clonal plasma cells to circulate in the peripheral blood and these cells are named circulating plasma cells (CPC)\textsuperscript{[3]}.

With the advancement of MM therapy, monoclonal antibodies targeting CD38 and SLAMF7 are widely used, yet these antibodies hamper the accuracy of serological detection, referred to the tests of M-protein\textsuperscript{[4]}. In addition, M-protein assays failed to mirror the response and progression of the disease for patients with oligo- or non-secretory MM. The rise of multi-parameter flow cytometry (MFC) technology has brought reliable methods for diagnosis, prognostic markers examination, minimal residual disease (MRD) monitoring, and, at the same time, detection of infinitesimal CPC in the peripheral blood of MM patients\textsuperscript{[5, 6]}. Additionally, the application of MFC in CPC detection could eliminate the need for repeated invasive bone marrow biopsies and, importantly, possess higher sensitivity than conventional slide-based methods\textsuperscript{[7]}. One of our previous studies identified CPC≥0.105% to be an independent risk factor for poor prognosis in MM\textsuperscript{[8]}, suggesting the predictive value of CPC, which was in line with the findings of some...
other groups\textsuperscript{[9–11]}. Nevertheless, most investigations regarding CPC to date were conducted in Caucasian populations\textsuperscript{[12–15]}. Given the racial and therapeutic heterogeneity, the prognostic implication of CPC, its kinetics, in particular, has not been fully elucidated in Chinese population. Hence, more extensive clinical studies are warranted to verify the relevance of CPC to the clinical features, efficacy and prognosis in MM patients.

The mutational landscape of tumor cells is currently one of the most intensively studied fields of research in MM. It is reported that an average of 1.6 mutations were detected per Mb in MM\textsuperscript{[16]}. High-throughput DNA sequencing, also known as next-generation sequencing (NGS), has been widely used in MM for the past few years, and mutations like KRAS, NRAS, TP53, FAM46C and DIS3 were observed recurrently. Yet, mutational landscape was heterogeneous in different studies\textsuperscript{[17–19]}, and the relationship between mutations and CPC levels in Chinese population has not been well explored. Previous studies identified high concordance in gene expression of BMPC and CPC by NGS technology\textsuperscript{[12, 14]}, suggesting that CPC are mainly derived from MM cells in the BM. In this work, NGS was applied to molecularly characterize the BM myeloma cells to determine the relationship between gene mutations and CPC levels and thus explore the mutations and pathways that regulate the egress of myeloma cells from the BM into the peripheral blood.

Here, we used MFC for CPC quantification and NGS technology for mutational landscape mapping to identify the correlation between CPC level and clinical characteristics as well as the mutations. This study aimed to validate the implication of CPC in efficacy evaluation and prognosis in Chinese population, and to further elucidate the relationship between genetic alteration and CPC quantification. This is one of the most extensive studies to date focusing on CPC in Chinese population, and, to the best of our knowledge, is the first to explore the molecular mechanisms affecting CPC levels in Chinese population.

Materials And Methods

Study population and ethics approval

A total of 301 patients newly diagnosed with MM from October 2015 to May 2021 at the First Affiliated Hospital of Nanjing Medical University were enrolled in the study. The diagnosis and treatment response assessment was conducted according to the revised International Myeloma Working Group (IMWG) criteria\textsuperscript{[20, 21]}. Patients with plasma cell leukemia were excluded. Clinical data were extracted from medical records. All patients were followed up until October 2021, with a median follow-up time of 29 months (range, 1–70 months). This retrospective study was conducted in accordance with the Declaration of Helsinki, and was approved by the institutional review boards of the First Affiliated Hospital of Nanjing Medical University Ethics Committee (No.2020-SR-589). Informed consents were obtained from all patients before enrollment into the study.

Multi-parameter flow cytometry
Mononuclear cells were isolated from blood or bone marrow samples by Ficoll gradient and were detected by 8-color flow cytometry to quantify CPC and BMPC. Details of the flow cytometry technique for CPC and BMPC detection have been described in our previous study\[^{8,22}\].

**Next-generation sequencing and analysis of mutations**

Bone marrow aspirates were obtained at diagnosis and were sorted by anti-CD138 magnetic microbeads (Miltenyi Biotec, Germany) for the purification of tumor cells. Genomic DNA (gDNA) was extracted from tumor cells using DNA extraction kits (QIAGEN, Germany) according to the manufacturer's instructions. The gDNA was fragmented by Enzyme Plus Library Prep Kit (iGenetech, China), and the libraries were prepared using probes and TargetSeq One Kit (iGenetech, China).

After the quality control, libraries were subjected to NovaSeq 6000 platform (Illumina, USA) for sequencing, targeting 387 genes involving pan-cancer driver genes and MM-related genes (genes included in this panel are listed in Supplementary Material), with an average sequencing depth of 1,000×. Reads were aligned, and data were subsequently converted and filtered. Mutations were annotated, analyzed and visualized by R package “maftools”\[^{23}\].

**Gene enrichment analysis**

Gene Ontology (GO) enrichment comprising “Biological Process (BP)”, “Cellular Component (CC)”, and “Molecular Function (MF)”, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment was applied to uncover the biological functions and signaling pathways of gene clusters. Gene enrichment and visualization were conducted using “clusterProfiler” package\[^{24}\] on R software (Version 4.1.1, R Foundation for Statistical Computing). GO terms and KEGG pathways with \(P < 0.05\) based on the cumulative hypergeometric distribution test were significantly enriched.

**Statistical analysis**

Mann-Whitney \(U\) tests or Kruskal-Wallis tests were used to determine the statistical significance between continuous variables, and \(\chi^2\) tests or Fisher’s exact tests were used for comparing categorical data. Pearson’s \(r\) was used to estimate the linear correlation between the CPC and bone marrow plasma cells (BMPC). Progression-free survival (PFS) and overall survival (OS) were plotted as Kaplan-Meier curves and were compared by log-rank test by R package “survival” and “survminer”. Data were analyzed by SPSS v23.0 (IBM Corp.) and R software. All tests were two-sided, and a \(P\) value < 0.05 was considered statistically significant.

**Results**

**Relationship between pre-therapeutic CPC level and clinical characteristics**
A total of 301 patients with NDMM were enrolled in this study, including 178 males and 123 females, with a median age of 56 years (range, 30–84 years). All patients underwent CPC assessment by MFC before treatment, and CPC was detectable in 170 patients (56.5%), with a median CPC of 0.02% for all patients (range, 0 ~ 19.86%).

To investigate the relationship between CPC and clinical features, patients were divided into CPC-low and CPC-high groups according to their CPC levels by the cut-off value of 0.105% determined in our previous study\(^\text{[8]}\). As shown in Table 1, patients in the CPC-high group had lower levels of hemoglobin \((P = 0.0025)\), as well as higher levels of lactate dehydrogenase (LDH) \((P = 0.0028)\) and \(\beta\)-2-microglobulin \((P < 0.0005)\).

There were significant differences in stages between the two groups according to either ISS or R-ISS staging system \((P = 0.0011\) and \(= 0.0023\), respectively). Significantly more patients in the CPC-high group presented stage III disease, according to both the above-mentioned staging systems, as compared to the CPC-low group \((P = 0.0007\) and \(= 0.0003\), respectively). It is of interest to note that the types of secreted monoclonal protein varied between groups \((P = 0.0084)\), with a higher proportion of IgA subtype in the CPC-low group \((P = 0.0153)\) and a higher proportion of light-chain subtype in the CPC-high group \((P = 0.0026)\). Although higher CPC portends the decreased adhesion of myeloma cells to the BM microenvironment, a process reported to be an underlying pathophysiological mechanism of extramedullary disease (EMD), the incidence of EMD was not statistically different between groups.
Table 1
Clinical characteristics of patients according to quantification of CPC.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>CPC-low (n = 210)</th>
<th>CPC-high (n = 91)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, median (IQR)</td>
<td>63 (54.3–68)</td>
<td>63 (56–67)</td>
<td>0.672</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td>0.762</td>
</tr>
<tr>
<td>Male</td>
<td>123 (58.6)</td>
<td>55 (60.4)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>87 (41.4)</td>
<td>36 (39.6)</td>
<td></td>
</tr>
<tr>
<td>Subtype, n (%)</td>
<td></td>
<td></td>
<td>0.0084</td>
</tr>
<tr>
<td>IgG</td>
<td>101 (48.1)</td>
<td>40 (44.0)</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>57 (27.1)</td>
<td>13 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Light chain</td>
<td>40 (19.1)</td>
<td>32 (35.2)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>12 (5.7)</td>
<td>6 (6.59)</td>
<td></td>
</tr>
<tr>
<td>D-S, n (%)</td>
<td></td>
<td></td>
<td>0.2392</td>
</tr>
<tr>
<td>□</td>
<td>12 (5.7)</td>
<td>2 (2.2)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>34 (16.2)</td>
<td>11 (12.1)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>164 (78.1)</td>
<td>78 (85.7)</td>
<td></td>
</tr>
<tr>
<td>ISS, n (%)</td>
<td></td>
<td></td>
<td>0.0011</td>
</tr>
<tr>
<td>□</td>
<td>38 (18.1)</td>
<td>8 (8.8)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>86 (41.0)</td>
<td>25 (27.5)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>86 (41.0)</td>
<td>58 (63.7)</td>
<td></td>
</tr>
<tr>
<td>R-ISS, n (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.0023</td>
</tr>
<tr>
<td>□</td>
<td>24 (12.7%)</td>
<td>6 (6.74%)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>135 (71.43%)</td>
<td>53 (59.55%)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>30 (15.87%)</td>
<td>30 (33.71%)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, g/L, median (IQR)</td>
<td>93 (73-111.8)</td>
<td>81 (68–98)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Albumin, g/L, median (IQR)</td>
<td>33.2 (28.4–38.4)</td>
<td>32.5 (26.3–37.7)</td>
<td>0.2782</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assessed on available data. Bold values indicate statistical significance (P< 0.05)

CPC, circulating plasma cells; IQR, interquartile range; D-S, Durie-Salmon staging system; ISS, International Staging System; R-ISS, revised International Staging System; BMPC, bone marrow plasma cells; MFC, multi-parameter flow cytometry; LDH, lactate dehydrogenase; EMD, extramedullary disease.
### Clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>CPC-low (n = 210)</th>
<th>CPC-high (n = 91)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH, U/L, median (IQR)</td>
<td>166 (139–211)</td>
<td>192 (151–252)</td>
<td>0.0028</td>
</tr>
<tr>
<td>Creatinine, µmol/L, median (IQR)</td>
<td>78.2 (58.9–113.8)</td>
<td>81.8 (65.9–188.8)</td>
<td>0.0759</td>
</tr>
<tr>
<td>Corrected calcium, mmol/L, median (IQR)</td>
<td>2.4 (2.3–2.5)</td>
<td>2.4 (2.3–2.6)</td>
<td>0.2501</td>
</tr>
<tr>
<td>β2-microglobulin, mg/L, median (IQR)</td>
<td>4.2 (3-7.9)</td>
<td>6.8 (3.8–13.2)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>BMPC (morphology), %, median (IQR)</td>
<td>17 (10.4–30.7)</td>
<td>26.8 (13.6–51.8)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>BMPC (MFC), %, median (IQR)</td>
<td>4.2 (1.2–9.4)</td>
<td>14.6 (6.5–25.1)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>EMD, n (%)</td>
<td>52 (24.8)</td>
<td>17 (18.7)</td>
<td>0.2491</td>
</tr>
<tr>
<td>Chomosomal abnormality, n (%)a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gain(1q)</td>
<td>89 (49.4)</td>
<td>49 (55.7)</td>
<td>0.3373</td>
</tr>
<tr>
<td>del(17p)</td>
<td>15 (8.3)</td>
<td>18 (20.5)</td>
<td>0.0046</td>
</tr>
<tr>
<td>t(4;14)</td>
<td>29 (16.1)</td>
<td>18 (20.5)</td>
<td>0.3799</td>
</tr>
<tr>
<td>t(11;14)</td>
<td>13 (7.2)</td>
<td>7 (8.0)</td>
<td>0.8304</td>
</tr>
<tr>
<td>t(14;16)</td>
<td>1 (0.6)</td>
<td>2 (2.3)</td>
<td>0.2518</td>
</tr>
<tr>
<td>Responsea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR or better</td>
<td>179 (91.8)</td>
<td>59 (74.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>VGPR or better</td>
<td>162 (83.1)</td>
<td>44 (55.7)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>CR or better</td>
<td>112 (57.4)</td>
<td>30 (38.0)</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

a Assessed on available data. Bold values indicate statistical significance (P<0.05)

CPC, circulating plasma cells; IQR, interquartile range; D-S, Durie-Salmon staging system; ISS, International Staging System; R-ISS, revised International Staging System; BMPC, bone marrow plasma cells; MFC, multi-parameter flow cytometry; LDH, lactate dehydrogenase; EMD, extramedullary disease.

Both morphology and flow cytometry showed a higher proportion of plasma cells in the bone marrow in the CPC-high group as compared to the CPC-low group (P<0.0005 for both) (Table 1). We computed a Pearson correlation coefficient for CPC and BMPC at initial diagnosis, and the Pearson's r estimates demonstrated a significant positive correlation between BMPC and CPC (r = 0.23, P<0.0001) (Fig. 1A).

Cytoplasmic light chain immunofluorescence with fluorescence in situ hybridization (clg-FISH) was performed in 268 patients, and patients in the CPC-high group showed a statistically higher incidence of del(17p) (P = 0.0046), whereas no differences were found for other types of chromosomal abnormality (Table 1). According to the latest mSMART[25], t(4;14), t(14;16), t(14;20), del(17p) and gain(1q) are defined...
as high-risk chromosomal abnormality (HRCA). We discovered a statistical difference in HRCA counts between the two groups, with 38.3%, 50% and 11.7% of patients in the CPC-low group that harbored 0, 1 or ≥2 HRCA(s), while it was respectively 28.4%, 47.7% and 23.9% in the CPC-high group ($P = 0.0256$) (Fig. 1B).

**Predictive value of pre-therapeutic CPC level for treatment response and prognosis**

Treatment responses were assessed after induction therapy, and clinical data of 274 patients were available for response evaluation. Response rates of $\geq$ partial response (PR), $\geq$ very good partial response (VGPR) and $\geq$ complete response (CR) in CPC-high patients were significantly lower as compared with those in the CPC-low group ($P < 0.05$) (Table 1, Fig. 1C). When comparing the CPC levels according to the treatment response ($\leq$PR, =VGPR, $\geq$CR), patients with the responses of $\leq$PR had higher CPC levels compared to those who achieved VGPR or $\geq$CR ($P = 0.0007$ and $< 0.0001$, respectively), while no statistical differences were found between patients with responses of VGPR and $\geq$CR ($P = 0.8445$) (Fig. 1D).

Survival analyses were subsequently performed to validate the prognostic implications of pre-therapeutic CPC in NDMM. The median PFS of patients in the CPC-low and CPC-high groups were 36 months and 19 months, and the median OS of the two groups was not reached and 35 months, respectively. A high level of CPC retained adverse effects on PFS and OS for patients with NDMM ($P < 0.0001$ for both PFS and OS).

**Predictive value of post-therapeutic CPC level for treatment response and prognosis**

Of the 144 patients with detectable CPC at diagnosis and with clinical data available for treatment response assessment, CPC turned negative in 124 (86.1%) patients during induction therapy and remained positive in the rest 20 (13.9%) patients, for whom the median CPC was 0.195% (range, 0.002%~3.25%).

To investigate the implication of post-therapeutic CPC for efficacy evaluation and prognosis, patients were grouped into CPC-negative and CPC-positive subgroups, with the former referring to patients presented with undetectable CPC after induction therapy and the latter denoting the ones with remaining CPC when induction therapy finished. The response rates of $\leq$PR, =VGPR, and $\geq$CR were 24.2%, 23.4% and 52.4% in the CPC-negative group, and were 80%, 10% and 10% in the CPC-positive group (Fig. 2A), illustrating a significantly worse efficacy conveyed by detectable CPC after induction therapy ($P < 0.0001$). Moreover, residual CPC after induction therapy anticipated poor outcome in MM, with a median PFS of 24 months vs 8.75 months ($P < 0.0001$) and median OS of not reached vs 11.5 months ($P < 0.0001$) for the CPC-negative and CPC-positive subgroups, respectively (Fig. 2B-C).
Implication of CPC for enhancing the accuracy of prognostic discrimination of the R-ISS staging system

The R-ISS is one of the most recognized and widely used staging systems for prognostic categorizing in MM. For the entire cohort, the median PFS for stage I, II or III based on the R-ISS staging system were 41 months vs 25 months vs 16 months ($P = 0.0037$ for all, $P = 0.0408$ between stage I and II, $P = 0.0913$ between stage II and III, $P = 0.0046$ between stage III and I). The median OS for the three stages was not reached vs not reached vs 42 months ($P < 0.0001$ for all, $P = 0.0935$ between stage I and II, $P = 0.0003$ between stage II and III, $P = 0.0013$ between stage III and I) (Fig. 2D-E). As we can see, in this study, the R-ISS staging system could predict the outcomes in general, yet failed to distinguish the PFS between stage I and II disease accurately, nor the OS between stage II and III disease. Since up to 67.6% of patients in our study were classified as R-ISS stage II, a more accurate prognostic stratification for these patients is warranted.

To evaluate the significance of CPC as a further biomarker for better prognosis prediction by the R-ISS system, we introduced the CPC level into the R-ISS staging system. Patients with R-ISS stage II disease were further grouped by CPC quantification using the aforementioned cut-off value, with those who had CPC $< 0.105\%$ at diagnosis defined as Stage IIa (R-ISS II + CPC-low) and the rest as Stage IIb (R-ISS II + CPC-high). Patients with R-ISS stage III and II disease were defined as Stage III and Stage II, respectively.

The median PFS for patients based on the new staging system was as follows: Stage II: 41 months; Stage IIa: 30 months; Stage IIb: 19 months and Stage III: 16 months ($P < 0.0001$ for all, $P = 0.2466$ between Stage II and IIa, $P < 0.0001$ between Stage IIa and IIb, $P = 0.4001$ between Stage IIb and III). The median OS for patients based on the new staging system was as follows: Stage II: not reached; Stage IIa: not reached; Stage IIb: 38 months and Stage III: 42 months ($P < 0.0001$ for all, $P = 0.3845$ between Stage II and IIa, $P = 0.0002$ between Stage IIa and IIb, $P = 0.3805$ between Stage IIb and III) (Fig. 2F-G). Accordingly, the introduction of CPC levels in the R-ISS staging system demonstrate more robust discrimination of prognosis for patients with NDMM.

NGS

NGS was performed in 143 patients, and mutations were detected in all patients, involving 337 genes. The median number of mutations was 17 (range, 1–34), and the median number of genes involved was 15 (range, 1–28), with the most frequently mutated genes being KRAS (29.4%), NRAS (23.1%), IGLL5 (19.6%), SYNE1 (18.9%) and AHNAK2 (17.5%). There was no statistical difference in the number of mutations between CPC-low and CPC-high patients, with a median of 17 (range, 1–34) in the former group and 16 (range, 9–30) in the latter one (Fig. 3A). We compared the CPC levels of patients with specific mutations to their wild-type counterparts and found that patients who bore mutations like TP53, BRAF, DNMT3A and TENT5C tended to have significantly higher CPC levels (Fig. 3B).
For the exploration of underlying biological functions or pathways involved in the CPC elevation, we eliminated mutations that were only presented in ≤ 2 patients and ranked the remaining mutations by the median CPC level in patients with corresponding mutations. The top 50 genes with the highest median CPC levels are shown in Fig. 4A. GO enrichment analysis, including BP, CC and MF, was performed, and the top terms enriched are presented in dot plots in Fig. 4B. Several GO terms regarding the modification and regulation of chromatin, which contains DNA, RNA, histones, etc., were significantly enriched in BP and CC categories. We also noticed that pathways associated with adhesion like focal adhesion and cell-substrate junction were statistically enriched in the CC category. The enriched MF terms were mainly involved in the catalytic activity of energy metabolism. The top 10 terms of GO enrichment and relevant genes, like TP53, ATM, EGFR, and BRCA1, were presented as a pathway-gene network in Fig. 3C. The enriched KEGG pathways were mainly of tumor signaling pathways (Supplementary Figure S1).

Discussion

The past decade has witnessed an ever-increasing focus on CPC, and the extensive application of MFC in CPC detection allows easy and non-invasive examination of tumor cells\cite{7,9,26}. According to our previous investigation, in which 108 patients were enrolled, CPC≥ 0.105% has been proved to be an independent risk factor for adverse prognosis in MM and was correlated with higher β2-microglobulin, LDH and BMPC (by morphological method)\cite{8}. To investigate the predictive implication of CPC on tumor burden and to analyze the relationship between CPC and treatment response and prognosis, we conducted a more extensive clinical study involving 301 patients, one of the most extensive clinical studies to date. As a result, we found that in addition to the β2-microglobulin and LDH as mentioned above, higher CPC was also associated with lower hemoglobin. Since hemoglobin, β2-microglobulin and LDH are all considered reliable biomarkers of tumor load in MM, thus suggesting that the release of plasma cells to peripheral circulation may be a result of a high disease burden. The percentage of atypical plasma cells in bone marrow is one of the most established parameters for the diagnosis and monitoring of MM, which involves invasive and painful procedures. Previous studies have analyzed the quantification of MM cells in paired samples of BM and peripheral blood. Korthals et al.\cite{27} reported that the MRD level of peripheral blood was 40-fold lower than that in BM aspirates when detected by IgH-PCR. A non-linear relationship was found between absolute CPC count and BMPC percentage in another study using MFC\cite{28}. In the present study, BMPC was detected by morphology and MFC, which confirmed that higher CPC was associated with elevated BMPC. We observed a positive linear correlation between BMPC and CPC (both by MFC), thereby verifying the value of CPC in predicting plasma cell quantification in BM, as such the detection of CPC could be used to monitor tumors without the need for repeated bone marrow biopsy.

It has been addressed in some literature that the levels of CPC were correlated with the prognosis in patients with plasma cell neoplasms, comprising newly diagnosed MM (NDMM)\cite{9}, relapsed or refractory MM (RRMM)\cite{10,11}, and pre-malignant neoplasms including monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM)\cite{26}. In this work, the cut-off value we worked out previously was applied to explore the correlation of CPC and their dynamics with efficacy and
prognosis so as to validate the feasibility and accuracy of CPC in clinical application. As a result, we found that high levels of CPC ($\geq 0.105\%$) before treatment predict poor response to therapy as well as adverse outcome in NDMM. It is noteworthy that the presence of post-therapeutic CPC, even rarely, indicated a low response rate to treatment, with up to 80% of patients having a response of PR or worse. In contrast, for patients with detectable CPC at diagnosis and negative CPC after treatment, 75.8% achieved remission of VGPR or better. Similarly, the remaining CPC portended a dismal prognosis with a median OS of only 11.5 months. Hence, the kinetics of CPC has the potential to be a significant biomarker for monitoring the progression and remission and for predicting the outcome of MM.

MM is a heterogeneous disease with diversified clinical features and outcomes$^{[29]}$. The revised International Staging System (R-ISS) is one of the most widely recognized system for prognostic categorizing$^{[30]}$, but up to half of the patients are classified as stage I disease, and the prognosis of these patients varies widely. A study from Mayo Clinic showed a similar prognosis between patients with R-ISS stage I disease accompanied by $\geq 5$ CPCs/$\mu$L and those with R-ISS stage II disease$^{[31]}$. When we introduced the level of CPC, at the aforementioned threshold, into the existing R-ISS system, we were excited to find that it could lead to more robust outcome stratification for a large number of patients with R-ISS stage II disease, thus reflecting the promising role of CPC in aiding the R-ISS system for more precisely prognostic anticipation.

Interestingly, we discovered a higher proportion of light-chain subtype and a lower percentage of IgA subtype in CPC-high patients than the CPC-low ones, which, to the best of our knowledge, is the first report of the differences in the pattern of subtype regarding CPC to date. This phenomenon was highly similar to that observed in plasma cell leukemia (PCL), that is, patients with PCL had the same tendency of subtype in comparison to MM patients without PCL$^{[32, 33]}$. Since PCL may represent the extreme situation of CPC elevation, the current understanding of PCL may have implications for CPC. PCL is defined by the accumulation of CPC that occupy up to 20% of the peripheral blood, or the absolute count of it reaches $2 \times 10^9$/L. PCL represents one of the most aggressive forms of MM and typically denoted a dismal outcome even in the era of novel agents$^{[32]}$. Mechanisms underlying the development of PCL remains unclear, while abundant literature have addressed the altered biology involving molecular and immunological phenotype of tumor cell in this disease$^{[34]}$. It is generally accepted that malignant plasma cells in PCL express CD19, CD20 and CD45 more frequently than those in MM$^{[35, 36]}$. Similarly, Sanoja-Flores et al.$^{[28]}$ displayed an upward expression of the biomarkers mentioned above in CPC than their BM counterparts. Therefore, we speculate that the similar phenotypes exhibited by CPC-high MM and PCL may partly explain their congruent subtype pattern. However, little is known about the mechanism by which the proportion of light-chain subtype is higher in PCL than that in MM. It is widely known that a complete immunoglobulin is composed of heavy and light chains, where the heavy chain is encoded by the IGH gene located on chromosome 14q32, and the two light chains, $\kappa$ and $\lambda$, are encoded on chromosomes 2p11 and 22q11, respectively$^{[37]}$. IgH translocation, considered the primary genetic event in MM, is currently thought to occur mainly during the class-switch recombination (CSR) along the B-cell development or even earlier$^{[38]}$. The incidence of IgH translocation is reported to be approximately 45% in
MM \footnote{25} and up to more than 80\% in PCL\footnote{33}. Since IgH translocation could yield the delete or silence of the IGHV gene necessary for IgH assembly\footnote{39}, we hypothesized, for the first time, that a higher proportion of IgH translocations in PCL may be a potential factor leading to the impairment of heavy chain synthesis, and hence, the presentation of more patients with light-chain subtype. Since only fusion probes for IgH translocations were used to observe the corresponding translocation in a subgroup of patients in the present study, we included only the incidence of t(4; 14), t(11; 14) and t(14;16) for analysis. Therefore, the percentage of IgH translocations we presented was lower than the actual incidence. Nevertheless, as expected, we still observed a higher rate of IgH translocation in CPC-high MM than the CPC-low MM (30.8\% vs 23.9\%). In brief, we speculate that the increased frequency of IgH translocations in CPC-high MM, as well as in PCL, result in a higher proportion of light-chain subtype, a speculation that needs to be confirmed by further studies.

Regarding the mechanisms by which CPC affects prognosis, we noticed a higher frequency of del(17p) in CPC-high MM than CPC-low MM in our cohort (20.5\% vs 8.3\%). Of note, Gundesen et al.\footnote{32} demonstrated an elevated del (17p) in PCL than that in MM patients (40\% vs 11\%). In addition, the phenotypes of CPC-high MM and PCL we mentioned above may represent, according to the phenotypic transformation during B-cell development\footnote{40}, a poorly differentiated cell population that has been shown to suggest inferior survival in MM\footnote{41}. These phenomena indicated that patients who carried CPC≥0.105\%, a threshold that even represents a minimal amount of CPC, have certain similarities with PCL patients, which partly explains the poor response and prognosis of these patients. A Chinese study by An et al.\footnote{42} also displayed that MM patients with a small amount of CPC had similar prognosis with PCL patients. Accordingly, the presence of even minimal CPC in Chinese MM patients represents a highly aggressive disease.

To further elucidate the association of CPC level with molecular features, NGS technology was employed to characterize the tumor cells in this study. To our knowledge, this is the first time that NGS has been used to molecularly characterize the tumor cells in a study of CPC in Chinese population. As a result, while more aggressive myeloma is thought to have a high mutational burden\footnote{43}, we found no significant differences in mutational burden between the CPC-low and CPC-high groups. When CPC levels were compared between patients who carried WT and mutant genes, patients with mutations involving TP53, BRAF, DNMT3A, APOBEC3C, ASCC3 and TENT5C, etc., tended to have significantly higher CPC levels. We have noticed that mutation of TP53, which is one of the most studied genes in MM, was related to a higher number of CPC, in line with our observation of a superior frequency of del (17p) in CPC-high patients by FISH assay. It has been reported that TP53 mutation is associated with the migration of MM cells from the bone marrow into peripheral blood, thereby facilitating the presentation of PCL\footnote{44}. Lee et al.\footnote{45} reported that mutation of TP53 was more frequent in PCL than in MM. These findings emphasize the role of TP53 in prompting the MM cells to migrate into the blood. To unveil the underlying pathways involved in the egress of MM cells towards the circulation, we analyzed the CPC levels corresponding to each mutation and performed enrichment for the mutated genes with the highest levels of CPC. As a result, pathways regarding the regulation of chromosome or chromatin as well as adhesion and junction
were significantly enriched. It has been reported that the CPC represent a subset of BM myeloma cells that driven by downregulated integrins and adhesion molecules to become a BM microenvironment independent subpopulation\[12,46\]; our results validate the role of adhesion and junction in CPC formation at the genetic level. The regulation of chromatin or chromosome involves plenty of physiological processes in cells, of which chromosomal instability is most extensively studied in MM. Chromosomal instability can result in copy number and structural changes of chromosomes, and act as an critical element in the development and progression of this disease\[43,47\], whereas the relationship between CPC and chromosomal instability as well as other procedures involved in chromatin or chromosome regulation is still poorly understood. Our findings proposed a suggested mechanism of CPC formation, and additional efforts are warranted to verify this hypothesis.

There are still some limitations in this study. The tumor cells subjected to NGS were sorted by the positive expression of CD138. Although CD138 is well recognized to be expressed on MM cells\[48\], it has been recently reported that there are still very few MM clones that present with CD138-negative and it is suggested that these CD138-negative cells may represent a group of cancer stem cells with a higher migration capacity\[49,50\]. Therefore, the application of NGS in combination with highly purified fluorescence-activated cell sorting (FACS) based on the specific aberrant phenotypes of patients or single-cell RNA sequencing may provide a more accurate method to characterize MM cells. In addition, limited to current technologies, the sensitivity of MFC is insufficient to detect CPC in a small half of the patients; hence, the detection of BM aspirates is still the golden standard for MRD monitoring, especially for patients with preferable responses. NGS technology has been proved to be extremely sensitive in CPC detecting\[14\], but its prohibitive cost makes it difficult to use for frequent disease assessment. Therefore, more sensitive flow cytometry, as well as less expensive sequencing techniques, are still warranted.

To conclude, we adopted the emerging technologies, comprising MFC and NGS, to analyze the relationship between CPC and the clinical characteristics as well gene mutations, in which the molecular characterization related to CPC levels has not been reported before in Chinese population. We demonstrated that levels of CPC can effectively reflect the tumor load, and CPC≥0.105% at diagnosis or detectable CPC after induction therapy indicate poor treatment response and adverse outcome. The introduction of the CPC level into the existing R-ISS system is proved to enable a more accurate risk stratification. We present the phenomenon and mechanic speculation, for the first time to the best of our knowledge, of the raised percentage of light-chain subtype in patients with higher CPC. Analysis of mutational landscape displayed the underlying mechanism in the CPC formation, potentially mediated by TP53 mutations and pathways involving chromatin or chromosome regulation and adhesion pathways. Overall, while bone marrow testing remains the gold standard in MRD detection for the majority of patients, the detection of CPC by MFC may provide a less-invasive, convenient and, more importantly, reliable approach for risk stratification and disease monitoring. Large-scale clinical research and further in-depth molecular mechanism exploration are required for a better understanding of CPC and its underlying mechanism, thus, shed light on promising therapies to overcome the adverse prognosis brought about by CPC.
Declarations

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Author contributions

Conception and design: LC, YX. Collection and assembly of data: YX, MX. Data analysis and interpretation: YX. Provision of study materials or patients: LC, RZ, YJ, QS. Supervision: LC. Administrative support: LC, JL, YW. Manuscript writing: YX drafted the manuscript, LC revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Availability of data and materials

The datasets and materials analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Ethical approval was obtained from the institutional review boards of the First Affiliated Hospital of Nanjing Medical University Ethics Committee. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008. Informed consents were obtained from all patients.

Consent for publication

All authors have read and approved the manuscript for publication.

Competing interests

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Figures

Figure 1

Association of CPC with clinical features, efficacy and prognosis at initial diagnosis.

(A) The Pearson correlation between BMPC and CPC of patients with NDMM. (B) Proportion of patients with different HRCA counts in the CPC-low and -high group. (C) Response rates (£PR, =VGPR or ¤CR) of patients after induction therapy in the CPC-low and -high group. (D) CPC levels in patients with different states of remission. (E, F) Kaplan-Meier survival curves for PFS (E) and OS (F) of patients with different levels of CPC.

CPC, circulating plasma cells; BMPC, bone marrow plasma cells; HRCA, high-risk chromosomal abnormality; PR, partial response; VGPR, very good partial response; CR, complete response; PFS, progression free survival; OS, overall survival.
Figure 2

Association of post-treatment CPC with efficacy and prognosis, and the risk stratification of R-ISS system in combination with CPC level.

(A) Response rates (PR, VGPR or CR) of patients with or without remaining CPC after induction therapy whose CPC were detectable at diagnosis. (B, C) Kaplan-Meier survival curves for PFS (B) and OS (C) of patients with or without remaining CPC after induction therapy. (D, E) Kaplan-Meier survival curves for PFS (D) and OS (E) of patients stratified by R-ISS staging system. (F, G) Kaplan-Meier survival curves for PFS (F) and OS (G) of patients stratified by R-ISS staging system combined with CPC levels. Patients with R-ISS stage disease were further grouped by CPC levels, with those who had CPC < 0.105% at diagnosis as R-ISS + CPC-low and the rest as R-ISS + CPC-high.

CPC, circulating plasma cells; PR, partial response; VGPR, very good partial response; CR, complete response; R-ISS, revised International Staging System; PFS, progression free survival; OS, overall survival.
Figure 3

Association of CPC level with mutational burden and gene mutations.

(A) Mutational load in patients with different levels of CPC. (B) Comparison of CPC levels between patients with specific mutations and their wild-type counterparts.

CPC, circulating plasma cells; WT, wild type.
Figure 4

Genes and enriched pathways associated with higher CPC levels.

(A) The top 50 genes with the highest CPC levels when eliminating mutations presented in £ 2 patients and ranked the remaining mutations by the median CPC level in patients with corresponding mutations. (B) Dot plot reflecting top terms in GO enrichment analysis regarding BP, CC and MF. (C) Cnetplot showing the pathway-gene network of the top 10 terms of GO enrichment and relevant genes.

CPC, circulating plasma cells; BP, Biological Process; CC, Cellular Component; MF, Molecular Function

Supplementary Files

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- Supplementary1.docx
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