Am J Blood Res 2020;10(3):26-45
www.AJBlood.us /ISSN:2160-1992/AJBR0115481

Review Article

Cell-free DNA for genomic profiling and minimal residual disease monitoring in Myeloma- are we there yet?

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Received April 26, 2020; Accepted June 11, 2020; Epub June 15, 2020; Published June 30, 2020

Abstract: Objective: Multiple myeloma (MM), a plasma cell neoplasm, afflicts elder individuals accounting for 10% of hematologic malignancies. The MM plasma cells largely reside within the bone marrow niche and are accessible through an invasive bone marrow biopsy, which is challenging during serial monitoring of patients. In this setting, cell free DNA (cfDNA) may have a role to ascertain the molecular aberrations at diagnosis and in assessment of residual disease during therapy. The aim of this review was to explore the utility and current status of cfDNA in MM. Method: PubMed was searched with terms including cell-free DNA, circulating-tumor DNA, Multiple Myeloma, diagnosis, genomic profiling, Minimal Residual Disease individually or in combination to shortlist the relevant studies. Result: cfDNA serves as a non-invasive source of tumor-specific molecular biomarker, ctDNA that has immense potential in facilitating management of cancer patients. The mutation detection platforms for ctDNA include hybrid capture and ultra-deep sequencing. Hybrid capture allows full length gene sequencing for mutation and CNV detection. The disease progression can be monitored by profiling prognostic somatic copy number alterations by ultra-low pass whole genome sequencing of ctDNA cost-effectively. Evolution of both the laboratory protocols and bioinformatics tools may further improve the sensitivity of ctDNA detection for better disease management. Only a limited number of studies were available in MM exploring the potential utility of cfDNA. Conclusion: In this review, we discuss the nuances and challenges associated with molecular evaluation of cfDNA and its potential role in diagnosis and monitoring of treatment response in MM.

Keywords: Cell free DNA, cfDNA, circulating tumor DNA, ctDNA, multiple myeloma, genomic profiling, deep sequencing, minimal residual disease, MRD

Introduction

Pathobiology of multiple myeloma (MM) has come a long way since its advent in terms of diagnosis, prognosis, risk stratification and disease monitoring. Multiple myeloma is primarily a hematological malignancy characterized by proliferation of neoplastic plasma cells, which are largely confined to the bone marrow but is also frequently associated with extramedullary disease. The pattern of distribution of malignant plasma cells in bone marrow compartment is essentially patchy in over 80% of the cases and thus a bone marrow aspirate and/or biopsy from a single site, as is the current practice, may not give complete information about the entire disease burden [1-4]. Recently, Rasche L et al [5] performed sequencing from multiple sites, including bone marrow, focal bone and soft tissue lesions from MM patients and demonstrated that the spatial heterogeneity is positively associated with regional outgrowth of tumor-subclones in over two-third of the patients. Also, the mutations in TP53 gene were primarily restricted to the focal lesions with the disease progression [1, 5]. Besides, in extramedullary disease, the myeloma subclones occupy niches outside the bone marrow and are not sampled by a conventional bone marrow biopsy and may lead to sampling bias [2, 3].

The residual tumor cells commonly referred to as minimal residual disease (MRD), may be present in a micro environmental niche that is not well sampled during the collection and testing of marrow aspirate. Both the spatial heterogeneity and variegated nature of MM

pose a challenge in MRD detection in the BM. This might lead to false negative results by missing the extra-medullary tumor cells disease burden, which may account for relapse in substantial proportion of MRD negative patients [6-8]. Another limitation of bone marrow-based approach is invasive nature of the procedure which further pose challenge in serial evaluation of patients at multiple timepoints of MRD monitoring. All these factors question the utility of bone marrow based studies to diagnose and monitor disease activity in multiple myeloma warranting the need for newer strategies for comprehensive disease characterization, tumor burden and MRD monitoring in MM. Exploring circulating tumor cells (CTCs) by multiparametricflowcytometry (MFC) or circulating DNA (ctDNA) by next generation sequencing (NGS) and imaging techniques, such as MRI and PET are few of the techniques to capture temporal and spatial genetic heterogeneity in MM [9-14].

Here, we systematically review the available data on the current status of ctDNA for assessing disease burden and MRD monitoring in Multiple Myeloma (MM). This includes current status of ctDNA analysis over repeated, invasive, and often uninformative tissue biopsies in MM with a future potential of response assessment and MRD monitoring.

Biology of cell free DNA

Circulating free tumor derived DNA (ctDNA) constitutes a fraction of the total cell free DNA (cfDNA) that is released by neoplastic cells in the blood and other body fluids [15, 16]. The ctDNA can be distinguished from cfDNA as the former contain cancer-specific somatic mutations, chromosomal abnormalities, copy-number alterations and epigenetic modifications [17]. These degraded short fragments of DNA harbor altered gene signatures present in both primary tumors and metastases. The first evidence of cfDNA was given by Mandel and Metais and was initially found in immune complexes derived from systemic lupus erythematosus (SLE) patients [18, 19]. Almost three decades later, tumor specific NRAS mutations were shown to be present in the plasma derived from patients with myeloid malignancy [20].

It is widely accepted that cfDNA are released into the bloodstream by both active and passive secretion including cellular degradation, apoptosis, and necrosis [15, 21]. Active secretion occurs mainly in metabolically active, live cells, and mainly includes nuclear expulsion from erythroid precursors, neutrophil extracellular trap release (NETosis) in response to various stimuli and excision repair mechanism [22]. Previous studies demonstrated the size of cfDNA in the order of ~166 bp, fragmentation of this length of DNA suggests that apoptosis and DNA methylation may be the primary source of cfDNA release [23-26]. The cfDNA has variable half-life ranging from 15 minutes to several hours and it is continuously cleared from the circulation by liver, spleen and kidney [27, 28].

Distribution and levels of cfDNA in health and disease

cfDNA is found to be present in both health and disease conditions, however their level is extremely elevated in malignancy. In normal subjects, cfDNA is pre-dominantly of hematopoietic origin, and derived from turnover of myeloid and erythroid precursors [21]. Endothelial cells, hepatocytes and neurons are other less common sources of cfDNA [22]. Apart from malignancy, there are numerous non-malignant pathological conditions in which increased levels of cfDNA are detected viz any tissue damage, inflammation, sepsis, post-transplant and in pregnancy (physiological) [29, 30]. In malignancy, the quantity of cfDNA is found to be correlated with type, grade and stage of tumor as seen in various studies [31, 32].

As a reference, 100 g of tumor load in a patient releases 3.3% of ctDNA into the circulation [33]. In healthy individuals, the concentration of cfDNA ranges from 0 to 100 ng/mL of blood (average ~30 ng/mL), whereas in cancer patients, the concentration ranges from 0 to over 1000 ng/mL of blood (average ~180 ng/mL) due to high cellular turnover rate [34, 35]. The pathophysiology of cfDNA is summarized in **Figure 1**.

Biological functions of cell free DNA

Cellular homeostasis

cfDNA release is an important mechanism to remove damaged nuclear material from cells

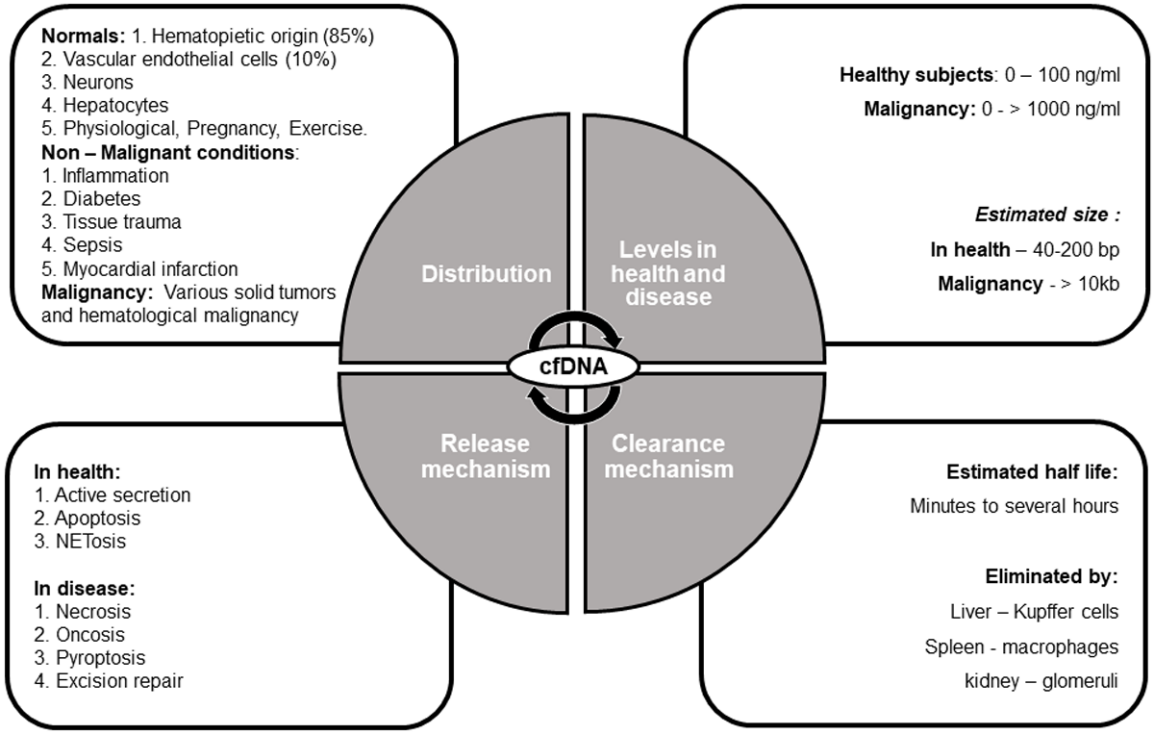

Figure 1. Pathophysiology of cell free DNA in health and disease.

as part of normal cellular homeostasis. Damaged DNA is mainly removed from the circulation via exosomes as oxidized mitochondrial DNA (mtDNA) maintaining the normal body homeostasis [21].

Cellular transformation

An important function of cfDNA is transformation as demonstrated experimentally by treating murine cells with serum from cancer patients and tumorigenesis of recipient cells in vivo model [36]. This led to the development of genometastasis hypothesis, where integration of cfDNA from diseased serum into healthy genome causes malignancy [37, 38].

Tumor metastasis

Presence of cfDNA in the circulation and microvasculature leads to trapping and immobilization of tumor DNA, contributing to the formation of pre-metastatic niche and spread of tumor [21, 23].

Immunomodulation and inflammation

Studies have shown the cytotoxic and pro-inflammatory actions of cfDNA in variable proportion, which regulates aberrant immune responses as well as mediates various inflammatory pathways [22, 39, 40].

cfDNA as diagnostic tool

In recent years, there has been a drastic increase in the number of publications referring to cfDNA/ctDNA analysis or liquid biopsy in various cancers [41, 42]. The first blood cfDNA liquid biopsy for epidermal growth factor receptor (EGFR) genotyping assay was approved by US-FDA for lung cancer patients in 2016 [43]. Since then the analysis of cfDNA is now frequently integrated into clinical trials [44-46]. Moreover, the application of cfDNA for diagnostics is rapidly evolving but is presently limited to betterment of treatment choices in late stage cancers.

Role in disease monitoring and tumor prognostication

The concentration of cfDNA in blood plasma may differ among patients and is shown to increase with increasing stage or type of cancer, and metastasis [47-49]. In fact, the increased levels of cfDNA are associated with poor survival in different cancer types [50-52]. It

may also be used for predicting patient risk stratification, therapy selection, prognostication, early relapse, monitoring emergence of resistance to treatment, and metastasis [53-59].

Utility over and above other techniques in disease evaluation

Till date, invasive tissue biopsies and radiological scans that involve radiation exposure have dominated the clinical assessment of cancer. In the current scenario, liquid biopsies come of age and among which, ctDNA is one of the most widely investigated markers in cancer patients. The advantages of cfDNA analysis over conventional tissue biopsies include: (1) Non-invasive technique; (2) Potential for cancer screening in asymptomatic individuals; (3) Early detection of cancer occurrence [60]; (4) Longitudinal study through serial sampling; (5) Monitoring of clonal evolution of malignant cells in real-time [61]; (6) Representative sampling of unreachable and non-resectable cancers [62, 63] and (7) Comprehensive characterization of tumor evolution and tumor dynamics [64-66].

Techniques for detection of cell free DNA

Since ctDNA is present at a very low variant allelic frequency (VAF; also known as Mutant AF) in the peripheral blood, high-end technologies are needed to detect such low VAFs. In a study done by Underhill *et al,* it was shown that the size selection of cfDNA fragments in plasma can be used to increase the amount of ctDNA [67]. Advancement of techniques like next generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR) have enabled detection of specific mutations present in ctDNA in blood with high sensitivity [68-70]. However, these newer techniques are still limited to detect small number of changes in ctDNA and yet to be validated in large prospective studies. Furthermore, there are certain analytic and post-analytic factors in the detection of cfDNA, that need thorough standardization viz. the experimental design, the choice of cfDNA detection method and analytical platform (e.g., digital PCR or NGS), data quality control methods, raw data processing and management (e.g., molecular bar-coding, in silico error suppression) etc. Nonetheless patient related confounding parameters like therapeutic or interventional protocols, co-morbidities, infections, lifestyle and dietary modifications may also influence the cfDNA concentration. The documented studies so far have reported wide variability in sensitivity and specificity in the assessment of cfDNA as they are based on diverse technical approaches [35, 71-74]. Both the pre-analytical and analytical steps involved in cfDNA analysis are summarized in **Table 1**.

The primary molecular based approaches for cfDNA quantitation include digital PCR and NGS (**Table 2**). Digital PCR is a highly-sensitive technology which can quantitatively monitor ctDNA for the detection of tumor-associated genetic mutations but is limited by the number of single candidate genetic loci [75-77]. On the contrary, to determine tumor mutation profiles or monitor tumor clonal evolution, the NGS-based platforms facilitate the coverage of entire sequence of a vast gene panel and allow identification of novel or epigenetic alteration. Despite the advantages of NGS-based analysis of ctDNA, the major limitation remains the cost-effectiveness and hands-on-time. For this reason, ultra-deep, high-coverage NGS-approach is still far from clinical use for ctDNA-based routine analysis.

Genomic landscape of MM

Multiple myeloma (MM) is characterized by a myriad of cytogenetic and molecular aberrations, which primarily include chromosomal translocations that involve the immunoglobin heavy chain locus and driver and/or secondary mutations in several oncogenic signaling pathways [78-87]. Since the first whole genome sequencing revealed the landscape of non-recurrent somatic alterations in MM [88], several NGS studies of the MM genome and exome have shed light on the spectrum of gene mutations associated with tumor progression [89-92]. Mutations have been reported most commonly in *KRAS* (23-26%), *NRAS* (20-24%) and *BRAF* (4-6%) genes that play a key role in MAPK pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) signaling and *FGFR3*, *TRAF3*, and *TP53* [88, 91-94]. Novel mutations in genes involved in RNA processing (DIS3) and protein homeostasis (FAM46C), regulator of *MYC* transcription (*FUBP1*), linker histones *HIST1H1B*, *HIST1H1D*, *HIST1H1E*, and *HIST1H2BK* have also been reported [89, 91, 95]. Using whole exome sequencing, Walker and group (2015) identified

Table 1. The pre-analytical and analytical steps involved in cfDNA isolation and analysis

Pre-analytical Considerations		
Plasma Collection (Tubes and Stability)	Plasma Centrifugation Procedure	Isolation of cfDNA (Kit and plasma input volume)
BD vacutainer tubes Stability: 4-6 hr Temp.: RT or 4°C	First centrifugation step, 1200-1600 × g for 10 minutes at 4°C	QIAGEN (QIAamp Circulating Nucleic Acid Kit) Type: Silica column based Plasma input volume: 1-5 mL
PAX gene tubes Stability: 7 days Temp.: RT (15-25°C)/24 h at 35°C	Second centrifugation step, 16,000 × g for 10 minutes at 4°C	Promega (Maxwell RSC ccfDNA Plasma kit) Type: Paramagnetic particles Plasma input volume: 1-5 mL
Streck tubes Stability: 14 days Temp.: RT (6-37°C)		Thermo Fisher (MagMAX Cell-Free DNA Isolation Kit) Type: Magnetic bead based Plasma input volume: 0.5-10 mL
NORGEN tubes Stability: 30 days Temp.: RT (15-25°C)/8 days at 37°C		MACHEREY-NAGEL (NucleoSpin Plasma XS) Type: Silica column based Plasma input volume: 0.2-0.72 mL
Roche tubes Stability: 7 days Temp.: RT (18-25°C)/16 h at RT (15-30°C)		EPIGENTEK (EpiQuik Circulating Cell-Free DNA Isolation Kit) Type: Magnetic bead based Plasma input volume: 0.1-1 mL
		ZYMO RESEARCH (Quick-cfDNA Serum and Plasma Kit) Type: Silica column based Plasma input volume: 0.2-10 mL
		NORGEN (Plasma/Serum Cell-Free Circulating DNA Purification) Type: Silica column based Plasma input volume: 0.01-10 mL
Analytical Considerations		
Quantification	Detection techniques	
NanoDrop 2000 (Thermo Fisher)/2100 Bioanalyzer DNA Quantification Kit (Agilent)/Quant-iT Pico Green dsDNA Assay Kit (Thermo Fisher)	qPCR/Real time qPCR Digital PCR Next Generation Sequencing	

Table 2. Various molecular approaches for the detection of genetic aberrations in cfDNA

Technique		Sensitivity	Aberrations detected
qPCR	ARMS-Scorpions PCR	0.05-0.1%	point mutations
	Clamping PCR	0.1-1%	point mutations
	TaqMan	0.1-1%	point mutations
Digital PCR	Beaming	0.01%	point mutations
	ddPCR	0.001%	point mutations
Targeted DNA sequencing	TAm-Seq	>2%	Point mutations and structural alterations in gene
	SAFE-SeqS	0.1%	Point mutations and structural alterations in gene
	CAPP-Seq	0.01%	Point mutations and structural alterations in gene
Whole genome sequencing (WES)	Digital karyotyping	0.001%	Genome-wide copy-number changes
	PARE	0.001%	Genome-wide rearrangements

Table 3. Data from various studies comparing the frequency of recurrent somatic gene mutations in multiple myeloma

Gene Mutation Frequency (%)			
Gene	Lohr et al 2014 [91] (n = 203)	Bolli et al 2014 [89] (n = 67)	Walker et al 2015 [96] (n = 463) (NCT01554852)
KRAS	23	20	21
NRAS	20	20	19
FAM46C	11	10	6
BRAF	6	12	7
TP53	8	12	3
DIS3	11	1	9
SP140	4	6	-
EGR1	4	6	4
TRAF3	5	2	4
ATM	4	3	3
CCND1	3	4	2
LTB	1	4	3
IRF4	2	-	3
FGFR3	2	-	3

15 gene mutations that were either actionable targets or carried prognostic value, which were incorporated into a mutation-based staging system [96]. The most frequently occurring gene mutations in multiple myeloma are listed in the **Table 3**.

Further technological advancement came with the development of a cost-effective genomics assay for myeloma, which was based on a moderate-depth capture sequencing platform, instead of Whole Genome Sequencing or Whole Exome Sequencing, for identification of chromosomal translocations, CNAs, and single-nucleotide variants [97]. In parallel, genomic classification of multiple myeloma was proposed based on comprehensive genotyping of patients by generating innovative clustering algorithms [98]. This study used custom target pulldown (TPD) analysis of a limited fraction of the genome as compared to WES and identified novel prognostic markers and disease subgroups [99]. Majority of the MM cases with hyperdiploidy status were characterized by the least number of gene mutations and CNAs and had a relatively good prognosis. Whereas, non-hyperdiploidy cases containing multiple subchromosomal CNAs and gene mutations showed a worse prognosis. More recently for improved stratification, chromosomal abnormalities detected by Next generation sequencing Seq-FISH and data from the Multiple Myeloma Research Foundation (MMRF) CoMMpass study was validated by R-ISS staging [100]. This study was undertaken because of the heterogeneity in methods and reporting of interphase FISH data in the MMRF CoMMpass study.

Cell free DNA in evaluation of genomic landscape of multiple myeloma

A few studies had recently documented the ctDNA based prediction of disease progression in MM patients by evaluation of the mutational spectrum and real-time monitoring of mutant clones [11-13, 55]. These studies demonstrated significantly higher levels of cfDNA in MM patients relative to controls and non-MM cancers. Using a high sensitivity targeted sequencing platform, a proof-of-concept study demonstrated the detection of mutations exclusively in the plasma but not in the bone marrow obtained from a subset of MM patients [13]. The existence of spatial and genetic heterogeneity in progressive MM disease was shown by sequential ctDNA quantitation using ddPCR by

longitudinal monitoring of specific clones, which are missed with single-site BM WES studies. The fractional abundance of specific clones correlated with the disease status and provided improved mutational characterization and therapeutic monitoring in MM. Moreover, the detection of plasma-based PIK3CA mutations raised the possibility of their association with extramedullary disease. Oberle A et al (2017) demonstrated an association between response to therapy and the presence of cfDNA clonotypic V(D)J rearrangements in a cohort of 27 MM patients [101].

A hybrid-capture-based liquid biopsy Sequencing (LB-Seq) method was evaluated for sequencing of gene targets including *KRAS, NRAS, BRAF, EGFR* and *PIK3CA*. This method included a variant filtering algorithm that enabled the detection of tumor-derived fragments available in cfDNA at very low variant allele frequencies (range 0.25-46%), albeit did not specifically reflect the typical mutational landscape in MM [12]. Another study used a targeted sequencing gene panel, spanning the exons and splice sites of 14 genes (*BRAF, KRAS, NRAS, TP53, IRF4, DIS3, CCND1, CYLD, EGR1, FAM-46C, PRDM1, SP140, TRAF3*, and *ZNF462*). The CAPP-seq ultra-deep targeted NGS approach was used for genotyping this gene panel that was specifically created to obtain maximum mutation recovery in plasma cell tumors. Also, the mutational profile of cfDNA and tumor genomic DNA (gDNA) of purified PCs from bone marrow aspirates was compared [102]. This proof-of-concept demonstrated the feasibility of ctDNA genotyping in real-time approach that could reliably detect both clonal and subclonal somatic mutations present in at least 5% of alleles in tumor PCs.

In a case report, the utility of ctDNA was demonstrated as a non-invasive qualitative (characterization of the tumor genome) and quantitative (tumor burden) biomarker in MM, especially in the setting of Extramedullary-MM patient with high risk oligosecretory disease [14]. In this longitudinal monitoring study of patient undergoing therapy, ctDNA specific mutation was demonstrated by the presence of *NRAS* Q61H that coincided with disease progression. This observation was further validated by alterations in the spectrum of single nucleotide variants (VAFs) and insertions/deletions in the ctDNA during the disease progression. This study provided evidence for the presence of clonal dynamics and tumor burden concomitant with drug resistance [14]. A recent Phase Ib trial of relapsed/refractory patients revealed a significant correlation with higher mutational fractional abundance in plasma with shorter overall survival whereas decrease in ctDNA levels at day five post-induction correlated with superior progression-free survival. This study claimed to be the first to demonstrate ctDNA in monitoring tumor burden for predicting disease outcome in MM [103].

Residual disease monitoring in MM

The major advancement in the treatment strategies of MM such as proteasome inhibitors and immunomodulatory agents have resulted in improved survival outcomes. According to the IMWG criteria, the minimal residual disease (MRD) negativity in the bone marrow is recommended for response assessment and correlates with improved progression-free survival and overall survival in myeloma [104]. Earlier, the myeloma guidelines included urine assessment for monitoring monoclonal FLCs, with the exception of patients with non-measurable M-protein levels in serum (<10 g/L by SPE) and urine (BJP <200 mg/24 h by UPE) [105-107]. The recent update to the IMWG consensus criteria firmly emphasized on the possibility of novel biomarkers of response in MM with improved sensitivity. Detection of MRD within the BM, either by Multicolor Flow Cytometry for phenotypic markers or by Next Generation Sequencing (NGS) technologies for genotypic aberrations is currently followed. The sensitivity of at least 10^{-5} is considered as sustained response when confirmed at least one year apart [108]. In a meta-analysis, MRD status emerged as a surrogate for PFS in newly diagnosed MM [109]. The continuous improvement in the methods for MRD testing have been witnessed over the past two decades, which allow sensitive detection as low as one in a million cells. A comparison of various techniques to detect MRD in Multiple myeloma is summarized in **Table 4**.

MRD detection by MFC allows detection and monitoring of neoplastic and normal plasma cells that can be distinguished by identifying aberrant expression of cell surface and intracellular protein markers [6]. Initially, three to four colors MFC was used for MRD detection,

Table 4. Comparison between different techniques for detection of Minimal Residual Disease in Multiple Myeloma (Adapted from [116, 137])

Method	Sample Type	Applicability	Sensitivity	Quantification	Clonal Evolution	Advantage	Limitations
NGS	Nucleic acid (DNA)	~90%	10^{-6}	Absolute	Detectable	Provides information on background repertoire	Not standardized, no guidelines for analysis and data interpretation, available in few labs, Expensive
Flow Cytometry (Next Gen. Flow)	BMA (Cells)	90-100%	3-4 colors-10^{-3}/10^{-4} 6-8 colors-10^{-4} 8-10 colors-10^{-5}	Absolute	Not applicable	Fast, reproducible, Accurate quantification well standardized, cost-effective	Large cell number needed (~6 million), Processing to be done on fresh samples
ASO-qPCR	Nucleic acid (DNA)	~80%	10^{-5}	Absolute	Not detected	More sensitive and specific than MFC, fresh sample not required, provides information on background repertoire	More time consuming and relatively laborious, requires patient specific primers, need of a bioinformatics analysis, Expensive
ddPCR	Nucleic acid (DNA)	Not reported	10^{-4}/10^{-5}	Absolute	Not detected	Standard curve Not needed, easy to perform	Dependent on ASO-primer, not standardized, no guidelines for analysis, data interpretation, Expensive
RT-qPCR	Nucleic acid (DNA)	90-95%	10^{-4}/10^{-5}	Absolute	Not detected	Well standardized, International guidelines for analysis and data interpretation	Dependent on ASO-primer, laborious, time consuming, affected by clonal evolution, large amount of diagnostic DNA, Expensive

Table 5. Current status of various studies on the utility of ctDNA in molecular characterization of MM

S.No.	Title of Study	Genes Targets by ctDNA	Outcome	Reference
1	ctDNA sequence analysis as an alternative to multiple myeloma bone marrow aspirates	Five genes (18 kb) (KRAS, NRAS, BRAF, EGFR, PIK3CA)	Ultra-deep sequencing of cfDNA to >20,000 × median coverage. Showed 96% concordance and >98% specificity when compared with matched BMA samples.	[12]
2	ctDNA analysis demonstrates spatial mutational heterogeneity that coincides with disease relapse in myeloma	Four genes (KRAS, NRAS, BRAF, TP53)	Mutations detected by OnTarget Mutation Detection (OMD) and ddPCR. High frequency of Plasma-Only mutations was observed in relapsed/refractory patients than newly diagnosed.	[13]
3	Whole-exome sequencing of cell-free DNA and circulating tumor cells in multiple myeloma	All genes and their exons	Screened and monitored tumor fraction and copy-number changes in cfDNA and CTCs using ultra-low pass whole-genome sequencing and whole-genome sequencing of matched CTCs, cfDNA and bone marrow biopsies from MM patients. ~99% concordance in clonal somatic mutations and ~81% concordance in copy number alterations between liquid and tumor biopsies.	[122]

which has currently expanded to 8-10 colors MFC in a single tube with monoclonal antibodies in dried formulation with sensitivity of 10^{-5} [110]. More recently, Next Generation Flow (NGF)-MRD has been optimized as a two-tube eight-color antibody panel. After bulk lysis, acquisition of $\geq 10^7$ cells/sample is recommended, and novel software tools for automatic plasma cell gating ease MRD detection. NGF-MRD showed better sensitivity than conventional eight-color flow-MRD [111]. Quality of sample and hemodilution impacts MFC based MRD detection results [112]. The sensitivity of NGF can be improved by increasing the number of cells analyzed. The minimum requirement by current consensus guidelines is 2 million and recommend 5 million events to be acquired to achieve a minimum sensitivity of 10^{-5}-10^{-6} [112]. The BMT CTN Myeloma Intergroup and IMWG flow MRD-negative response criterion was reported to be highly translational and sensitive for the evaluation of treatment efficacy in MM [7, 113]. Recently, the utility of a non-invasive approach combining immune-magnetic beads and flow cytometry for enrichment and detection of circulating myeloma cells for monitoring MRD in MM patients was shown [114].

In regard to the molecular approaches for MRD assessment, initially, polymerase chain reaction-based amplification of the V(D)J clonal rearrangements was used for MRD assessment on BM samples for qualitative information. Later, clonal-size based methods (PAGE, Gene Scanning) and allele specific oligonucleotide PCR (ASO-RQ-PCR) were used for quantitation [115]. Minimal residual disease negativity by deep sequencing emerged as a major prognostic factor in MM [116]. In a retrospective study, patients with MM who underwent autologous stem-cell transplantation were evaluated by sequencing of all rearranged immunoglobulin gene segments present in a myeloma clone and the prognostic value of sequencing-based MRD detection became evident. NGS-based approach was more sensitive for low level MRD detection as compared to multiparameter flow cytometry, real-time quantitative ASO-PCR and droplet digital PCR (ddPCR) [77, 117]. Digital PCR based MRD detection is still in the phase of standardization and optimization with varying definition of positivity in different studies [75, 76]. The sensitivity and accuracy of ddPCR are almost comparable to ASO-PCR [76]. Unlike qASO-PCR, ddPCR does not require a standard curve construction and gives absolute number of copies of the target sequence. Both ASO-PCR and ddPCR cannot detect clonal evolution and therefore have limited applicability.

A limitation of the molecular approach is that consensus primers for clonality identification are used in the diagnostic sample followed by evaluation of follow-up sample for MRD detection. Also, there is an absolute need for the patient specific primers and probes. In NGS approaches, MRD detection can be enabled once the clonality of the sequence in the diagnostic sample is established. In contrast to this, in ASO-PCR even if the clonality is established there are many hurdles that hinder its applicability in MRD detection which includes, sequence mismatch against consensus primer and diversity of the CDR region. NGS can detect almost all clones and subclones and hence can monitor clonal evolution. Unlike ASO-PCR, additional advantage of NGS is that it is independent of a standard curve.

CtDNA in disease monitoring and MRD assessment in myeloma

Recent technological developments in detection of MRD by utilizing peripheral blood instead of bone marrow have emerged [118, 119]. Usually cell-free fraction of peripheral blood (serum/plasma) and the cellular part (PBMCs) are used to detect MRD in peripheral blood. In a study by Korde et al (2014), myeloma associated clonotypes by NGS of the immunoglobulin VDJ segments were detected in 13/14 plasma samples (i.e. cfDNA) at diagnosis, while only 1/13 plasma samples after therapy showed myeloma associated clonotype, while monoclonal protein was still detected in 12/13 serum samples by immunofixation and/or serum electrophoresis [118]. Although, detection of myeloma-specific clonotypes in the peripheral blood plasma was shown at diagnosis; however, after two cycles of combination therapy these clonotypes could not be quantified using standard volumes of peripheral blood plasma as the tumor load in the plasma is several folds lower than in the BM [118]. Study of MRD detection in PB using NGS of clonotypic VDJ gene rearrangements by Oberle et al (2017) showed that

myeloma clonotypes were absent in PB samples despite the presence of M-protein [101]. Furthermore, this study showed discordance in 30% of the samples in plasma cfDNA and PB leukocytes in MRD-positive cases. Hence, MRD analysis needs to be performed in both the compartments of PB (plasma and leukocytes) [101].

A correlation was shown between ctDNA and tumor burden in non-responders compared with less than half of responder myeloma patients. However, Mazzotti et al (2018) demonstrated no correlation in paired bone marrow and blood samples (ctDNA) for MRD by NGS utilizing only Ig gene rearrangements [120]. In a long-term study on blood-based MRD monitoring in MM, cfDNA detection of VDJ rearrangement by ASO-qPCR was used to demonstrate its utility as a prognostic marker [121]. Also, the length of cfDNA fragments was shown to be associated with treatment response of patients. Overall, myeloma clonotypes are present at a much higher level in BM samples than in PB samples, therefore, MRD testing using PB samples still poses many challenges.

Recently, advancement in the analysis of ctDNA was based on a two-step sequencing approach [122]. The first step, developed by the team at Broad and Dana-Farber Cancer Institute was called “ultra-low pass” whole genome sequencing (ULPS). This method is cost-effective in identification of blood samples with tumor DNA fraction of at least 5-10%, which allows more comprehensive genetic analysis. This was followed by whole exome sequencing (analyzing the protein-coding regions of the genome) of the filtered samples in the second step. As opposed to NGS alone, wherein the emphasis is on increasing sequencing depth, ultra-low pass whole-genome sequencing allows sequencing of the DNA at ultra-low depth as low as 0.1X. This two-step approach is unique and novel technique for screening and monitoring tumor fraction and copy-number variations in patient samples [122]. The ULPS requires low amount of sample to be analyzed as compared to conventional NGS and requires high-end computational analysis to interpret the results. In contrast to traditional array-based technique for getting molecular information, ULPS provides accurate and cost-effective genetic information. Before proceeding with genomic analysis of patient sample, ULPS provides tumor quality and content information to guide for further steps. By this QC step, researchers can identify samples with high purity for further genomic characterization. The current status of various studies for the utility of ctDNA in molecular characterization of MM is summarized in **Table 5**.

Challenges and pitfalls in cfDNA analysis in MM

Akin to the technical challenges involved in the isolation and characterization of plasma cells in MM, still several other methodological issues are in the standardization phase and therefore limit the clinical use of cfDNA analysis in the current scenario [123, 124]. In regard to the pre-analytical procedures starting from sample collection timing, isolation of the plasma or serum from the blood, cfDNA extraction and quantification, and the storage condition for the cfDNA are critical for the data interpretation. In a study, the stability of cfDNA was compared in standard EDTA and other specialized blood collection tubes (including Streck, Roche, PAXgene, Norgen and CellSave tubes). Ten metastatic cancer patients with *KRAS*-mutation served as a source of blood samples, which was collected and stored for three days. It was demonstrated that the cfDNA levels were the least stable in EDTA tubes (≤6 hours), whereas cfDNA could be recovered from all the specialized tubes after 48-72 h, irrespective of the temperature [125]. The choice of specialized blood collection tubes is less critical if the samples are processed within a few hours.

In a comparative study, the isolation efficiency of cfDNA using various commercially available kits including QIAamp circulating nucleic acid kit (QIA), Maxwell RSC ccfDNA Plasma Kit (RSC), PME free-circulating DNA Extraction Kit (PME), the EpiQuick Circulating Cell-Free DNA Isolation Kit (EQ), and NEXTprep-Mag cfDNA Isolation Kit (NpMV1/2) was performed [125]. Both the total cfDNA concentration as well as the ctDNA fraction (*KRAS* mutation) was quantitated by ddPCR. Both QIA and the RSC kits had comparable isolation efficiencies of both non-mutated total cfDNA and *KRAS* mutated ctDNA, whereas the yield generated by other kits was relatively less. The advantage of RSC kit was its fully automated protocol over the labor-intensive QIA kit.

A multicentric Innovative Medicines Initiative consortium CANCER-ID (http://www.cancer-id.eu) recently documented a comparison of different technologies for cfDNA purification, quantification, and characterization [126]. The suitability of mononucleosomal DNA as a surrogate for cfDNA as a process quality control from nucleic acid extraction to mutation detection was demonstrated. The key challenges of cfDNA based tumor profiling still include the low overall yield (<10 ng/ml plasma) and low percentage of ctDNA fraction (<5%) relative to the total cfDNA.

Apart from the technical challenges, other practical issues in ctDNA analysis include the source of ctDNA origin, signal stability, specificity, sensitivity and limit of detection. The identification of tumor-specific somatic mutations is restricted to the hot-spot regions used to discriminate the tumor DNA from healthy DNA. In addition, the variable amounts of ctDNA detected in the bloodstream poses sensitivity issues in the detection of early-stage cancers. Thus, alternative strategies have been investigated towards improvement of the specificity and sensitivity of ctDNA detection.

The sensitivity of cfDNA analysis has been improved by undertaking several considerations. The size selection approaches of cfDNA, which are believed to reflect the tissue of origin, tumor-derived cfDNA are considerably shorter in size. Either physical methods or using bioinformatics tools, this challenge of short length has been overcome. Many studies focused on physical sorting of cfDNA which includes electrophoresis [127-130] while in-silico analysis of the fragmentation pattern of ctDNA require different size selection algorithms to select for appropriate size, which exhibits preferred end-coordinates [131, 132]. When the tumor fraction in the total cfDNA sample is low, targeted approach for detecting point mutations are used such as qPCR, digital PCR and targeted DNA sequencing. These targeted approaches are highly sensitive and enhance the depth of mutational analysis. In contrast, when the tumor fraction is high, whole genome analysis-based approaches (such as whole genome sequencing and whole exome sequencing) are used which are less sensitive than targeted approaches [133]. Therefore, for ctDNA monitoring, targeted approaches may prove to be more beneficial. As tumors continuously evolve during the course of the disease and their heterogeneity pattern change upon exposure to selective influence of different therapies, therefore the sensitivity and efficiency of NGS based technologies are continuously being improved over time.

Future directions and conclusion

The cell-free DNA is a potential non-invasive biomarker for early cancer detection and can facilitate monitoring of myeloma patients. It is proposed that a combi-panel of multiple biomarkers could serve as a promising approach for disease monitoring. Such multi-panels could include the analysis of tumor-specific mutations in cfDNA using NGS, which can be combined with the identification of genetic alterations in cfDNA using ultra-low pass coverage WGS. In addition, the methylation profiling of cfDNA and the concurrent quantification of protein biomarkers can be add-ons for a more comprehensive cancer profiling. Together all these could increase the sensitivity and specificity of liquid biopsy in MM, especially in the early stages of the disease. To overcome the various hurdles in the translational potential of cfDNA in clinical practice, the unresolved issue of primary location of the disease could be achieved by the analysis of epigenetic markers. It is suggested that the highly tissue-specific DNA methylation profile is representative of cfDNA tissue of origin although further targeted studies are warranted [134]. To achieve this goal, multiple research groups may collaborate in a global consortium approach, which may cover a wide population cohort.

The cfDNA genotyping in MM may have an immediate clinical application as it could be incorporated into clinical trials. This may facilitate the identification of patients carrying actionable mutations, their longitudinal genetic monitoring during targeted therapy administration and MRD estimation. To enhance the utility of ctDNA as a routinely applicable biomarker for disease monitoring in MM, analyzing ctDNA epigenetic modifications and immune signatures may boost its potential for early detection in MM [135-137]. The need of the hour is an improved understanding of the kinetics of ctDNA production in myeloma to monitor MM disease burden using peripheral blood in routine clinical practice.

In conclusion, identifying tumor-specific mutations (ctDNA) at diagnosis and by sequential tracking of plasma-only mutations may be useful for prognostication that can help clinicians guide suitable treatment for individual MM patients. Moreover, ctDNA analysis holds great potential in prediction of MM disease progression, treatment outcome monitoring, tumor dynamics monitoring, MRD assessment or early relapse prior to its actual clinical occurrence.

Acknowledgements

This work was supported by grant from Department of Biotechnology, Govt. of India to Prof. Ritu Gupta [Grant: BT/MED/30/SP11006/2015]. AtulBasnal would like to thank University Grants Commission, Govt. of India for CSIR-UGC-Senior Research Fellowship.

Disclosure of conflict of interest

None.

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