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Research Article

The Clinical Value of Circulating Cell Free DNA in Multiple Myeloma

Tong Yang[1], Yun Lin[2*],Weimin Chen[2], Zhihong Wang[2], Tiannan Wei[1], Jin Shang[2]

[1]Department of Hematology, Shengli Clinical Medical College, Fujian Medical University, Fuzhou 350000, China;[2]Department of Hematology, Fujian Provincial Hospital, Fuzhou 350000, China

ABSTRACT

Purpose: To evaluate the feasibility of the detection of circulating cell free DNA (cfDNA) and the prognostic value of cfDNA in patients with Multiple Myeloma (MM).

Experimental design: All samples were screened hematopoietic malignancy associated genetic mutations from cfDNA and Bone Marrow (BM), using the Next Generation Sequencing (NGS). The mutated genetic, checked in both cfDNA and BM, were monitored at the different time points during the treatment by droplet-based digital PCR (ddPCR).

Results: Three patients were included in our prospective study. Two patients detected DNMT3A mutations and one patient detected GNAQ mutation. The abundance of mutations decreased as the disease remission, and increased as the disease progresses. The detection of cfDNA has shown to predict relapses before paraprotein.

Conclusion: cfDNA is a sensitive tool for monitoring compared to Para protein levels. The utility of cfDNA for monitoring disease is Promising.

Keywords: DNA; Mutation; Multiple myeloma; DNA sequencing

INTRODUCTION

As the second common hematologic malignancy, multiple myeloma (MM) is characterized by recurrent cytogenetic and molecular abnormalities. In recent decades, autologous stem cell transplants [1,2] and novel treatments such as immune modulatory drugs (ImIDs) [3-5], proteasome inhibitors (PIs) [6-8], and monoclonal antibodies [9-11] have improved the treatment of MM, which has led to longer survival times as a result of changing the natural history of the disease [12]. Approximately 60% to 80% of patients achieve a complete response [13]. However, some patients still face the problem of relapse. Relapse due to undetected is the leading cause of death. As a result, investigators have examined ways of detecting minimal residual disease (MRD) in order to detect relapses. In clinical practice, the most common way to detect MRD is multipara Meter Flow Cytometer (MFC) [14-17]. However, myeloma cells can change their surface antigen patterns after initial diagnosis, which may result in false negative results [18]. Therefore, a new method of monitoring disease progression is urgently needed.

In 1948, Mandel and Metais [19] detected circulating cell free DNA (cfDNA) in the blood stream of cancer patients. In 1977, Leon et al. [20] demonstrated that serum levels of cfDNA in cancer patients were higher than in healthy individuals. Following this, Stroun et al. [21,22] described a portion of plasma cfDNA was derived from tumor (ctDNA) and was carrying its molecular characteristics. In recent years, there have large developments on the use of cfDNA, for follow-up and treatment of cancer patients [23-25]. And newly developed technologies such as droplet-based digital PCR (ddPCR) and Next-generation sequencing (NGS) increased largely the detection sensitivity, specificity and precision of the analysis of sequences, allowing their detection in cfDNA [26]. In recent years, cfDNA analysis was used for prognosis and monitoring MM, but the results was unclear [27,28].

In our study, we detected mutations from cfDNA and paired BM samples using NGS to characterize the mutational profile in individual MM patients and monitored specific mutants in sequential cfDNA samples by ddPCR. The aim of the study was to evaluate the feasibility of the detection of cfDNA and the prognostic value of cfDNA in patients with multiple myeloma, used the method of NGS combined with ddPCR.

MATERIALS AND METHODS

Sample extraction and data

From January 2019 to March 2022, Bone Marrow (BM) and Peripheral Bloods (PB) of all MM patients, never receiving treatment, were collected in our hospital. All patients were selected based on IMWG 2014 criteria [29], estimated survival time greater than 5 years. All patients signed an informed consent form which approved by the ethics committee. Blood samples and BM were collected

Correspondence to: Yun Lin, Department of Hematology, Fujian Provincial Hospital, Fuzhou 350000, China, E-mail: sllinyun@163.com

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before the first cycle of chemotherapy treatments. All samples were screened hematopoietic malignancy associated genetic mutations from cfDNA and BM, using the Next-generation sequencing. As the high price of the NGS, the mutated genetic, checked in both cfDNA and BM, were monitored at the different time points during the treatment by ddPCR. Para protein was checked at the same time to compare the fractional abundance (FA) of mutations. The following data were collected in a prospective database: clinical characteristics (gender, age, ECOG-PS), biological data (type of Myeloma, ISS and DS at diagnosis, Cytogenetic abnormalities) and follow-up data (Para protein fractional abundance (FA) of mutation data of relapse data of death or last follow-up).

Gene target sequencing

Genomic DNA extraction: Peripheral bloods and BM of all subjects were collected in cell-free DNA storage tubes and EDTA tubes respectively. Genomic DNAs were extracted using QiaAmp Blood DNA Mini kits (Qiagen, CA, USA). The concentration and quality of genomic DNAs were examined with a NanoDrop® ND1000 (Thermo Fisher Scientific, MA, USA), the Qubit® 3.0 Fluor meter (Thermo Fisher Scientific, MA, USA) and 1% agarose gel electrophoresis.

DNA library preparation: In order to prepare the paired-end DNA sequencing libraries, genomic DNA was sheared using a Covaris™ (Woburn, MA, USA) sonicator, followed by peak detection, end repair, poly A-tailing, adaptor ligation, and amplification.

The paired-end DNA sequencing libraries were prepared through genomic DNA shearing, use a Covaris™ (Woburn, MA, USA) sonicator, followed by peak detection, end repair, poly A-tailing, paired-end adaptor ligation, and amplification. Covaris fragmented the qualified genomic DNA sample randomly and the fragment sizes of the library are mainly 250-300 bp. After End-Repair and A-tailing were applied, the adapters were ligated with unique barcodes specific to Illumina technology for sequencing and amplification. According to the instructions provided by the manufacturer, we used the KAPA Hyper Prep kit for these steps.

Targeted sequencing: A sure select custom design (Agilent Technologies, Inc., Santa Clara, CA) was used to target whole exons of 393 most frequently occurring hematologic genes. Each captured library was then loaded on novase6000 platform (Illumina, Inc., San Diego, CA) at the Wuhan Kindstar Global gene Technology (Kindstar, Wuhan, China). For high sensitivity and accuracy, each sample was sequenced at 1500-2500 × depth.

Data analysis

For base-calling, raw image files were processed with Illumina base calling software using default parameters and 150 bp pair-end reads were generated for each individual. Burrows-Wheeler Aligner (BWA, v0.7.17) was used to align paired-end reads to the human reference genome (GRCh37/HG19). Data quality control (QC) was performed throughout the whole pipeline, from clean data to mapping data to variant calling. Genome Analysis ToolKit (GATK, v4.1.1.0) was used to realign insertions and deletions, calibrate quality scores, and identify variants with Picard-tools (v4.1.1.0) removing duplicate reads. ANNOVAR (v201804) was used to annotate mutations.

As a result of sequence alignment and variant calling, synonymous and intronic variants with a minor allelic frequency (MAF) ≥ 1% in the 1000 Genomes Project, the dbSNP database, and the Exome Aggregation Consortium (ExAC) database were discarded. NGS reads were visualized using an integrated genomic viewer (IGV).

Variant classification

It was crucial to ensure high-quality clinical data by accurately classifying somatic genetic alterations detected *via* next-generation sequencing (NGS). Based on guidelines from the Association for Molecular Pathology (AMP), the American Society of Clinical Oncology, and the College of American Pathology, somatic sequence variants can be categorized based on their clinical significance. A four-tiered system to categorize somatic sequence variations based on their clinical significances is proposed: tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants of unknown clinical significance; and tier IV, variants deemed benign or likely benign. Tiers I to III must be reported in descending order of clinical importance. It is not recommended to include tier IV or benign/likely benign variants/alterations in the report.

Then, we placed verified germ line variants into the following categories according to guidelines from the American College of Medical Genetics and Genomics (ACMG) and Association of Molecular Pathology (AMP): pathogenic (P), Likely Pathogenic (LP), Variant of Uncertain Significance (VUS), Likely Benign (LB) and Benign (B).

Detection of droplet digital PCR (ddPCR)

Compared to traditional PCR and qPCR, digital PCR allows higher precision due to the unique sample partitioning step and Poisson statistical data analysis. Therefore, digital PCR is particularly useful for applications that require the detection of small amounts of input nucleic acid or finer resolution of target amounts, for instance, detecting rare sequences, analyzing Copy Number Variations (CNVs), and analyzing gene expression.

The Droplet Digital PCR (ddPCR) technique uses water-oil emulsion droplets to perform digital PCR. Samples are fractionated into 20,000 droplets, and PCR amplification occurs in each droplet. As with most standard TaqMan probe-based assays, ddPCR technology uses similar reagents and workflows. The technique uses fewer samples than other commercially available digital PCR systems, reducing costs and conserving valuable samples.

QX200 droplet digital PCR system workflow

Genomic DNA extraction: QIAamp Circulating Nucleic Acid Kit (Qiagen, CA, USA) was used to extract genomic DNA.

Prepare PCR-ready samples prior to starting ddPCR: Create a prepared sample by combining the DNA sample, primers, and probes with ddPCR supermix. Load 20 µl of the prepared sample into each well.

Droplet generation: Droplet generation begins with preparation of nucleic acid samples, which may be performed with primers, fluorescent probes (such as TaqMan probes with FAM and HEX or VIC), and a proprietary supermix. In the QX200 Droplet Generator, samples are partitioned into 20,000 Nano liter-sized droplets using proprietary reagents and microfluidics. Samples are then placed into the QX200 Droplet Generator, which utilizes proprietary reagents and microfluidics to partition the samples into 20,000 Nano liter-sized droplets. Droplets created by the QX200 Droplet Generator are uniform in size and volume.

PCR amplification of droplets: The droplets are transferred into 96-well plates for PCR amplification using a thermal cycler (Table 1).

Table 1: The temperature and times of PCR amplification.

Temperature	Time	Cycle
95°C	10 min	
94°C	30 sec	40
60°C	1 min	
98°C	10 min	
4°C	1 min	

Droplet reading: Samples are placed in the QX200 Droplet Reader after PCR amplification of the nucleic acid target in the droplets. Each droplet is analyzed separately using a two-color detection system (set to detect either HEX or VIC), enabling multiplexed analysis for different targets. PCR-positive and PCR-negative droplets are counted using the droplet reader and QuantaSoftTM software.

Analyze results: There is an increase in fluorescence in positive droplets containing at least one copy of the target compared to negative droplets. QuantaSoft software measures the number of positive and negative droplets in a sample for each fluorophore (for example, FAM and HEX). After fitting the fraction of positive droplets to a Poisson distribution, the absolute initial copy number of the target DNA molecule in the input reaction mixture is determined.

RESULTS

Patients' characteristics

So far, three patients were included in this prospective study. The patient's characteristics were summarized in Table 2.

Associated genetic mutations

BM and PB of 3 patients were collected before the first cycle of chemotherapy treatments. Then, DNA extracted from the PB and BM. We checked genetic mutations from cfDNA and BM in all these patients by NGS. In patient 1, we found FGFR3 mutation (4.8%) and DNMT3A mutation (1.2%) in BM and DNMT3A mutation (0.5%) in cfDNA. In patient 2, NRAS mutation (4.3%), DNMT3A mutation (1.2%) and KMT2C mutation (0.4%) were detected in BM and DNMT3A mutation (1.4%) was detected in cfDNA. In patients 3, GNAQ mutation (1.8%) and FGFR3 mutation (20.5%) were detected in BM and GNAQ mutation (3.6%) was detected in cfDNA.

Mutation in cfDNA can monitor patient progress

To determine the target mutations, we selected mutations that were detected both in BM and cfDNA. For the monitoring of disease, three patients were evaluated with mutation quantitation by ddPCR. We found the fractional abundance (FA) of mutation had a decline when disease was in remission. The FA of mutation were also checked when paraprotein were negative. When disease keep in complete remission, the FA of mutations were also not detected. One patient had earlier detectable cfDNA compared to para protein. As the disease relapsed, the FA of previously identified mutations rapidly increased. So far, there are two patients still keep disease in complete remission (CR), which the FA of mutation still not to be checked (Table 3).

Table 2: Characteristics of patients enrolled in this study.

	Patient 1	Patient 2	Patient 3
Gender	F	M	M
Age	68	60	70
ECOG-PS	0	0	0
Type of myeloma	IgG-λ	IgG-κ	IgG-λ
ISS at diagnosis	I	II	III
DS at diagnosis	IIIA	IIIA	IIIA
Cytogenetic abnormalities			
Chromosome	Normal	Normal	Normal
RB1	84%	ND	73%
D13S319	84%	ND	70%
IGH	74%	ND	69%
CCND1/IGH	ND	ND	ND
IGH/MAF	ND	ND	ND
IGH/MAFB	ND	ND	ND
IGH/FGFR3	84%	ND	34%
IGH/CCND3	ND	ND	ND
1q21	75%	ND	ND
P53/CEP17	ND	ND	ND

Table 3: The continuous variation of mutations and paraprotein.

Patient 1	2019.1	2019.03	2019.06	2019.08	2019.09	2020.02	2020.07	2020.12	2021.05	2021.09	2022.01
Paraprotein	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative
DNMT3A	0.50%	0.32%	0.28%	0.12%	ND	ND	2.86%	3.22%	3.68%	3.89%	3.22%
Patient 2	2019.04	2019.1	2020.05	2020.09	2020.12	2021.06					
Paraprotein	Positive	Negative	Negative	Negative	Negative	Negative					
DNMT3A	1.40%	0.48%	ND	ND	ND	ND					
Patient 3	2018.1	2019.03	2019.1	2020.01	2020.06	2020.12	2021.05				
Paraprotein	Positive	Positive	Negative	Negative	Negative	Negative	Negative				
GNAQ	3.60%	0.36%	0.02%	ND	ND	ND	ND				

DISCUSSION

CfDNA (Circulating Cell Free DNA) is a type of double-stranded DNA that circulates in blood. Unlike bone marrow biopsy or primary tissue biopsy, it can be easily accessed through venipuncture. Additionally, cfDNA may be used in solid tumors as well as hematological malignancies to monitor disease progression. With the development of DNA sequencing technology, especially the development of NGS, cfDNA becomes more feasible as more gene mutations are discovered in malignant tumors.

The spatial and temporal limitations of single-site bone marrow biopsy are highlighted by multifocal tumor deposition and intra clonal heterogeneity in MM. A liquid biopsy, such as cfDNA, offers an interesting alternative since tumor cells and genetic material typically leak into the bloodstream. Some studies have shown that cfDNA can be used to obtain the molecular profile of myelomas instead of the bone marrow aspirate, and they have supported the concept of cfDNA as a prognostic marker [30-32]. So we selected mutations of cfDNA to monitor the state of disease and the response of treatment. Besides that, mutations of cfDNA was sensitive detected than BM. Li found that cfDNA samples (53%) had a higher rate of mutations of BRAF, KRAS, and NRAS compared to BM samples (34%) [33]. Mithraprabhu found that cfDNA samples had a higher proportion of TP53 mutations than BM samples [34,35]. In our study, we found the same results. In our three patients, blood samples of two patients had a higher proportion of mutations compared with BM samples.

Presently, MM is an incurable disease with treatment aimed at extending event-free progression. Monitoring the response of treatment in MM is essential to determining the best way to optimize patient management. Currently, serial measurement of para proteins and serum free light chains is used to monitor disease relapse or assess MRD. However, several objectives have been used to evaluate cfDNA's effectiveness and reliability [36]. Rusted found that proportion of the mutations in cfDNA correlated well with serum para protein [37]. Furthermore, we demonstrate that circulating cfDNA has prognostic value. In our studies, we found the FA of mutation had a decline when disease was in remission. The FA of mutation were also checked when para protein were negative. When disease keep in complete remission, the FA of mutations were also not detected. Besides that, mutations were earlier detected in cfDNA compared to para protein in one relapsed patients. These studies showed that cfDNA may better indicate the tumor burden than para protein.

The results can also find in Diffuse Large B Cell Lymphoma (DLBCL) and acute leukemia. They observed the detection of disease-specific rearrangement in peripheral blood cfDNA by NGS has shown to predict relapses in diffuse-large B-cell lymphoma (DLBCL) before established radiological staging methods demonstrated evidence of disease recurrence [38,39]. Short et al. analyzed paired plasma cfDNA and bone marrow (BM) samples from acute leukemia patients using NGS. Two patients showed cfDNA mutations (IDH1 and ASXL1) during remission that later appeared in the BM following morphological relapse three and fourteen months later. Furthermore, Jilani et al. [40] studied the detection of FLT3-ITD in cfDNA and BM samples of newly diagnosed AML, MDS, and ALL patients using PCR [41]. Thus, cfDNA can be useful for identifying early relapses and monitoring tumor evolution.

CONCLUSION

In our study, we found that cfDNA is a sensitive tool for monitoring compared to Para protein levels. The utility of cfDNA for monitoring disease is Promising. Considering the limited sample size, large prospective trials are required to determine the effectiveness of using cfDNA in clinical practice. MM is an incurable disease with treatment aimed at extending event-free progression. Monitoring the response of treatment in MM is essential to determining the best way to optimize patient management. Circulating Cell Free DNA (CfDNA) is a type of double-stranded DNA that circulates in blood. Unlike bone marrow biopsy or primary tissue biopsy, it can be easily accessed through venipuncture. The fractional abundance (FA) of mutation had a decline when disease was in remission. The FA of mutation were also checked when Para protein were negative. The abundance of mutations decreased as the disease remission, and increased as the disease progresses. The detection of cfDNA has shown to predict relapses before Para protein. cfDNA is a sensitive tool for monitoring compared to Para protein levels. The utility of cfDNA for monitoring disease is Promising.

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AUTHOR NOTES

Tong Yang and Yun Lin contributed equally to this work.

CONTRIBUTIONS

Tong Yang identified patients, collect and analysed the data, wrote the manuscript; Yun Lin designed and performed the experiments, analysed and interpreted data, wrote the manuscript; Weiming Chen, Zhihong Wang, Tiannan Wei, Jin Shang identified and managed patients, collected clinical data. All authors read and approved the final manuscript.

ETHICS DECLARATIONS

Ethics approval and consent to participate

Our study had been approved by the appropriate ethics committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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