Title page

Title: Ultra-low depth sequencing of plasma cell DNA for the detection of copy number aberrations in multiple myeloma

Short title: Ultra-low depth sequencing of plasma cell DNA in MM

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Abstract

Cytogenetic abnormalities are powerful prognostic factors in multiple myeloma (MM) and are routinely analyzed by FISH on bone marrow (BM) plasma cells (PC). Although considered the gold standard, FISH experiments can be laborious and expensive. Therefore, array-CGH (aCGH) has been introduced as an alternative approach for detecting copy number aberrations (CNAs), reducing the number of FISH experiments per case and yielding genome-wide information. Currently, next generation sequencing (NGS) technologies offer new perspectives for the diagnostic workup of malignant disorders. In this study, we examined ultra-low depth whole genome sequencing (LDS) as a valid alternative for aCGH for the detection of CNAs in BM PCs in MM. To this end, BM aspirates obtained in a diagnostic setting from 20 MM cases were analyzed. CD138+ cell-sorted samples were subjected to FISH analysis. DNA was extracted for subsequent aCGH and LDS analysis. CNAs were detected by aCGH and LDS in all but one case. Importantly, all CNAs identified by parallel first generation aCGH analysis were also detected by LDS, along with six additional CNAs in five cases. One of these additional aberrations was in a region of prognostic importance in MM and was confirmed using FISH. However, risk stratification in these particular cases was unaffected. Thus, a perfectly concordant prognostication between array-CGH and LDS was observed. This validates LDS as a novel and cost-efficient tool for the detection of CNAs in MM.

Keywords: Molecular cytogenetics, Multiple myeloma, Prognostic factor, Ultra-low depth sequencing

Introduction

Plasma cell neoplasms (PCN) are characterized by the accumulation of clonal plasma cells (PC) that typically secrete a monoclonal immunoglobulin. Multiple myeloma (MM) is a bone marrow-based multi-focal PCN with a clonal bone marrow (BM) PC percentage of $\geq 10\%$ or biopsy-proven plasmacytoma combined with at least one additional myeloma-defining event¹. It is a heterogeneous disease with a high variability in clinical outcome. Patients with low-risk MM may be asymptomatic and have an overall survival (OS) of > 10 years, whereas the OS for high-risk patients is < 2 years². Aside from clinical biomarkers [e.g. presence of osteolytic lesions, renal failure, anemia and levels of beta-2-microglobulin ($\beta 2M$) and serum albumin], chromosomal abnormalities are imperative to define prognostic groups in MM³. MM cases can be classified according to their primary event as hyperdiploid (HD) - defined as having more than 47 chromosomes in MM and often characterized by numerical gains of odd number chromosomes - and non-hyperdiploid (NHD) often characterized by translocations in the IGH locus². While hyperdiploidy is associated with longer OS, the prognostic impact of *IGH* translocations depends on the translocation partner. Additional hallmark genomic aberrations include loss of 17p and 1p and gain of 1q, all associated with adverse outcome⁴. Most relevant in identifying high-risk patients are $t(4;14)(p16;q32)$, $t(14;16)(q32;q23)$ and $del(17)(p13)$, recommended by the International Myeloma Work Group (IMWG) as the minimum required genetic testing panel. A more comprehensive panel includes examination for $t(11;14)(q13;q32)$, $t(14;20)(q32;q13)$, $del(1)(p)$, $gain(1)(q)$ and ploidy status^{2,5,6}. Moreover, emerging data suggest a potential of cytogenetic signatures for therapeutic stratification of MM patients⁷.

The current gold standard for detecting these cytogenetic events in MM is fluorescence in situ hybridization (FISH). However, this approach can be costly and labor-intensive, depending on the number of regions to be investigated, and provides only limited information. Currently, array-comparative genomic hybridization (aCGH) is often performed in parallel with a reduced FISH panel for the detection of genome-wide copy number aberrations (CNA) and balanced translocations, respectively. As such, the number of FISH experiments as well as the cost of diagnostic testing in MM can be reduced. However, BM samples often yield limited numbers of plasma cells after purification,

at times insufficient for the high input requirements of aCGH. Alternatively, ultra-low depth whole genome sequencing (LDS) is a cost-effective approach for large-scale genomics with high throughput and low input and hence could improve CNA detection, as demonstrated already in the field of constitutional genetics⁸.

In this study, we examined whether LDS could be an inexpensive alternative for aCGH for the clinical diagnostics of MM. For this purpose, purified PCs from 20 MM cases were analyzed by aCGH and LDS in parallel. Copy number profiles obtained by both techniques were compared to each other and to gold standard FISH analysis.

Material and methods

Sample selection

BM aspirates and peripheral blood samples were collected at the University Hospitals Leuven from an unselected series of 20 patients on the occasion of diagnostic workup for MM¹. The study was approved by the Ethical Committee (Belgian number B32220141013, study S56534/ML10468, date of approval April 4, 2014) and all patients provided written informed consent.

Plasma cell isolation

Within 48 hours after sample collection, PCs were isolated from BM aspirates using the EasySep™ Human CD138 Positive Selection Kit (StemCell Technologies Inc., Vancouver, Canada), enabling a PC purity of at least 90%. DNA was then isolated by phenol-chloroform extraction and quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Life Technologies, Waltham, MA).

Fluorescence in situ hybridization

Purified PCs (for two cases in combination with cytoplasmic immunoglobulin staining) were prepared for FISH using cytospin⁹ and examined with following FISH probes; IGH rearrangements were detected using a break-apart probe (LSI IGH DC BA, Abbott, Chicago, IL) and subsequently, translocation partners were identified using dual-fusion probes (4p16: LSI IGH (SG)/FGFR3 (SO) (DC DF), 11q13: LSI IGH (SG)/CCND1-XT (SO) (DC DF), 16q23: LSI IGH (SG)/CMAF (SO) (DC DF), all from Abbott); numerical probes were used to assess del(17)(p13) (LSI TP53 (SO)/CEP17 (SG), Abbott) and gain(1)(q21)(CKS1B (SG)/CEP1 (SO), homemade + Abbott). Ploidy was evaluated based on three probes D1Z5, D9Z1 and D17Z1, as previously described¹⁰. Cases with two out of these three centromeres gained were

described as hyperdiploid, cases with only D9Z1 gained as “presumably hyperdiploid”. Additional FISH probes used to confirm aberrations detected by LDS are listed in Supplemental Table 1. Probes were evaluated by averaging the counts of two independent observers, each scoring a minimum of 50 nuclei, and interpreted using a 10% cut-off for fusion or break-apart probes and a 20% cut-off for numerical probes, as defined by the European Myeloma Network (EMN)^{11,12}.

Library preparation and sequencing

Input DNA was sheared into 150 bp fragments by the Covaris M220 (Covaris, Woburn, MA) and fragment sizes were confirmed using the DNF-486 High Sensitivity NGS Fragment Analysis Kit. Libraries were prepared from 50 or 10 ng DNA on the Sciclone GC Liquid Handling Workstation using the KAPA HTP Library Prep Kit (Roche, Basel, Switzerland) and amplified by 7 or 13 PCR cycles respectively. Subsequently, they were sequenced (35 bp, single-end reads) on the HiSeq 4000 platform (Illumina, CA, USA).

LDS CNA calling

Copy numbers were called using QDNAseq¹³, implementing a read depth-based approach in non-overlapping genomic bins – set to 100 kb in this study. In short, copy number estimates are obtained by counting the number of sequencing reads mapping to each bin and comparing this to read counts in a reference genome. Resulting log2 ratios are then interpreted using cut-off log2 values for gains (0.1375) and losses (-0.152), calculated theoretically based on aforementioned EMN recommendations for detecting numerical abnormalities using FISH. Subclonal aberrations (in 5 – 20% of purified PCs) were called using correspondingly calculated thresholds (gains: [0.036 ; 0.1375], losses: [-0.037 ; -0.152]) and were only reported if validated by FISH and in case of prognostic importance. Only events present in at least three consecutive bins were considered, generating a theoretical detection limit of 300 kb. The feasibility of LDS to detect CNAs down to 5% was validated in serial dilution experiments on a mixture of two samples, each harboring one gain or loss (data not shown, available upon request). Data visualization and interpretation was improved by the development of a CytoSure software plugin by Oxford Gene Technologies, allowing a rapid and reliable analysis analogous to array-CGH.

Array-CGH

Array-CGH was done on 50 – 500 ng of purified PC samples using Cytosure Haematological Cancer +SNP (8x60K) arrays (Oxford Gene Technologies, Oxford, UK), with same-sex reference pools as controls. Dye-swap experiments were carried out to correct for dye bias. Arrays were analyzed with the CytoSure Interpret software (OGT, Oxford, UK) using the same thresholds as defined for LDS. Interpretation and visual revision of CNA profiles was done as described previously^{14,15}. Where necessary, diploid baseline values were adjusted based on FISH results.

RESULTS

Patient characteristics

We have analyzed BM samples of 20 patients with MM, four of which were included at the time of progression or relapse, while the remaining cases were newly diagnosed. The PC percentage in BM aspirates ranged from 9 to 97% with a median of 30,6% (Table 1). In one patient, BM collection was insufficient for PC separation, despite being done in a localized lesion. As this aspirate contained 43% PC, it was subjected to downstream analysis as such. Initial risk assessment was done using FISH

according to IMWG guidelines² and identified 11 patients with standard- and 9 with high-risk disease. Of the latter, four cases were standard-risk at diagnosis but acquired adverse alterations at relapse (i.e. gain of 1q and/or deletion of 17p13).

Detection of CNAs in diagnostic MM cases

FISH

For all MM cases, a comprehensive FISH panel was performed at diagnosis, assessing IGH translocations, del(17)(p13), gain(1)(q21) and ploidy status (Fig 1). Using this panel, abnormal patterns were observed in 19 patients, 12 (63%) of which with HD and 7 (37%) with NHD. Translocations involving the IGH locus were present in 12 patients. The incidence of individual aberrations detected by FISH is shown in Fig 1. Additional FISH analysis of case MM17 to find the IGH translocation partner revealed a subclonal t(8;14)(MYC).

LDS

All samples were sequenced at a mean depth of coverage of 0.15X with an average of 501 uniquely mappable reads per bin. Using QDNAseq, genomic aberrations were detected in 19 cases (all but MM31), with an average of 10 CNAs per sample and CNA sizes ranging from 600 kb to 172 Mb for focal CNAs. Although the theoretical resolution of this assay is 300 kb, aberrations as small as 100 kb were detected by LDS in our daily practice and confirmed using FISH (e.g. region 14q32/*IGH*), implying a possibly lower detection limit.

Array-CGH

Array analysis revealed genomic abnormalities in the same 19 patients as LDS with an average of 10 CNAs per sample and CNA sizes ranging from 600 kb to 151 Mb for focal aberrations. The theoretical resolution of our array is 200 kb for deletions and 270 kb for duplications.

Comparison of FISH, array-CGH and LDS

Concordance of FISH versus LDS and array-CGH

Comparing CNAs observed by FISH, array-CGH and LDS revealed a high concordance between all techniques. Obviously, array-CGH and LDS allowed comparison of genome-wide copy number data, while FISH only interrogated numerical aberrations in three defined regions and ploidy status. Routine FISH analysis identified CNAs – with or without translocations – in 13 cases and unbalanced translocations in two (MM25 and MM38), that were all confirmed by LDS/aCGH. The remaining five cases carried no aberrations (MM6) or only balanced (MM4, MM27, MM31 and MM35) translocations according to FISH, though LDS/aCGH revealed additional (secondary) CNAs not included in the FISH panel in four (Supplemental Table 2). Del(17)(p13) was observed in four cases (MM3, MM26, MM38 and MM53) by aCGH and LDS and were validated in FISH in 85%, 70%, 41% and 73% of PCs respectively. Of course, no consensus has been reached yet on the most clinically relevant cut-off for del(17)(p13).

Genome-wide comparison LDS versus aCGH

A genome-wide comparison of CNAs detected by LDS and aCGH shows a nearly perfect concordance, quantified by the fraction of the genome with identical CNA calls by both techniques (Fig 2). On average, LDS and aCGH were concordant in 98.2% of loci, with all aberrations detected by aCGH confirmed by LDS. However, in five cases, LDS revealed additional CNAs, one of which in a region of prognostic importance (MM29, gain(1)(q21)). As this patient already carried a t(4;14)(p16;q32), this did not change the prognosis. Ploidy was initially incorrectly estimated by aCGH in two and by LDS in one case, according to FISH results. Chromothripsis was observed in three patients by both LDS and aCGH.

Risk stratification

In line with the high level of agreement in CNAs detected by FISH, array-CGH and LDS, risk stratification based on these cytogenetic features showed a perfect concordance between all three techniques (Fig 1). Of note, risk stratification for aCGH and LDS results included the reduced FISH panel for IGH translocations.

Implementation

After extensive validation, we implemented LDS in our routine diagnostic setting for MM. Of 293 MM cases obtained from April 2018 to August 2019, LDS analysis was successful in 287 (98 %). Of note, in 38 cases (13%), DNA collection was limiting for aCGH, though sufficient for LDS. LDS failure could be attributed to suboptimal PC purification performed > 48 hours after sample collection for five cases and to lack of CD138 expression in one case, as confirmed by flow cytometry in a subpopulation of PCs. Prognostication could be rescued by additional FISH experiments in five cases, and LDS on a new bone marrow sample in one case.

Discussion

The prognostic assessment of MM patients relies on the presence of cytogenetic features, investigated by FISH on purified PCs as the golden standard. Currently, the diagnostic work-up of MM by several (cyto)genetic labs combines FISH with aCGH to reduce the number of labor-intensive and costly FISH experiments. Alternatively, ultra-low depth whole genome NGS (LPWGS) could allow a less expensive detection of CNAs, as validated in several malignancies, including a small pilot study in MM^{16–18}. At our institution, average total costs were reduced by 33%. As such, the aim of this study was to evaluate the feasibility of introducing LDS in clinical diagnostics of MM as an alternative for aCGH. To this extent, we have examined BM samples of 20 patients with newly diagnosed or relapse MM. As a part of our routine clinical work-up, we performed FISH on purified PCs for the interrogation of IGH translocations, del(17)(p13), gain(1)(q21) and ploidy status. For a genome-wide examination of genomic imbalances, aCGH and LDS were carried out in parallel and compared LDS data interpretation was facilitated by the Cytosure software plugin. While aCGH required at least 100 ng DNA, including dye-swap experiments, LDS generated high-quality data from as little as 2.5 ng. Of note, more advanced arrays are optimized to handle lower DNA inputs, yet still well above LDS input requirements. Since implementing LDS in routine diagnostic setting, 293 cases have been analyzed, 38 (13%) of which had insufficient DNA for aCGH analysis and were successfully resolved using LDS. Moreover, LDS is less labor-intensive and can be done at roughly two thirds of the cost of FISH alone.

Overall, there was an excellent agreement between CNAs observed by aCGH and LDS with concordant calls in over 98% of loci. Interestingly, LDS could detect all CNAs that were observed by aCGH, as well as six additional aberrations in five cases, validated by FISH. These aberrations were specific for MM and could possibly alter stratification, but did not for these particular cases. In fact, there was a perfect concordance in risk stratification by all three methods. This confirms the technical feasibility of using LDS as an alternative to aCGH for the identification of genome-wide CNAs in MM. Of note, the lower sensitivity of aCGH may be attributed to the low quality of the first-generation arrays used in this study in particular or to non-uniform probe spacing and the admixture of non-informative probes in aCGH in general. Though theoretically the resolution of aCGH is higher, the robustness of LDS enables a reliable detection of similar and even smaller aberrations and is not limited to predefined probe positioning. Recently, novel NGS strategies were reported enabling the detection of both CNAs and translocations using a single assay, either genome-wide¹⁹ or using a custom targeted NGS panel^{20–22}. While there is no denying the technical advantage of identifying structural rearrangements and CNAs simultaneously, these methods are still limited to research context and either require larger DNA input amounts or higher sequencing depths as compared to our strategy. Roughly estimated, this would increase the cost 1.5 to twofold (Supplemental Table 3). Therefore, we believe that the

approach described in this study remains a cost-effective, high-throughput asset for routine MM diagnostics in a clinical setting.

In the context of refining the existing cytogenetic risk model in MM (e.g. through the definition of a cytogenetic prognostic index²³, the characterization of a high-risk 'double-hit' subgroup²⁴, the incorporation of chromothripsis as an adverse factor²⁵), finding the most (cost-)efficient approach for routine clinical testing is imperative. This study demonstrates the potential strength of a dual analysis using FISH and LDS for the detection of translocations and CNAs respectively.

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Data sharing statement

The data that support the findings of this study are available in the supplementary material of this article.

Figure legends

Figure 1. Comparison of the minimal testing panel results and consequent risk stratification using FISH (upper), array-CGH (middle) and LDS (lower). For each patient, events observed by FISH, array-CGH and LDS are shown in the first, second and third row, respectively. Presence of cytogenetic events is indicated in red, absence in green. Discrepancies are indicated with a black rectangle. Risk categories were determined for each method separately according to IMWG guidelines. Risk stratification for LDS and aCGH included the reduced FISH panel for IGH translocations.

Figure 2. Genome-wide comparison of autosomal copy number gains (red) and losses (green) detected by aCGH (upper row) and LDS (bottom row for each patient).

Tables

Table 1. Clinical patient characteristics.

Patient	Age (y)	Sex	ISS	Anemia ^c	Serum Albumin (g/L)	B2M (mg/L)	Paraprotein (g/L)	PC in BM aspirate (%)
MM2	78	M	I	N	38.1	2.79	28.7	13.3
MM3	69	M	NA	Y	40.2	NA	28.2	97
MM4	72	M	III	N	38.6	6.6	39.2	43 ^a
MM6	68	F	II	N	30.9	2.54	12.4	14
MM9	65	M	II	N	25.5	4.28	46.7	47
MM10	80	F	N	N	38.9	N	40.4	25
MM13	72	M	II	Y	34.1	5.43	47.1	48.3
MM17	68	F	II	Y	33.9	3.88	47.4	66.7
MM18	68	F	NA	Y	37.8	NA	39.4	30
MM22	70	M	II	Y	33.1	4.85	11.6	18
MM25	66	M	II	Y	27.1	4.42	80	31.3
MM26	55	F	III	Y	30.5	7.98	69	75.3
MM27	72	M	NA	N	40.1	NA	29.7	32.3
MM29	74	M	NA	N	41.3	NA	14.3	21.3
MM31	57	M	II	N	44.7	5.48	25.2	33.7
MM33	72	F	II	Y	42.3	4.12	17.1	20.7
MM35	53	M	I	N	39.4	2.81	39.1	20.3
MM38	60	F	I	Y	42.6	2.86	28.2	9 ^b
MM46	57	F	I	N	48.8	1.98	/ ^d	13.3
MM53	50	F	I	N	38.4	2.92	28.3	73.3

^a % PC in localized lesion instead of BM aspirate. ^b A higher percentage locally, ditto in the bone marrow biopsy. ^c Anemia: a hemoglobin value of <100g/L. ^d Oligosecretory kappa restrictive MM.

ISS, International Staging System for plasma cell myeloma; B2M, Serum beta-2 microglobulin; PC, plasma cells; M, Male; F, Female; NA, not available.

The four MM patients which were included at the time of progression or relapse are depicted in **bold**.

Figure 1. Comparison of the minimal testing panel results and consequent risk stratification using FISH (upper), array-CGH (middle) and LDS (lower). For each patient, events observed by FISH, array-CGH and LDS are shown in the first, second and third row, respectively. Presence of cytogenetic events is indicated in red, absence in green. Discrepancies are indicated with a black rectangle. Risk categories were determined for each method separately according to IMWG guidelines. Risk stratification for LDS and aCGH included the reduced FISH panel for IGH translocations.

Figure 2. Genome-wide comparison of autosomal copy number gains (red) and losses (green) detected by aCGH (upper row) and LDS (bottom row for each patient).