Circulating tumor DNA as a liquid biopsy in plasma cell dyscrasias

Multiple myeloma (MM) is a clinically and genetically heterogeneous malignant proliferation of plasma cells (PCs) with a typical multifocal distribution in the bone marrow (BM) and occasional extra-medullary dissemination.[1] Advances in the genetic knowledge of MM are increasingly translated into biomarkers to refine diagnosis, prognostication and treatment of patients.[2]

MM genotyping has so far relied on the analysis of purified PCs from the bone marrow (BM) aspirate, which may fail in capturing the postulated spatial heterogeneity of the disease and imposes technical hurdles limiting its transfer in the routine and clinical grade diagnostic laboratory. In addition, longitudinal monitoring of disease molecular markers may be limited by patient discomfort caused by repeated BM samplings during disease course.

Table 1A. Somatic non-synonymous mutations discovered by cfDNA genotyping and their validation in tumor gDNA.

ID Sample	Genes	CHR	Absolute position*	REF	VAR	cDNA change§	Protein change	cfDNA allele fraction	gDNA allele fraction
ID1	*CYLD*	chr16	50820803	A	T	c.1987A>T	p.R663W	0.95%	26.75%
ID2	*KRAS*	chr12	25380276	T	A	c.182A>T	p.Q61L	25.01%	44.72%
ID3	*NRAS*	chr1	115258747	C	A	c.35G>T	p.G12V	3.08%	63.07%
ID5	*KRAS*	chr12	25380279	C	T	c.179G>A	p.G60D	1.05%	15.42%
ID7	*FAM46C*	chr1	118166229	T	C	c.739T>C	p.Y247H	3.82%	53.38%
ID7	*NRAS*	chr1	115256529	T	C	c.182A>G	p.Q61R	6.72%	54.57%
ID7	*TRAF3*	chr14	103363617	A	-	c.839_839delA	p.E280fs*3	9.66%	76.97%
ID8	*CYLD*	chr16	50813911	G	A	c.1474G>A	p.G492S	0.87%	3.93%
ID11	*KRAS*	chr12	25398281	C	T	c.38G>A	p.G13D	4.39%	16.82%
ID12	*NRAS*	chr1	115256529	T	C	c.182A>G	p.Q61R	3.33%	35.14%
ID13	*NRAS*	chr1	115256530	G	T	c.181C>A	p.Q61K	32.52%	19.11%
ID15	*DIS3*	chr13	73337723	C	T	c.1993G>A	p.E665K	37.86%	86.29%
ID15	*TP53*	chr17	7578269	G	A	c.580C>T	p.L194F	36.29%	81.79%
ID17	*TP53*	chr17	7577610	T	A	c.673-2A>T	p.224?	8.84%	79.53%
ID18	*IRF4*	chr6	394920	G	T	c.316G>T	p.D106Y	1.48%	39.08%
ID18	*TRAF3*	chr14	103336686	A	G	c.148A>G	p.K50E	0.29%	4.86%
ID19	*FAM46C*	chr1	118165764	G	C	c.274G>C	p.D92H	0.68%	6.98%
ID19	*NRAS*	chr1	115256521	A	C	c.190T>G	p.Y64D	0.65%	9.97%
ID21	*NRAS*	chr1	115256529	T	G	c.182A>C	p.Q61P	0.54%	26.06%
ID21	*TP53*	chr17	7578406	C	T	c.524G>A	p.R175H	0.73%	38.91%
ID26	*FAM46C*	chr1	118165699	G	C	c.209G>C	p.R70P	1.22%	5.16%
ID26	*FAM46C*	chr1	118166036	C	G	c.546C>G	p.D182E	5.35%	18.83%
ID26	*NRAS*	chr1	115256529	T	C	c.182A>G	p.Q61R	16.08%	32.59%
ID26	*NRAS*	chr1	115256530	G	T	c.181C>A	p.Q61K	11.55%	15.04%
ID27	*DIS3*	chr13	73337723	C	T	c.1993G>A	p.E665K	0.64%	51.36%
ID27	*TRAF3*	chr14	103363719	C	T	c.941C>T	p.S314F	0.42%	33.81%
ID28	*BRAF*	chr7	140453136	A	T	c.1799T>A	p.V600E	1.43%	32.88%
ID29	*KRAS*	chr12	25398281	C	T	c.38G>A	p.G13D	11.36%	43.4%

Table 1B. Somatic non-synonymous mutations discovered in tumor gDNA genotyping and missed in plasma cfDNA.

ID Sample	Gene	CHR	Absolute position*	REF	VAR	cDNA change§	Protein change	cfDNA allele fraction	gDNA allele fraction
ID3	*TP53*	chr17	7577570	C	T	c.711G>A	p.M237I	–	3.31%
ID3	*TP53*	chr17	7577121	G	A	c.817C>T	p.R273C	–	1.83%
ID6	*CYLD*	chr16	50785530	C	T	c.520C>T	p.174Q*	–	2.44%
ID8	*CYLD*	chr16	50785572	C	T	c.562C>T	p.188Q*	–	4.88%
ID8	*KRAS*	chr12	25380275	T	A	c.183A>T	p.Q61H	–	1.14%
ID8	*NRAS*	chr1	115256530	G	T	c.181C>A	p.Q61K	–	2.55%
ID14	*CYLD*	chr16	50828193	G	A	c.2540G>A	p.W847*	–	4.96%
ID18	*SP140*	chr2	231176307	C	A	c.2502C>A	p.Y834*	–	2.43%
ID18	*ZNF462*	chr9	109686963	G	T	c.770G>T	p.R257L	–	3.5%
ID19	*KRAS*	chr12	25398285	C	T	c.34G>A	p.G12S	–	1.46%
ID19	*NRAS*	chr1	115258747	C	G	c.35G>C	p.G12A	–	3.58%

CHR: chromosome; REF: reference allele; VAR: variant allele. *Absolute chromosome coordinates of each variant based on the hg19 version of the human genome assembly. §cDNA change determined on the following RefSeq: NM_015247.2 for *CYLD*, NM_033360.3 for *KRAS*, NM_002524.4 for *NRAS*, NM_017709.3 for *FAM46C*, NM_003300.3 for *TRAF3*, NM_014953.3 for *DIS3*, NM_000546.5 for *TP53*, NM_002460.3 for *IRF4*, NM_004333.4 for *BRAF*, NM_007237.4 for *SP140*, NM_021224.4 for *ZNF462*.

Circulating tumor DNA is shed into the peripheral blood (PB) by tumor cells and can be used as source of tumor DNA for the identification of cancer-gene somatic mutations, with obvious advantages in terms of accessibility. In addition, the systemic origin of cell-free DNA (cfDNA) allows catching the entire tumor heterogeneity.[3] Tumor cfDNA was identified in MM patients by preliminary studies tracking the clonotypic V(D)J rearrangement as disease fingerprint,[4] or genotyping a highly restricted set of cancer genes that were not specifically addressed to resolve the typical MM mutational landscape.[5-7] We developed a CAPP-seq ultra-deep targeted next-generation sequencing (NGS) approach to genotype a gene panel specifically designed to maximize the mutation recovery in plasma cell tumors, and compared the mutational profiling of cfDNA and tumor genomic DNA (gDNA) of purified PCs from BM aspirates in a consecutive series of patients representative of different clinical stages of PC tumors ranging from monoclonal gammopathy of undetermined significance (MGUS), to smoldering MM, and symptomatic MM.

The study was based on a series of 28 patients with PC disorders, whose clinical and molecular characteristics were consistent with an unselected cohort of PC dyscrasia patients (*Online Supplementary Table S1*) [two had MGUS, five smoldering MM (SMM), and 21 symptomatic MM]. The study was conducted according to good clinical practice and the ethical principles outlined in the Declaration of Helsinki. All patients provided written informed consent. The following material was collected: cfDNA isolated from plasma; tumor gDNA from CD138$^+$ purified BM PCs for comparative purposes, and germline gDNA extracted from PB granulocytes after Ficoll gradient separation, to filter out polymorphisms. The sampling was done in 25 newly diagnosed and three relapsed/refractory treated patients. A targeted resequencing gene panel, including coding exons and splice sites of 14 genes (target region: 31 kb: *BRAF, CCND1, CYLD, DIS3, EGR1, FAM46C, IRF4, KRAS, NRAS, PRDM1, SP140, TP53, TRAF3, ZNF462; Online Supplementary Table S2*) was specifically designed and optimized to allow a priori the recovery of at least one mutation in 68% (95% confidence interval: 58-76%) of patients, based on literature data.[8-10] Ultra-deep NGS was performed on MiSeq (Illumina) using the CAPP-seq library preparation strategy (NimbleGen).[11] The somatic function of VarScan2 was used to call non-synonymous somatic mutations, and a stringent bioinformatic pipeline was developed and applied to filter out sequencing errors (detection limit $3x10^{-3}$). The sensitivity and specificity of plasma cfDNA genotyping were calculated in comparison with tumor gDNA genotyping as the gold standard. Details of the experimental procedures are given in the *Online Supplementary Methods.*

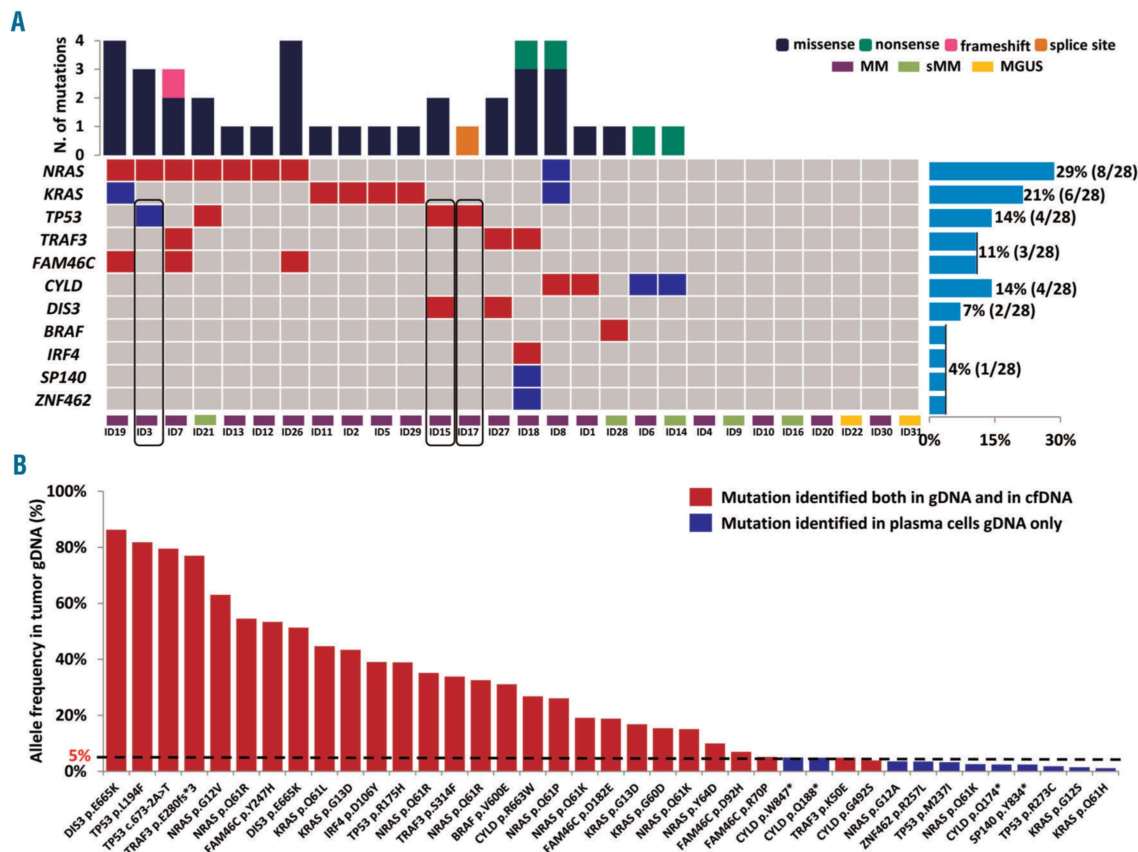

Figure 1. Overview of the mutations identified in the PC dyscrasia series. (A) Mutations detected in plasma cfDNA and confirmed in tumor gDNA are filled in red; mutations detected in tumor gDNA only are filled in blue. Each column represents one tumor sample and each row represents one gene. The fraction of tumors with mutations in each gene is plotted (right). The number and the type of mutations in a given tumor are plotted above the heat map. Patients positive for del(17p) are framed in black. (B) Bar graph of the allele frequencies in tumor gDNA of the variants that were discovered in plasma cfDNA (red bars) or missed in plasma cfDNA (blue bars). The dashed line tracks the 5% allelic frequency threshold.

cfDNA was detectable in plasma samples with an average of ~11 000 haploid genome-equivalents per mL of plasma (range: 19-52562 hGE/mL; median: 6617 hGE/mL). The amount of cfDNA correlated with clinic-pathological parameters reflecting tumor load/extension, including BM PC infiltration (Spearman's rho coefficient=0.42, *P*=0.02; *Online Supplementary Figure S1A*), and clinical stage. Indeed patients presenting with ISS stage 3 had significantly higher amounts of cfDNA compared with MGUS/SMM samples and MM cases at ISS stages 1-2 (*P*=0.01; *Online Supplementary Figure S1B*, Mann-Whitney test). Conversely, we did not observe differences in cfDNA concentration between newly diagnosed and relapsed/refractory MM patients (*data not shown*). More than 90% of the target region was covered >1000X in all plasma samples, and >2000X in 23/28 (*Online Supplementary Figure S2* and *Online Supplementary Table S3*). Overall, within the interrogated genes, 18/28 (64%) patients had at least one non-synonymous somatic mutation detectable in cfDNA (Figure 1A and Table 1A); 28 total variants were identified, with a range of 1-4 mutations per patient. Quite consistent with the typical spectrum of mutated genes in MM, plasma cfDNA genotyping revealed somatic variants of *NRAS* in 25%; *KRAS* in 14%; *TP53, TRAF3* and *FAM46C* in 11%, respectively, *CYLD* and *DIS3* in 7%, respectively, and *BRAF* and *IRF4* in 4% of cases, respectively. Variants in *NRAS, KRAS* and *BRAF* genes occurred in a mutually exclusive manner, and they overall involved 43% of patients. *TP53* mutations were positively associated with the deletion of the remaining allele as revealed by fluorescence in situ hybridization on purified PCs (*P*=0.02, Fisher-exact test). Overall, the molecular spectrum of mutations discovered in tumor cfDNA reflected previous observations in genomic studies based on PC genotyping (see representative example for the two most frequently mutated genes in *Online Supplementary Figure S3*), thus supporting the tumor origin of the mutations identified in cfDNA.

To validate the tumor origin of mutations discovered in cfDNA and to derive the accuracy of our approach in resolving tumor genetics, the genotype of cfDNA was matched with that of gDNA from purified BM PCs in all the patients. Sequencing of tumor gDNA identified 39 somatic mutations in 20/28 (71.4%) patients (Figure 1A). cfDNA genotyping correctly identified 72% of mutations (n=28/39) that were discovered in tumor PCs (*Online Supplementary Figure S4A*); overall the variant allele frequencies in plasma samples correlated with those in tumor biopsies (Pearson correlation coefficient=0.58, *P*=9.6e-05; *Online Supplementary Figure S4B*) and with the degree of bone marrow involvement (Pearson correlation coefficient=0.5, *P*=0.006). Specifically, of the 28 mutations correctly identified in tumor cfDNA, four were detected in two SMM patients out of a total of 7 biopsy-confirmed mutations (4/7, 57%) in three SMM patients, and 24 were detected in 16 MM cases out of a total of 32 biopsy-confirmed mutations (24/32, 75%) in 17 MM cases. Notably, BM PC confirmed mutations not discovered in cfDNA (n=11) had a low representation in the tumor (median allelic frequency: 2.5%; range: 1.1-4.96%) (Table 1B, Figure 1B). Since circulating tumor DNA is diluted in cfDNA from normal cells,[12,13] variants that are already rare in tumor gDNA are much less represented in plasma and may fall below the sensitivity threshold of the CAPP-seq under the experimental conditions adopted in this work. Consistently, based on ROC analysis, cfDNA genotyping has the best performance in detecting tumor PC confirmed mutations when they are represented in at least 5% of the alleles of tumor plasma cells (*Online Supplementary Figure S4C*). Above this threshold, cfDNA genotyping detected 100% of biopsy-confirmed mutations. Noteworthy, cfDNA genotyping was still able to detect almost half (10/21) of low-abundance mutations in tumor PCs (i.e., allelic frequency <20%), indicating a good capacity of tumor cfDNA to mirror also the subclonal composition of the tumor. Of course, these data concerning the sensitivity of cfDNA genotyping refer to the depth of coverage used in the paper, and higher depth may allow a better overlap of gDNA and cfDNA. In none of the cases cfDNA genotyping identified additional somatic mutations not detected in the purified BM PCs, thus suggesting that, as far as our limited patient cohort is concerned, the genotype of PC collected from a single tumor site is already representative of the entire tumor genetics. Alternatively, spatial genomic heterogeneity, supported by very recent findings in MM,[14] may exist but involving minor subclones not sufficiently represented to be detectable in plasma.

Our results provide the proof of principle that circulating tumor cfDNA genotyping is a feasible, non-invasive, real-time approach that reliably detects clonal and subclonal somatic mutations represented in at least 5% of alleles in tumor PCs. Despite the genetic heterogeneity characterizing MM, and the inclusion in the study cohort of seven patients at pre-malignant/asymptomatic disease stages, the designed gene-panel employed in our study proved to be very effective, in that it allowed the recovery of at least one mutation in tumor gDNA of 20/28 (71%) cases. To the best of our knowledge, this is the first gene panel specifically created to maximize mutational recovery in MM patients by using an affordable number of genes, and by virtue of this potentially effective and manageable even in clinical practice in a hopefully near future.

One of the original findings of the study is that cfDNA genotyping can resolve tumor genetics also in cases at early disease stages as SMM patients, who may benefit the most from this non-invasive approach. Indeed, among asymptomatic patients cfDNA genotyping could allow a non-invasive longitudinal molecular monitoring of clonal evolution and the identification of the switch point on which the disease acquires high-risk genetic features. This has been prevented so far by the unfeasibility of serial BM sampling in the clinical routine.

An immediate clinical application of cfDNA genotyping in MM could be the incorporation of this minimally-invasive method in clinical trials for the identification of patients carrying actionable mutations and their longitudinal genetic monitoring during targeted therapy administration or for the estimation of minimal residual disease.

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References

1. Kumar SK, Rajkumar V, Kyle RA, et al. Multiple myeloma. Nat Rev Dis Primers. 2017;3:17046.
2. Lionetti M, Neri A. Utilizing next-generation sequencing in the management of multiple myeloma. Expert Rev Mol Diagn. 2017;17(7):653-663.
3. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol. 2014;32(6):579-586.
4. Oberle A, Brandt A, Voigtlaender M, et al. Monitoring multiple myeloma by next-generation sequencing of V(D)J rearrangements from circulating myeloma cells and cell-free myeloma DNA. Haematologica. 2017;102(6):1105-1111.
5. Kis O, Kaedbey R, Chow S, et al. Circulating tumour DNA sequence analysis as an alternative to multiple myeloma bone marrow aspirates. Nat Commun. 2017;8:15086.
6. Mithraprabhu S, Khong T, Ramachandran M, et al. Circulating tumour DNA analysis demonstrates spatial mutational heterogeneity that coincides with disease relapse in myeloma. Leukemia. 2017;31(8):1695-1705.
7. Rustad EH, Coward E, Skytoen ER, et al. Monitoring multiple myeloma by quantification of recurrent mutations in serum. Haematologica. 2017;102(7):1266-1272.
8. Bolli N, Avet-Loiseau H, Wedge DC, et al. Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. Nat Commun. 2014;5:2997.
9. Kortuem KM, Braggio E, Bruins L, et al. Panel sequencing for clinically oriented variant screening and copy number detection in 142 untreated multiple myeloma patients. Blood Cancer J. 2016;6:e397.
10. Lohr JG, Stojanov P, Carter SL, et al. Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. Cancer Cell. 2014;25(1):91-101.
11. Newman AM, Bratman SV, To J, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014;20(5):548-554.
12. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol. 2013;10(8):472-484.
13. Abbosh C, Birkbak NJ, Wilson GA, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. Nature. 2017;545(7655):446-451.
14. Rasche L, Chavan SS, Stephens OW, et al. Spatial genomic heterogeneity in multiple myeloma revealed by multi-region sequencing. Nat Commun. 2017;8(1):268.