Monitoring multiple myeloma by quantification of recurrent mutations in serum

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ABSTRACT

Circulating tumor DNA is a promising biomarker to monitor tumor load and genome alterations. We explored the presence of circulating tumor DNA in multiple myeloma patients and its relation to disease activity during long-term follow-up. We used digital droplet polymerase chain reaction analysis to monitor recurrent mutations, mainly in mitogen activated protein kinase pathway genes *NRAS*, *KRAS* and *BRAF*. Mutations were identified by next-generation sequencing or polymerase chain reaction analysis of bone marrow plasma cells, and their presence analyzed in 251 archived serum samples obtained from 20 patients during a period of up to 7 years. In 17 of 18 patients, mutations identified in bone marrow during active disease were also found in a time-matched serum sample. The concentration of mutated alleles in serum correlated with the fraction in bone marrow plasma cells (r=0.507, n=34, $P<0.002$). There was a striking covariation between circulating mutation levels and M protein in ten out of 11 patients with sequential samples. When relapse evaluation by circulating tumor DNA and M protein could be directly compared, the circulating tumor DNA showed relapse earlier in two patients (3 and 9 months), later in one patient (4 months) and in three patients there was no difference. In three patients with transformation to aggressive disease, the concentrations of mutations in serum increased up to 400 times, an increase that was not seen for the M protein. In conclusion, circulating tumor DNA in myeloma is a multi-faceted biomarker reflecting mutated cells, total tumor mass and transformation to a more aggressive disease. Its properties are both similar and complementary to M protein.

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Introduction

Multiple myeloma is caused by proliferation of monoclonal plasma cells in the bone marrow and is the second most common hematologic malignancy.[1] The treatment options have improved markedly in recent years and led to prolonged survival.[2] However, the disease is still considered to be incurable. The typical course of multiple myeloma is repeated treatment responses followed by increasingly aggressive relapses. Ultimately, the disease becomes refractory to all treatment and the patient dies.

To assess disease progression and treatment response, clinicians rely on monitoring of the monoclonal immunoglobulin (M protein) secreted by the tumor cells as a biomarker for tumor mass.[3,4] However, some patients escape the traditional monitoring. Between 1-3% of patients have non-secretory multiple myeloma and no

detectable M protein.[5,6] Furthermore, 10% of newly diagnosed myeloma patients have oligo-secretory disease, defined as a baseline level of M protein that is too low to evaluate treatment response reliably by traditional methods.[3,7] These patients are challenging to monitor and are, therefore, often denied access to clinical trials.[7]

A promising new cancer biomarker is circulating tumor DNA (ctDNA), which may be extracted from serum or plasma.[8] DNA fragments are released from cancer cells as well as normal cells in the body during apoptosis and necrosis.[9,10] The cancer-derived fragments may be identified if they contain tumor-specific mutations or other genetic aberrations.[8] In studies of solid tumors, ctDNA has provided information about tumor mass and residual disease, as well as information about the tumor genome that could otherwise only have been obtained by a tumor biopsy.[11-15] Information about ctDNA in multiple myeloma lags behind as only a single study has so far been published.[16]

The somatic mutational landscape of multiple myeloma has been described in several studies.[17-21] Out of more than 6,000 genes in which coding mutations have been identified, 13 are mutated more frequently than predicted from the background mutation rate, suggesting that they are implicated in the development of the disease.[17,18] Among these recurrently mutated genes, *NRAS*, *KRAS* and *BRAF* in the mitogen activated protein (MAP) kinase pathway are most frequently mutated, occurring in bone marrow plasma cells from approximately 50% of patients at diagnosis. Moreover, activating mutations in the MAP kinase pathway are of interest because they are potential therapeutic targets.[22-25]

In this study, we explored ctDNA as a biomarker of multiple myeloma and focused on mutations in recurrently mutated genes including *NRAS*, *KRAS* and *BRAF*. We measured the concentrations of specific mutations in serum through several responses and relapses for up to 7 years in 20 patients and found a remarkable covariation with the concentration of M protein. However, in terminal aggressive disease, ctDNA appears to reflect the development of the disease better.

Methods

Study design and patients

We conducted a retrospective study measuring ctDNA in archived serum samples from patients with multiple myeloma. Mutations of interest were identified in a bone marrow biopsy or purified bone marrow plasma cells and subsequently measured in serum by mutation-specific digital droplet polymerase chain reaction (ddPCR). Patients were included based on the following criteria: (i) presence of one or more mutations in genes recurrently mutated in myeloma[17,18] and (ii) availability of relevant serum or plasma samples. Twenty patients from two sources were included in this study: one previously published study of the *BRAF*^{V600E} mutation in myeloma and an on-going whole exome sequencing (WES) study.[26] A flowchart describing the patients' inclusion in detail is presented in the Online Supplementary Material (*Online Supplementary Figure S1*).

Clinical data were obtained from the patients' records and archived blood smears were evaluated for the presence of plasma cells. All patients had given written consent. The study was approved by the Regional Committee for Medical and Health Research Ethics (2016/821).

Details about the following experimental procedures are provided in the *Online Supplementary Methods*.

Detection of mutations in serum by digital droplet polymerase chain reaction

Serum (n=249) and citrate-plasma (n=2) samples were obtained from the Norwegian Multiple Myeloma Biobank. DNA was extracted from a median sample volume of 1.8 mL (range, 0.4-3 mL) using a QiaAmp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany). To detect mutations, we used the ddPCR system QX100/200 from Bio-Rad Laboratories (Hercules, CA, USA).[27] Detailed assay information is presented in *Online Supplementary Table S1* and raw data examples in *Online Supplementary Figure S2*. Patients' samples were considered to be mutation-positive if the mutant concentration in the sample was higher than the 95% confidence interval of the assay-specific false positive rate (*Online Supplementary Table S2, Online Supplementary Figure S3*). The estimated number of mutant copies required in a sample to be considered mutation-positive ranged from 0.84 to 2.96 copies of mutated DNA (median 1.4). The quantity of mutated DNA in positive samples was reported in copies per mL of serum.

Whole exome sequencing

WES of purified plasma cells and matched germline controls was performed as previously described.[26] The target coverage of >100x was achieved for 85% of exonic regions. The limit of detection of WES was a mutated allele fraction of 2-4 % in the bone marrow sample.

Statistical analysis

Bivariate correlations were performed by the Spearman correlation rank test. The level of statistical significance with two-tailed *P*-values was $P<0.05$. Statistical analyses were carried out in SPSS v. 21 (IBM Corporation, Armonk, NY, USA).

Results

A summary of clinical and mutational data for each patient is given in Table 1.

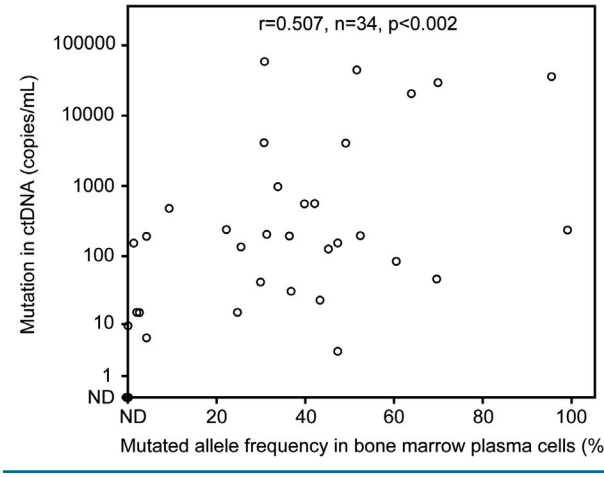

Figure 1. Correlation between mutation levels in bone marrow plasma cells and serum samples. Time-matched bone marrow and serum samples were obtained within 10 days of each other. Purified bone marrow plasma cells were analyzed by WES. In three cases in which WES was negative, positive results from the more sensitive ddPCR of bone marrow plasma cells were reported instead. Serum samples were analyzed by ddPCR.

Relation between tumor mutations in serum and bone marrow plasma cells

We started by determining whether mutations found in bone marrow plasma cells could be detected in time-matched serum samples by ddPCR, and found that this was the case for 17 of 18 patients (34 of 35 mutations). We examined the quantitative relationship between the concentrations of circulating mutated DNA and the allele fractions of the same mutations in bone marrow plasma cells. There was a moderate positive correlation between the two (r=0.507, n=34, P<0.002) (Figure 1). Thus, the concentration of a mutation in serum reflects the fraction of tumor cells harboring the same mutation.

Relation between levels of recurrent mutations and M protein in serum

Eleven patients had sequential serum samples available, spanning a median of 50 months (range, 8-90). In these patients, we monitored the concentration of mutated DNA over time in relation to tumor mass and treatment response as evaluated by M protein concentration. All 11 patients had a MAP kinase pathway mutation and two had at least one additional mutation (Table 1, patients 1-11). Most of these mutations were highly present in the bone marrow at diagnosis, with 75-100% mutation-positive plasma cells by immunohistochemistry or >50% mutated allele fraction by WES. Slightly lower MAP kinase mutated allele fractions of 34% and 26% were found at diagnosis in patients 2 and 5, respectively, and patient 11 had 25-50% *BRAF*^{V600E}-mutated cells by immunohistochemistry. No diagnostic bone marrow samples were available from patients 9 and 10.

The concentrations of MAP kinase mutations in serum showed marked covariation with M protein levels. For example, patient 1 (Figure 2A) was monitored by M protein as well as circulating *BRAF*^{V600E} mutation during 51 months, from diagnosis through five relapses until death. Every change in disease activity, as reflected by the M protein level, was accompanied by similar changes in serum *BRAF*^{V600E} mutation levels. Similar observations were made in ten of the 11 patients with available sequential samples (Figures 2 and 3 and *Online Supplementary Figure S4*). The observed covariation in ten patients was confirmed by a formal correlation analysis of 210 time-matched measurements of M protein and circulating MAP kinase pathway mutation with correlation coefficients ranging from 0.63 to 0.96 (*Online Supplementary Table S3*). Only in patient 10 (*Online Supplementary Figure S4B*) was there no correlation. In this patient the *BRAF*^{V600E} mutation became undetectable after being present at a very low concentration (<10 copies/mL) at an early time point.

An important aspect of ctDNA analysis in myeloma is its sensitivity, compared to conventional methods, to detect low levels of disease. When looking at the ability to

Table 1. Summary of clinical data and mutations.

Patient	Mutation(s)	Sex	Age (years)	Survival (months)	N. of treatments	M-Protein	ISS-stage	Hb (g/dL)	Ca-corr (mmol/L)	Creatinine (μmol/L)	Bone disease
1	BRAF V600E	M	69	51	6	IgA kappa	-	-	-	-	-
2	NRAS Q61K	F	66	40	4	IgA kappa	3	8.7	2.53	105	Yes
3	KRAS Q61H	F	77	35*	2	IgG kappa	2	11.9	2.71	58	No
4	FAM46C S27Y, IRF4 K123R, KRAS A146P	F	54	52	6	IgG kappa	2	11.9	2.31	71	-
5	KRAS Q61R, TP53 Y236N	M	58	9	2	IgG kappa	1	15.3	2.42	54	-
6	BRAF V600E	F	81	64	2	IgG kappa	3	12.8	2.51	94	Yes
7	BRAF V600E	M	57	77	10	IgG lambda	-	11.9	-	107	No
8	BRAF V600E	F	68	104*	3	IgG lambda	1	14.2	2.26	60	Yes
9	KRAS Q22K	F	61	107	9	IgG lambda	2	10.9	2.36	61	Yes
10	BRAF V600E	M	54	79	6	Lambda	1	14.1	2.53	70	Yes
11	BRAF V600E	M	75	22	3	Lambda	3	7.8	3.53	248	No
12	NRAS G12D	F	48	58*	4	IgA kappa	-	10.9	-	82	Yes
13	NRAS Q61K	M	67	55*	2	IgA kappa	3	9.9	3.2	81	-
14	DIS3 H788R, NRAS Q61R	M	73	24	3	IgA kappa	1	14.5	2.31	93	Yes
15	NRAS G12A	M	68	23*	2	IgG kappa	1	14.1	2.23	30	-
16	NRAS Q61R	M	61	33*	2	IgA lambda	2	9.4	-	-	Yes
17	NRAS Q61K	M	50	32*	2	IgA kappa	2	12.3	2.76	97	No
18	KRAS Q22K	F	83	28*	3	IgA kappa	3	12.6	2.27	63	Yes
19	BRAF V600E, NRAS Q61K	F	69	46*	1	IgA lambda	2	10	2.44	79	Yes
20	BRAF V600E	M	64	42	2	Lambda	2	11.3	2.85	125	Yes

Clinical parameters are reported from the time of diagnosis of multiple myeloma. Survival is calculated from the date of diagnosis to the date of death or last observation. M: male; F: female; *: patient still alive; -: missing data; ISS: International staging System; Ca-corr: albumin-corrected serum calcium; Hb: hemoglobin.

predict relapse, we found that serum mutation levels tended to increase before or at the same time as M protein in most cases in which the two methods could be compared (Figures 2 and 3 and *Online Supplementary Figure S4*). Notably, a relapse from complete remission in patient 2 (Figure 2B) was detected by ctDNA 9 months before M protein became detectable. Relapses were also detected earlier by ctDNA in patient 1 (Figure 2A), although with somewhat shorter lead-times. On the other hand, in patient 11 (*Online Supplementary Figure S4F*) the second relapse was heralded by an increase in urine M protein 4 months before ctDNA became detectable. Furthermore, ctDNA often became undetectable during periods of remission even though low levels of M protein were still detectable, or the concentration of ctDNA would fluctuate around the limit of detection. In summary, ctDNA showed relapse earlier in two patients (3 and 9 months), later in one patient (4 months) and in three patients there was no difference.

In patient 3 (Figure 2C), we initially detected a *KRAS*^{Q61H} mutation in plasma cells by WES as well as by ddPCR of serum. Light chain escape occurred at the second relapse, 38 months after the start of treatment, when the tumor cell secretion converted from IgA-κ to κ chains only. Despite this change, we could monitor the disease by the serum concentration of *KRAS*^{Q61H} mutation.

In two patients, we monitored one or two recurrent mutations in addition to the MAP kinase pathway mutations (Figure 3A,B). Patient 4 (Figure 3A) had an *IRF4* mutation highly present in bone marrow plasma cells at the last relapse. At diagnosis, this mutation was not detected in plasma cells by WES, but a few copies were found by ddPCR of plasma cells and serum. The concentration of the mutation in serum increased abruptly after initiation of therapy and covaried with M protein level for the rest of the disease course. Conversely, a *FAM46C* mutation present at a 60% allele fraction in plasma cells at diagnosis became undetectable in both serum and plasma cells during the disease course. In patient 5 (Figure 3B), the concentrations of M protein and *KRAS* and *TP53* mutations followed similar patterns in serum, despite a plasma cell allele fraction of only 4% for the *TP53* mutation at diagnosis.

Altogether, we monitored 14 mutated clones in 11 patients. Twelve of the mutations were detectable in serum at each relapse and covaried with M protein, whereas two mutations became undetectable during the disease course *(FAM46C*^{S279N} in patient 4 and *BRAF*^{V600E} in patient 10). These observations suggest that the serum concentration of recurrent mutations over long periods of time reflect the changes in total tumor mass in most myeloma patients.

Serum mutation levels in aggressive disease

In patients 1, 4, and 5 (Figure 2A, Figure 3 A,B) we noticed a marked increase in serum mutation levels in the

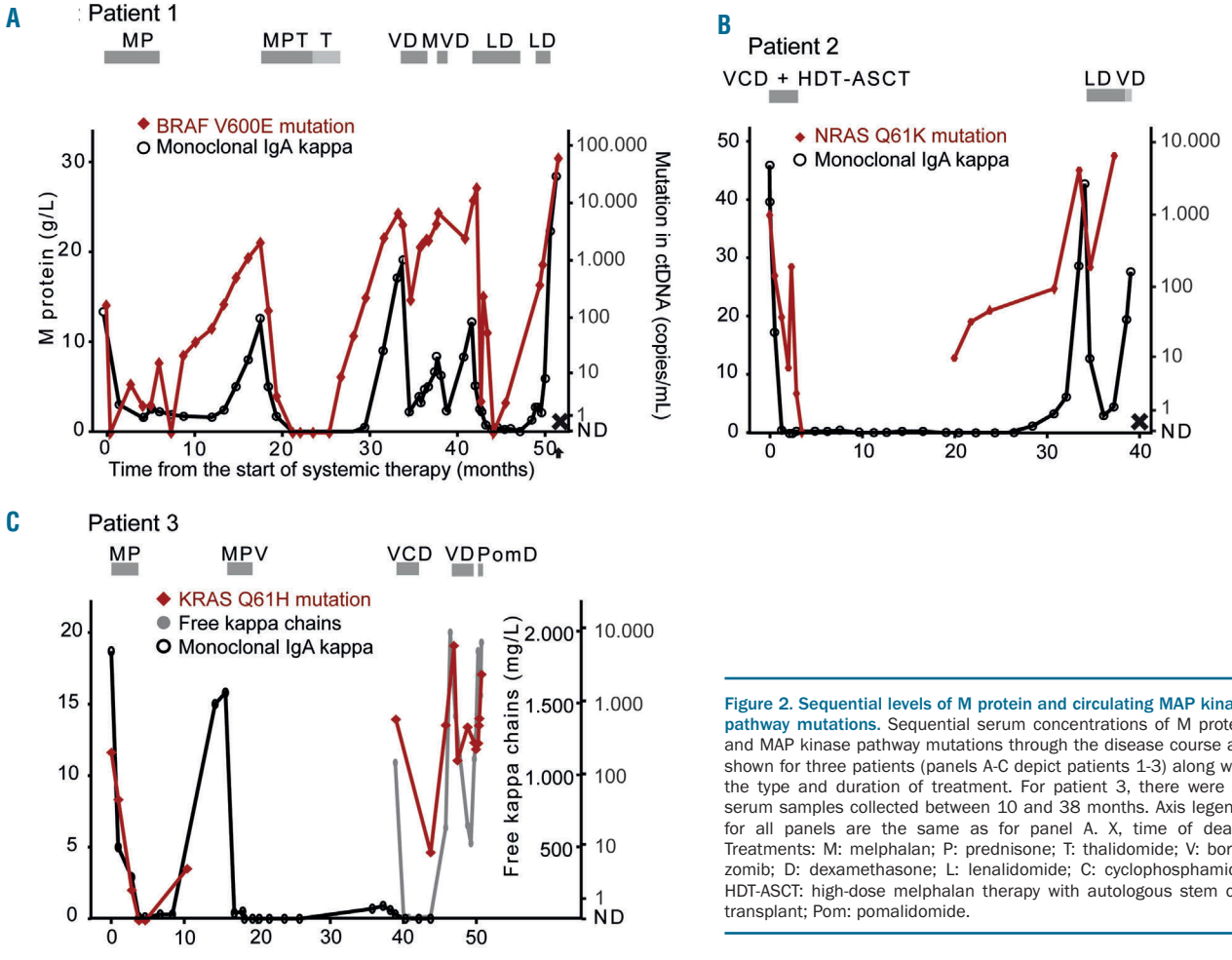

Figure 2. Sequential levels of M protein and circulating MAP kinase pathway mutations. Sequential serum concentrations of M protein and MAP kinase pathway mutations through the disease course are shown for three patients (panels A-C depict patients 1-3) along with the type and duration of treatment. For patient 3, there were no serum samples collected between 10 and 38 months. Axis legends for all panels are the same as for panel A. X, time of death. Treatments: M: melphalan; P: prednisone; T: thalidomide; V: bortezomib; D: dexamethasone; L: lenalidomide; C: cyclophosphamide; HDT-ASCT: high-dose melphalan therapy with autologous stem cell transplant; Pom: pomalidomide.

terminal phase of the disease. At that time the patients had treatment refractory disease and remained alive only for a few weeks or months. To further analyze the dynamics of ctDNA over time, we compared the peak levels of mutations and M protein at each relapse (Figure 4). To facilitate the comparison between patients, we normalized the concentrations of M protein and the ctDNA as indicated in the legend to Figure 4. Only one mutation per patient is shown in Figure 4, however, in patients 4 and 5, mutations in *IRF4*, *TP53* and *KRAS* behaved in the same manner indicating that they were all characteristics of the same aggressive clone. The discrepancy between ctDNA and M protein in patients 1, 4 and 5 was particularly evident in the terminal phase when the ratio of ctDNA to M protein was up to 400-fold higher than at the start of treatment.

We analyzed several aspects of these patients which can contribute to the marked increase in serum concentrations of mutations. Patient 1 and 5 had plasma cells with immature morphology, whereas patients 1 and 4 had secondary plasma cell leukemia, with >20% plasma cells in blood. No plasma cells were found in blood from patient 5. In two of the patients there was >10% increase in mutated allele fraction in bone marrow plasma cells from the start of treatment to the time of terminal disease (34-49% in patient 2 and 26-52% in patient 5). Thus, several factors may have contributed to the increased concentrations of ctDNA that were evident after transformation to a more aggressive disease.

Discussion

We studied the serum concentrations of recurrent mutations identified in bone marrow plasma cells from 20 patients with multiple myeloma. Our comprehensive series of samples covering the entire disease course from diagnosis to death of several patients provides a unique insight into the dynamics of ctDNA in relation to disease activity. The most striking findings were a marked covariation with the concentration of M protein, the gold standard biomarker to monitor tumor mass in multiple myeloma, and increasing concentrations of ctDNA relative to M protein as the disease became more aggressive.

To explain the increase of ctDNA, it is useful to discern between tumor mass and activity of the cells. The latter includes a number of functional aspects, such as proliferative rate and degree of adherence to the bone marrow environment. M protein is a typical tumor mass marker as long as the mechanisms of production and secretion of

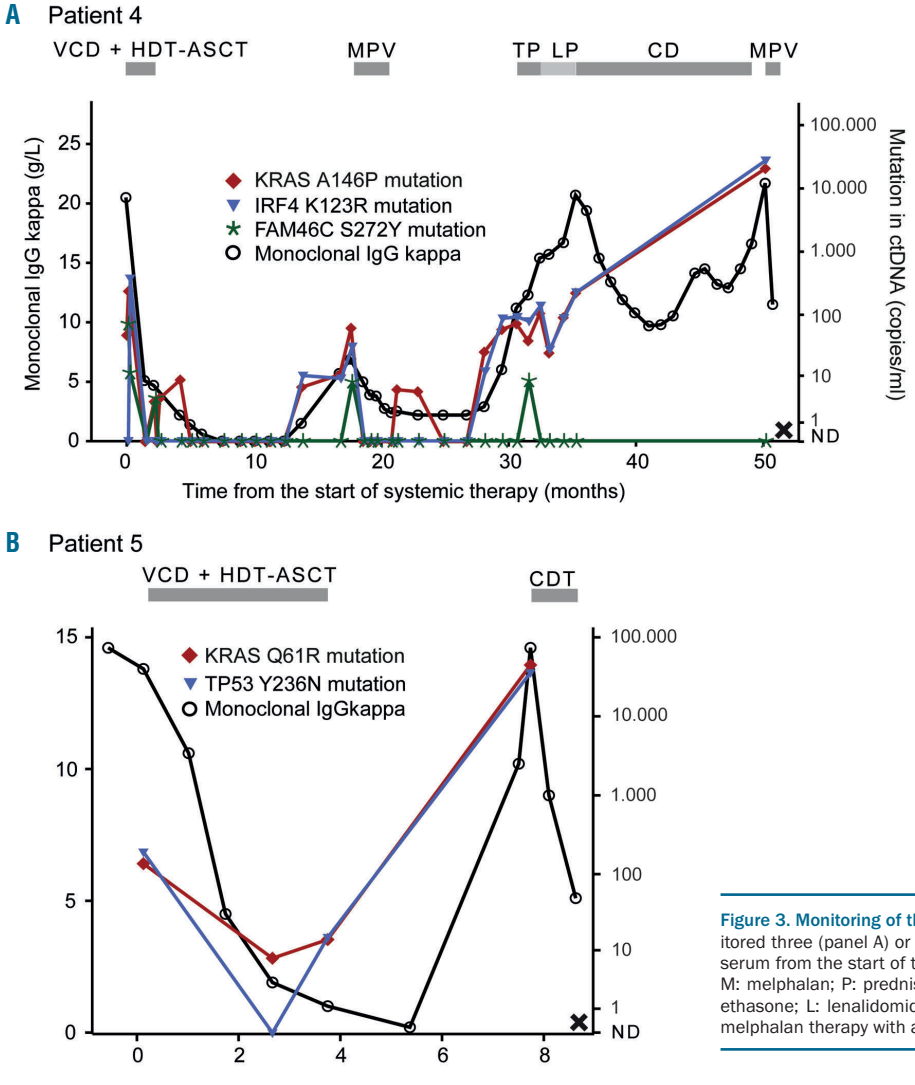

Figure 3. Monitoring of three or two mutations in serum. Here, we monitored three (panel A) or two (panel B) recurrent mutations by ddPCR of serum from the start of treatment until the terminal phase. Treatments: M: melphalan; P: prednisone; T: thalidomide; V: bortezomib; D: dexamethasone; L: lenalidomide; C: cyclophosphamide; HDT-ASCT: high-dose melphalan therapy with autologous stem cell transplant

immunoglobulin are intact. As shown, ctDNA and M protein seem to reflect tumor mass equivalently during long periods of the disease when the secretory mechanisms are operative and cellular functions relatively stable. This picture changes, however, when there is transformation to a more proliferative disease with high turnover of cells and perhaps a larger fraction of non-secretory cells. Furthermore, myeloma cells may be present in the circulation as shown in two of our patients. Although its clinical significance is unclear, ctDNA seems to reflect disease activity and progression differently from M protein.

Our serum samples were stored for up to 11 years before analysis. Despite reports of DNA degradation during protracted sample storage,[28] we found no statistically significant correlation between DNA yield and storage time, as shown in the methods section. Furthermore, it is recommended that ctDNA is analyzed in plasma rather than serum because of DNA released from leukocytes during sample preparation.[29] However, to our knowledge, serum and plasma have not been directly compared in a clinical setting, and previous studies have successfully used stored serum samples.[30,31] The close covariation between ctDNA and M protein found in our study adds to the evidence that stored serum can provide meaningful results and is a valuable material for the study of ctDNA.

A weakness of this study was the low number of patients, limiting the generalizability of our results. Another weakness was the low and variable volume of serum available for analysis at each time-point, as reported in the methods section and elaborated in the *Online Supplementary Methods.* Because the ability of ddPCR to detect low levels of mutations is primarily limited by the sample volume and concentration of DNA, the sensitivity of our ctDNA measurements varied and was suboptimal in many samples. The potential to detect early relapse and minimal residual disease by ctDNA was, therefore, most likely under-estimated in our study.

Mithraprabhu *et al.* recently reported the detection and monitoring of ctDNA in myeloma patients.[16] Their design differed from ours as they sequenced DNA from plasma as well as bone marrow plasma cells, targeting recurrently mutated regions in the *NRAS*, *KRAS*, *BRAF* and *TP53* genes. Interestingly, they found 24% of mutations exclusively in plasma, consistent with the spatial heterogeneity of multiple myeloma previously demonstrated by multi-region DNA sequencing of bone marrow plasma cells.[32,33] They also monitored specific mutations by ddPCR in three to six sequential samples from seven patients[16] and our results are essentially in agreement with their observations.

There are also apparent discrepancies between the studies. We detected 97% of mutations in serum when they had been identified in a time-matched bone marrow sample, whereas the corresponding number was only 39% (38/97) in the study by Mithraprabhu *et al.*[16] This may be explained by the high sensitivity of their procedure as the majority of mutations they detected in bone marrow plasma cells had a mutated allele fraction between 0.01 and 1%. In comparison, the limit of detection by WES of bone marrow plasma cells in our study was 2-4 % mutated allele fraction, which is in line with previous studies using WES.[17,18]

There are several potential applications of ctDNA in multiple myeloma. The mechanisms by which M protein and ctDNA are released into the bloodstream appear to be independent of each other. Thus, monitoring the disease using ctDNA may be possible in situations in which M protein is not a reliable biomarker, such as in light chain escape and non-secretory or oligo-secretory disease.[6,7,16] Furthermore, non-invasive detection of specific mutations may be useful to guide the use of targeted drugs such as BRAF or MEK inhibitors in patients with *BRAF*, *NRAS* or *KRAS* mutations.[22-24]

In principle, any tumor-specific DNA sequence such as a somatic mutation or a translocation breakpoint could be monitored by ddPCR.[12,34,35] Alternatively, targeted sequencing may be applied directly to plasma or serum DNA to detect several targets simultaneously.[13,30,36] This approach has the potential to describe tumor clonal evolution over time and its relation to clinical phenomena such as drug resistance[37,38] and may be preferred in many situations. The choice of method will depend on the purpose.

Altogether, this study provides detailed insight into the development of ctDNA levels over long periods of time in a limited number of patients. Circulating tumor DNA appears to be a multi-faceted biomarker of mutated cells, total tumor mass and transformation to a more aggressive disease in patients with multiple myeloma. However, several important questions remain unanswered, including the potential of ctDNA in minimal residual disease assessment and early detection of relapse.

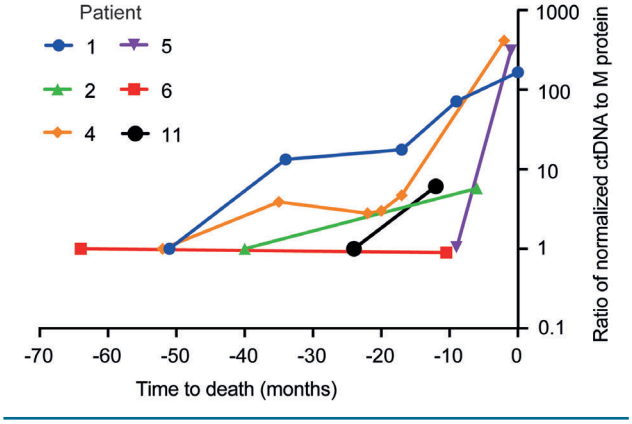

Figure 4. Ratio between serum levels of recurrent mutations and M protein during long-term follow-up. Diagnostic serum concentrations of M protein and MAP kinase mutations were normalized to one for each patient. Subsequent data are from the time points of peak M protein level at relapses, before a new treatment was started. The ratio of normalized mutation level to M protein was calculated for each data point. Patients were included in the figure if the diagnostic and at least one peak value of ctDNA and M protein were available. Patient 3 was excluded because of light chain escape, and patient 9 (*Online Supplementary Figure S4A*) was excluded because most peak values of ctDNA were too low to be confidently quantified.

Acknowledgments

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References

1. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med. 2011;364(11):1046-1060.
2. Naymagon L, Abdul-Hay M. Novel agents in the treatment of multiple myeloma: a review about the future. J Hematol Oncol. 2016;9(1):52.
3. Durie BG, Harousseau JL, Miguel JS, et al. International uniform response criteria for multiple myeloma. Leukemia. 2006;20(9): 1467-1473.
4. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. 2014;15(12):e538-548.
5. Kyle RA, Gertz MA, Witzig TE, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc. 2003;78(1):21-33.
6. Chawla SS, Kumar SK, Dispenzieri A, et al. Clinical course and prognosis of non-secretory multiple myeloma. Eur J Haematol. 2015;95(1):57-64.
7. Larson D, Kyle RA, Rajkumar SV. Prevalence and monitoring of oligosecretory myeloma. N Engl J Med. 2012;367(6):580-581.
8. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem. 2015;61(1):112-123.
9. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001;61(4):1659-1665.
10. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. Cell. 2016;164(1-2):57-68.
11. Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;368(13):1199-1209.
12. Garcia-Murillas I, Schiavon G, Weigelt B, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med. 2015;7(302):302ra133.
13. 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.
14. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med. 2016;8(346):346ra392.
15. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6(224):224ra224.
16. 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 Jan 3. [Epub ahead of print]
17. 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.
18. Walker BA, Boyle EM, Wardell CP, et al. Mutational spectrum, copy number changes, and outcome: results of a sequencing study of patients with newly diagnosed myeloma. J Clin Oncol. 2015;33(33):3911-3920.
19. Bolli N, Avet-Loiseau H, Wedge DC, et al. Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. Nat Commun. 2014;5:2997.
20. Kortum KM, Mai EK, Hanafiah NH, et al. Targeted sequencing of refractory myeloma reveals a high incidence of mutations in CRBN and Ras pathway genes. Blood. 2016;128(9):1226-1233.
21. Lionetti M, Barbieri M, Todoerti K, et al. Molecular spectrum of BRAF, NRAS and KRAS gene mutations in plasma cell dyscrasias: implication for MEK-ERK pathway activation. Oncotarget. 2015;6(27): 24205-24217.
22. Heuck CJ, Jethava Y, Khan R, et al. Inhibiting MEK in MAPK pathway-activated myeloma. Leukemia. 2016;30(4):976-980.
23. Hyman DM, Puzanov I, Subbiah V, et al. Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. N Engl J Med. 2015;373(8):726-736.
24. Andrulis M, Lehners N, Capper D, et al. Targeting the BRAF V600E mutation in multiple myeloma. Cancer Discov. 2013;3(8): 862-869.
25. Sharman JP, Chmielecki J, Morosini D, et al. Vemurafenib response in 2 patients with posttransplant refractory BRAF V600E-mutated multiple myeloma. Clin Lymphoma Myeloma Leuk. 2014;14(5): e161-163.
26. Rustad EH, Dai HY, Hov H, et al. BRAF V600E mutation in early-stage multiple myeloma: good response to broad acting drugs and no relation to prognosis. Blood Cancer J. 2015;5:e299.
27. Huggett JF, Cowen S, Foy CA. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem. 2015;61(1):79-88.
28. Sozzi G, Roz L, Conte D, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. J Natl Cancer Inst. 2005;97(24):1848-1850.
29. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: preanalytical considerations. Clin Chim Acta. 2013;424: 222-230.
30. Roschewski M, Dunleavy K, Pittaluga S, et al. Circulating tumour DNA and CT monitoring in patients with untreated diffuse large B-cell lymphoma: a correlative biomarker study. Lancet Oncol. 2015;16(5):541-549.
31. Diaz LA, Jr., Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486(7404):537-540.
32. Weinhold N, Chavan SS, Heuck C, et al. High risk multiple myeloma demonstrates marked spatial genomic heterogeneity between focal lesions and random bone marrow; implications for targeted therapy and treatment resistance. Blood. 2015;126 (23):20.
33. Melchor L, Jones JR, Lenive O, et al. Spatiotemporal analysis of intraclonal heterogeneity in multiple myeloma: unravelling the impact of treatment and the propagating capacity of subclones using whole exome sequencing. Blood. 2015;126 (23):371.
34. Krumbholz M, Hellberg J, Steif B, et al. Genomic EWSR1 fusion sequence as highly sensitive and dynamic plasma tumor marker in Ewing sarcoma. Clin Cancer Res. 2016;22(17):4356-4365.
35. Reinert T, Scholer LV, Thomsen R, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2016;65(4):625-634.
36. Schreuer M, Meersseman G, Van Den Herrewegen S, et al. Quantitative assessment of BRAF V600 mutant circulating cell-free tumor DNA as a tool for therapeutic monit oring in metastatic melanoma patients treated with BRAF/MEK inhibitors. J Transl Med. 2016;14:95.
37. Russo M, Siravegna G, Blaszkowsky LS, et al. Tumor heterogeneity and lesion-specific response to targeted therapy in colorectal cancer. Cancer Discov. 2016;6(2):147-153.
38. Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013;497(7447):108-112.