Introduction

Multiple myeloma is caused by proliferation of monoclonal plasma cells in the bone marrow and is the second most common hematologic malignancy. The treatment options have improved markedly in recent years and led to prolonged survival. 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. However, some patients escape the traditional monitoring. Between 1-3% of patients have non-secretory multiple myeloma and no

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.

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.
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.\(^3\) 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.\(^11-13\) Information about ctDNA in multiple myeloma lags behind as only a single study has so far been published.\(^14\)

The somatic mutational landscape of multiple myeloma has been described in several studies.\(^7,21\) 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.\(^7,13\) 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.

### Results

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

### 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 0.64 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.\(^28\) 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).

![Figure 1. Correlation between mutation levels in bone marrow plasma cells and serum samples.](image-url)
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 MAP kinase mutations in serum 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

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<th>Table 1. Summary of clinical data and mutations.</th>
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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 where 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 S4) 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<sup>Q61H</sup> 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<sup>Q61H</sup> 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<sup>S279N</sup> in patient 4 and BRAF<sup>V600E</sup> 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
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
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, 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. 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. 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. 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 multiregion DNA sequencing of bone marrow plasma cells. They also monitored specific mutations by ddPCR in three to six sequential samples from seven patients 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 59% (38/97) in the study by Mithraprabhu et al. 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.

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. 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.

In principle, any tumor-specific DNA sequence such as a somatic mutation or a translocation breakpoint could be monitored by ddPCR. Alternatively, targeted sequencing may be applied directly to plasma or serum DNA to detect several targets simultaneously. This approach has the potential to describe tumor clonal evolution over time and its relation to clinical phenomena such as drug resistance 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.

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References


