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Clinical Implications of Circulating Tumor DNA in Multiple Myeloma and Its Precursor Diseases

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Background: Genetic alterations play a pivotal role in multiple myeloma (MM) development and therapeutic resistance. Traditionally, the genetic profiling of MM requires invasive bone marrow (BM) procedures; however, these procedures are associated with patient discomfort and cannot fully capture the spatial and temporal heterogeneity of the disease. Therefore, we investigated the clinical implications of liquid biopsy using targeted deep sequencing.

Methods: We analyzed the genetic profiles of circulating tumor DNA (ctDNA) by targeted deep sequencing from 102 patients, including those with monoclonal gammopathy of undetermined significance (MGUS, N=7), smoldering MM (N=6), and symptomatic MM (N=89).

Results: The number of ctDNA mutations increased with disease progression from MGUS to MM, with averages of 1.0 mutations in MGUS, 1.8 mutations in smoldering MM, and 1.9 mutations in MM, respectively. Shared mutations between BM and ctDNA were more prevalent in MM (68.9%) than in MGUS (25.0%). RAS/RAF and *TP53* mutations were significantly enriched in MM ctDNA. Specific mutations were associated with clinical features in patients with MM: hypercalcemia and *TET2* (P=0.006), renal insufficiency and *NRAS* (P=0.012), paramedullary myeloma and *TP53* (P=0.02), and extramedullary myeloma and *NRAS* (P=0.007). *TET2* mutations significantly affected 2-yr progression-free survival (hazard ratio=7.11, P=0.003). Serial ctDNA profiling accurately predicted treatment response in patients with MM.

Conclusions: Our findings highlight the potential of liquid biopsy for understanding MM progression and prognosis utilizing a minimally invasive approach, paving the way for its integration into personalized treatment strategies and real-time disease monitoring.

Key Words: Cell-free DNA, Circulating tumor DNA, High-throughput nucleotide sequencing, Liquid biopsy, Myeloma

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INTRODUCTION

Multiple myeloma (MM) is a malignancy of differentiated, mature lymphoid-derived plasma cells (PCs) characterized by significant genetic heterogeneity, mutational burden, and high-risk genomic lesions, including t(4;14) 14q32, del(17p), and del(13q) [1, 2]. Almost all MMs evolve from a premalignant plasma cell clonal disorder termed monoclonal gammopathy of undetermined significance (MGUS); however, this precursor condition remains undetected in several clinical cases [3]. Smoldering MM (SMM) is an indolent stage between MGUS and MM, with a 10-fold higher risk of progression to MM compared with that of MGUS [4].

Genetic alterations are crucial to MM progression. Next-generation sequencing (NGS) has helped identify recurrent mutations in cancer-related genes, including RAS and *TP53*, with clonal heterogeneity [5, 6]. Specifically, mutations in the RAS/RAF/MAPK pathway genes (*BRAF*, *NRAS*, or *KRAS*) occur more frequently in MM than in SMM [7]. However, molecular profiles of MM genomes derived from single-site bone marrow (BM) biopsies represent only a small fraction of the BM compartment. Large-gauge needles used for these biopsies cause patient discomfort and complications, such as bleeding and infection. Additionally, monitoring using positron emission tomography/computed tomography (PET/CT) imaging may also fail to capture the intrinsic spatial and temporal heterogeneities of MM [8]. The invasive nature of BM biopsies and the radiation exposure associated with PET/CT hinder the frequent application of these procedures during therapy. Therefore, a less invasive technique capable of capturing clonal heterogeneity in clinical settings is required.

Circulating tumor DNA (ctDNA) consists of small DNA fragments released from apoptotic or necrotic tumor cells [9]. Genetic alterations present in tumor tissues can be accurately recapitulated using liquid biopsies targeting ctDNA [10]. ctDNA levels are higher in MM than in other tumors, and high concordance has been observed between mutations in BM aspirates and ctDNA [11, 12]. Recent studies have explored ctDNA analysis for treatment outcomes and tumor load monitoring in MM [13, 14]; however, ctDNA-based liquid biopsy has not yet been extensively validated in clinical settings.

In this study, we evaluated the clinical implications and prognostic relevance of liquid biopsies targeting ctDNA in patients with MGUS, SMM, or newly diagnosed MM (NDMM). Notably, we performed targeted deep sequencing of ctDNA collected from treatment-naive patients, as well as an exploratory analysis using serially collected ctDNA from patients with MM before and after treatment.

MATERIALS AND METHODS

Patients and samples

Patients newly diagnosed as having PC dyscrasia, including MGUS, SMM, and MM, between March 2019 and September 2021 were eligible for this study. We retrospectively identified 118 eligible patients who agreed to participate and provided peripheral blood (PB) and BM samples at the time of the diagnosis of PC dyscrasia at Seoul St. Mary's Hospital, Korea. Among them,16 patients were excluded because of inadequate QC for targeted NGS analysis. The final cohort included seven patients with MGUS, six with SMM, and 89 with MM (Supplemental Data Fig. S1). We analyzed data in March 2023. This study was approved by the Institutional Review Board of Seoul St. Mary's Hospital (KC24TISI0257) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. Detailed methods on ctDNA extraction, NGS protocol, identification of somatic variants, DNA copy number analysis, subcohort selection, and statistical analysis are described in the Supplemental Data section. The raw FASTQ files are available from the corresponding authors upon reasonable request.

Definition of clinical features

The diagnosis of MGUS, SMM, or MM was established based on the International Myeloma Working Group criteria [15]. Hypercalcemia was defined as a serum calcium level >11.0 mg/dL, anemia as an Hb level <10.0 g/dL, and renal failure as a serum creatinine level >2 mg/dL [15]. Paramedullary myeloma was defined as plasmacytomas originating from the bone in continuity with the BM, whereas extramedullary myeloma referred to soft tissue plasmacytomas or PC infiltration at anatomical sites distant from the BM. All patients with SMM and MM underwent PET/CT screening with [^{18}F]Fluorodeoxyglucose (FDG) to identify bone lytic lesions and paramedullary or extramedullary myeloma. PC leukemia was defined as ≥5% circulating PCs in the PB [16]. Myeloma-extended sites, as described in previous reports [17, 18], included paramedullary myeloma, extramedullary myeloma, and PC leukemia. NDMM staging was performed according to the International Staging System [19]. High-risk cytogenetic abnormalities were defined as the presence of one or more of the following based on FISH results using BM samples collected at diagnosis: del(17p), t(4;14), t(14;16), and 1q gain/

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amplification [20, 21].

RESULTS

Patient characteristics

Mean ages, sex distributions, and clinical features such as renal insufficiency, bone lytic lesions, and MM subtypes are summarized in Table 1. Kappa chain-type MM was more prevalent than lambda chain-type MM. Myeloma-extended sites, including paramedullary myeloma, extramedullary myeloma, and PC leukemia, were identified in 31.5% of patients with MM.

Table 1. Baseline clinicopathologic characteristics

Characteristic	MGUS (N=7)	SMM (N=6)	MM (N=89)	*P*
Age, mean±SD	67.6±12.4	63±7.2	64.8±9.3	0.667
≥ 65 yrs, N (%)	4 (57.1)	3 (50.0)	51 (57.3)	1.000
Sex				1.000
Male	4 (57.1)	3 (50.0)	45 (50.6)	
MM-related presentation				NA
Hypercalcemia	NA	NA	7 (7.9)	
Renal insufficiency	NA	NA	14 (15.7)	
Anemia	NA	NA	58 (65.2)	
Bone lytic lesion	NA	NA	68 (76.4)	
Type of myeloma				0.817
IgG	3 (42.9)	4 (66.7)	45 (50.6)	
IgA	1 (14.3)	1 (16.7)	23 (25.8)	
Others	3 (42.9)	1 (16.7)	21 (23.6)	
Light chain-type				0.139
Kappa	2 (28.6)	5 (83.3)	54 (60.7)	
Lambda	5 (71.4)	1 (16.7)	35 (39.3)	
AL amyloidosis				0.761
No	6 (85.7)	6 (100.0)	80 (89.9)	
Yes	1 (14.3)	0 (0.0)	9 (10.1)	
ISS*				0.370
I	NA	4 (66.7)	32 (37.2)	
II	NA	2 (33.3)	31 (36.0)	
III	NA	0 (0.0)	23 (26.7)	
Not available	NA	0 (0.0)	3 (3.4)	
Myeloma-extended sites				NA
No	NA	NA	61 (68.5)	
Yes	NA	NA	28 (31.5)	
Paramedullary myeloma				NA
No	NA	NA	66 (74.2)	
Yes	NA	NA	23 (25.8)	
Extramedullary myeloma				NA
No	NA	NA	81 (91.0)	
Yes	NA	NA	8 (9.0)	

(Continued to the next page)

Table 1. Continued

Characteristic	MGUS (N=7)	SMM (N=6)	MM (N=89)	*P*
Plasma cell leukemia				NA
No	NA	NA	85 (95.5)	
Yes	NA	NA	4 (4.5)	
Cytogenic profile				0.426
Standard risk	4 (57.1)	4 (66.7)	32 (36.0)	
High risk	3 (42.9)	2 (33.3)	55 (61.8)	
Not available	0 (0.0)	0 (0.0)	2 (2.2)	
Abnormal cytogenic profile				
t(4;14)	0 (0.0)	0 (0.0)	20 (22.5)	0.221
del(17p)	3 (42.9)	0 (0.0)	22 (24.7)	0.185
t(14;16)	0 (0.0)	0 (0.0)	2 (2.2)	0.999
t(11;14)	1 (14.3)	0 (0.0)	16 (18.0)	0.834
del(13q)	3 (42.9)	4 (66.7)	52 (57.3)	0.662
1gain/amp(1q21)	2 (28.6)	2 (33.3)	28 (31.5)	0.999
t(14;20)	1 (14.3)	0 (0.0)	21 (23.6)	0.561
Unknown	0 (0.0)	0 (0.0)	2 (2.2)	
β2-Microglobulin				0.552
<5.5 μg/mL	5 (71.4)	6 (100.0)	63 (70.8)	
≥5.5 μg/mL	1 (14.3)	0 (0.0)	23 (25.8)	
Unknown	1 (14.3)	0 (0.0)	3 (3.4)	
Albumin				0.242
<3.5 g/dL	1 (14.3)	1 (16.7)	38 (42.7)	
≥3.5 g/dL	6 (85.7)	5 (83.3)	51 (57.3)	
Lactate dehydrogenase				0.479
<Upper limit of normal	5 (71.4)	6 (100.0)	70 (78.7)	
≥Upper limit of normal	2 (28.6)	0 (0.0)	19 (21.3)	
Hb, mean±SD, g/L	118±18	121±09	98±22	0.003
Absolute neutrophil count, mean±SD, $\times 10^9$/L	4.6±1.2	2.2±0.5	3.6±2.4	0.157
Absolute lymphocyte counts, median (range), $\times 10^9$/L	1.7 (0.7 – 2.3)	1.6 (1.5 – 3.4)	1.6 (0.6 – 12.9)	0.607
Platelet count, mean±SD, $\times 10^9$/L	228.3±47.8	234.0±53.2	201.5±84.0	0.471
GFR, mean±SD, mL/min/1.73 m^2	60.0±33.5	84.4±21.6	66.9±33.0	0.369

*Fisher's exact test between two groups of SMM and MM
Abbreviations: AL, amyloid light chain; SD, standard deviation; ISS, International Staging System; GFR, glomerular filtration rate; NA, not available; MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma.

Genetic alterations in ctDNA samples

We used targeted deep sequencing (Supplemental Data Table S1) to analyze ctDNA samples from 102 patients at the time of diagnosis, including those with MGUS (N=7), SMM (N=6), and MM (N=89). The median ctDNA concentrations for MGUS, SMM, and MM were 10.8 ng/mL (range, 6.5–199.5), 9.6 (range, 1.9–13.1) ng/mL, and 15.3 ng/mL (range, 2.8–523.5), respectively; however, the higher concentration in patients with MM than in those with MGUS or SMM was not statistically significant (Supplemental Data Table S2). The median sequencing depth across the entire target genome was 2,163× (range, 674× –3,045×; Supplemental Data Table S2).

In total, we identified 226 somatic mutations, including 174 single nucleotide variants and 52 indels, across the 102 pa-

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tients (Supplemental Data Table S3). Non-silent mutations in at least one target gene, such as *KRAS*, *BRAF*, *DNMT3A*, and *TP53*, were detected in 76.5% of patients (78/102) [5, 22, 23]. Non-silent mutation positivity rates in BM samples were 100% for both MGUS and SMM and 94% for MM. In contrast, rates in ctDNA were 57%, 67%, and 79%, respectively, suggesting an association between ctDNA mutation positivity and disease progression (P=0.041).

The average number of ctDNA non-silent mutations increased with disease progression: 1.0 in MGUS, 1.8 in SMM, and 1.9 in MM. When comparing BM and ctDNA mutation profiles, none of the patients with SMM (0 out of 2) had shared mutations, whereas 25.0% of patients with MGUS (1 out of 4) and 68.9% of patients with MM (42 out of 61) had at least one shared mutation between BM and ctDNA. Among the 102 patients, 11 (10.8%) harbored at least one copy number alteration (CNA). These CNAs were identified in 10 patients with MM and one patient with MGUS (Supplemental Data Table S4). Recurrent copy number gains were observed in *MYC* (N=3), *MCL1* (N=3), *NIPA2* (N=3), and *STK11* (N=2), whereas other CNAs were observed as singleton events.

Differences in ctDNA profiles according to disease progression

Recurrent genetic alterations were identified in 10 genes across multiple ctDNA samples (>5%; Fig. 1A; Supplemental Data Table S5): *KRAS* (N=15, 14.7%), *NIPA2* (N=13, 12.7%), *TP53* (N=11, 10.8%), *GNAS* (N=11, 10.8%), *ZFHX3* (N=9, 8.8%), *NRAS* (N=9, 8.8%), *NOTCH1* (N=8, 7.8%), *MLH1* (N=8, 7.8%), *BRAF* (N=7, 6.9%), and *MYC* (N=6, 5.9%). Notably, 29 (32.6%) patients harbored at least one RAS/RAF (*KRAS*, *NRAS*, and *BRAF*) activating mutations. Among *KRAS* mutations, p.G12D/C/S/V (N=8) was the most frequent, followed by p.Q61R/L/H (N=4), p.G13D (N=3), and p.Y64D (N=2) (Fig. 1B). Singleton events accounted for the remaining *KRAS* mutations. For *NRAS*, p.Q61R/H/K (N=6) was the most common mutation, followed by p.G13V/R (N=2) and p.G12D (N=1) (Fig. 1C). Among *BRAF* mutations, p.V600E (N=3) was the most frequent, followed by

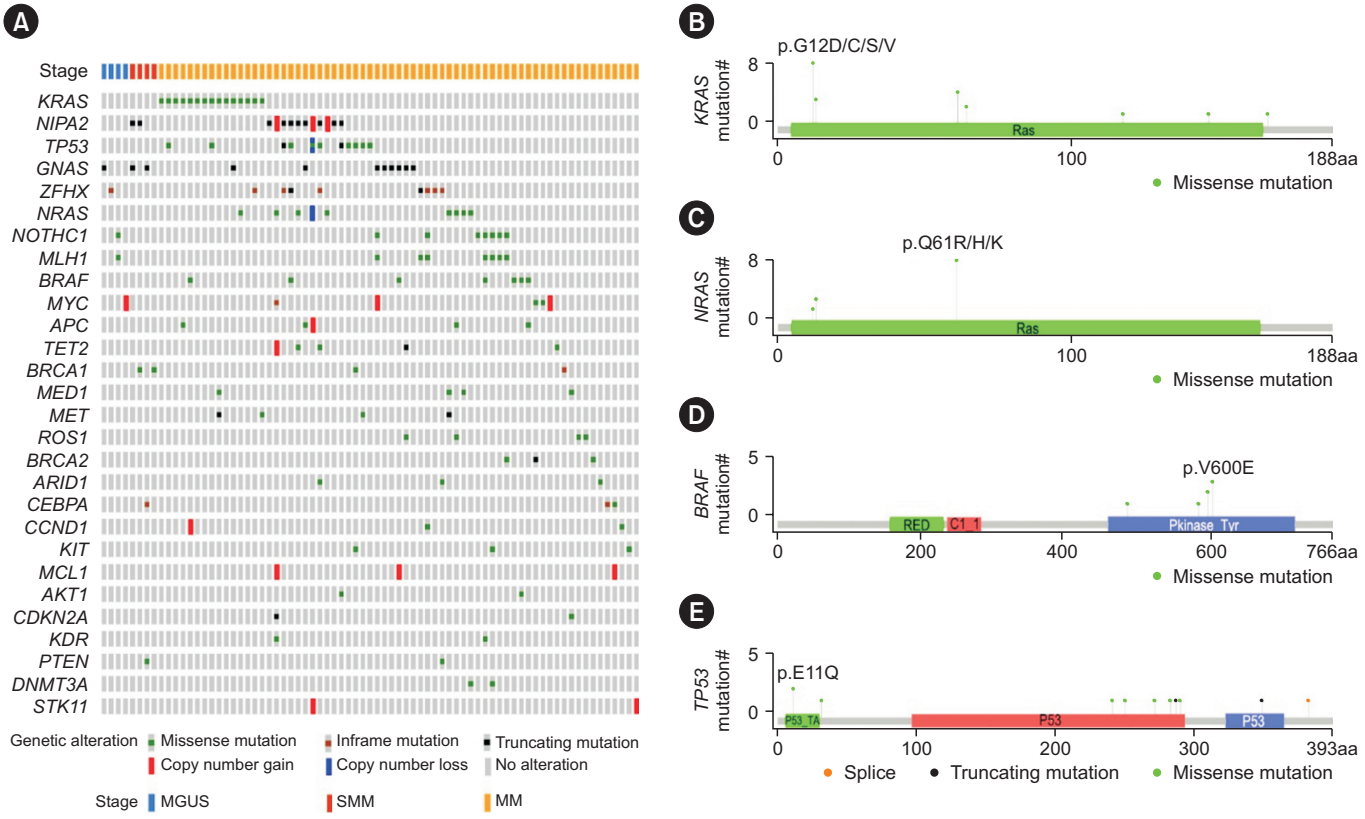

Fig. 1. Clonal genomic landscape of ctDNA in plasma cell dyscrasia. (A) Distribution of MM putative driver somatic mutations from next-generation sequencing of ctDNA. (B) Location of *KRAS* mutations. (C) Location of *NRAS* mutation. (D) Location of *BRAF* mutation. (E) Location of *TP53* mutation.
Abbreviations: MM, multiple myeloma; SMM, smoldering MM; MGUS, monoclonal gammopathy of undetermined significance.

p.D594G/N (N=2), p.N581I (N=1), and p.K483E (N=1) (Fig. 1D). RAS/RAF-activating mutations were significantly enriched in MM genomes but were not detected in MGUS or SMM genomes (P=0.017).

Recurrent mutations in *TP53* were scattered across the entire coding region (Fig. 1E) and were exclusively detected in MM genomes (12.7%; N=13; Fig. 1A). These findings suggest that mutations in *KRAS*, *NRAS*, *BRAF*, and *TP53* may contribute to the progression of MM from precursor diseases. In contrast, mutations in *GNAS* were detected not only in MM (9.0%; N=8) but also in MGUS (14.3%; N=1) and SMM (33.3%; N=2) genomes (Fig. 1A), indicating that *GNAS* mutations may occur during the early stages of MM progression.

Clinical correlation between ctDNA at diagnosis with MM

In ctDNA from patients with MM, recurrent genetic alterations were detected in 12 genes (occurring in >5% of patients, Supplemental Data Table S5): *KRAS* (16.9%), *NIPA2* (12.4%), *TP53* (12.4%), *NRAS* (10.1%), *GNAS* (9.0%), *ZFHX3* (9.0%), *NOTCH1* (7.9%), *MLH1* (7.9%), *BRAF* (7.9%), *MYC* (5.6%), *APC* (5.6%), and *TET2* (5.6%). We investigated the association between these 12 genes and clinicopathological characteristics, including hypercalcemia, renal insufficiency, anemia, bone lytic lesions, amyloidosis, myeloma-extended sites, neutropenia, and thrombocytopenia. Simple correlation analysis revealed that genetic alterations in five genes were positively related to specific clinicopathological characteristics (Supplemental Data Table S6): hypercalcemia and *TET2* (P=0.006), renal insufficiency and *NRAS* (P=0.012), bone lytic lesions and *TP53* (P=0.050), paramedullary myeloma and *TP53* (P=0.020), and extramedullary myeloma and *NRAS* (P=0.007), *MYC* (P=0.012), or *APC* (P=0.012). Using Fisher's exact test, the relationships between hypercalcemia and *TET2* (P=0.048), renal insufficiency and NRAS (P=0.031), paramedullary myeloma and *TP53* (P=0.030), and extramedullary myeloma and *NRAS* (P=0.032) remained significant (Table 2). Multivariate analysis of clinical variables with a univariate P-value <0.05 indicated that none were associated with clinicopathological characteristics (Supplemental Data Table S7).

Prognostic implication of ctDNA

We performed a survival analysis of patients with MM to investigate the prognostic impact of genetic alterations in ctDNA. After excluding five patients who did not receive treatment because of loss to follow-up or early death, the median follow-up duration was 28.4 months [95% confidence interval (CI), 25.7–32.4] from treatment initiation. The 2-yr overall survival (OS) and progression-free survival (PFS) rates were 89.5% (95% CI, 82.8–96.7) and 54.9% (95% CI, 44.9–67.1), respectively.

Univariate analysis helped identify significant associations between poor OS and four genes: *APC* (P=0.006), *NIPA2* (P=0.009), *TET2* (P=0.011), and *NRAS* (P=0.030) (Fig. 2A and Supplemental Data Table S8). High lactate dehydrogenase levels (P=0.020) and ineligibility for transplantation (P=0.007)

Table 2. Association between recurrently mutated genes and clinicopathologic characteristics of multiple myeloma

Gene	Major presentation at diagnosis of multiple myeloma											
	Hypercalcemia			Renal insufficiency			Paramedullary myeloma			Extramedullary myeloma		
	No (N=82)	Yes (N=7)	*P*	No (N=75)	Yes (N=14)	*P*	No (N=66)	Yes (N=23)	*P*	No (N=81)	Yes (N=8)	*P*
KRAS	15 (18.3%)	0 (0%)	0.597	13 (17.3%)	2 (14.3%)	1.000	10 (15.2%)	5 (21.7%)	0.333	15 (18.5%)	0 (0%)	0.342
NIPA2	9 (11.0%)	2 (28.6%)	0.207	9 (12.0%)	2 (14.3%)	0.682	7 (10.6%)	4 (17.4%)	0.465	9 (11.1%)	2 (25.0%)	0.257
TP53	10 (12.2%)	1 (14.3%)	1.000	10 (13.3%)	1 (7.1%)	1.000	5 (7.6%)	6 (26.1%)	0.030	10 (12.3%)	1 (12.5%)	1.000
NRAS	7 (8.5%)	2 (28.6%)	0.146	5 (6.7%)	4 (28.6%)	0.031	5 (7.6%)	4 (17.4%)	0.229	6 (7.4%)	3 (37.5%)	0.032
GNAS	7 (8.5%)	1 (14.3%)	0.495	7 (9.3%)	1 (7.1%)	1.000	7 (10.6%)	1 (4.3%)	0.675	7 (8.6%)	1 (12.5%)	0.545
ZFHX3	8 (9.8%)	0 (0%)	1.000	8 (10.7%)	0 (0%)	0.347	5 (7.6%)	3 (13.0%)	0.421	8 (9.9%)	0 (0%)	1.000
NOTCH1	7 (8.5%)	0 (0%)	1.000	7 (9.3%)	0 (0%)	0.591	4 (6.1%)	3 (13.0%)	0.369	6 (7.4%)	1 (12.5%)	0.495
MLH1	7 (8.5%)	0 (0%)	1.000	7 (9.3%)	0 (0%)	0.591	4 (6.1%)	3 (13.0%)	0.369	6 (7.4%)	1 (12.5%)	0.495
BRAF	7 (8.5%)	0 (0%)	1.000	7 (9.3%)	0 (0%)	0.591	6 (9.1%)	1 (4.3%)	0.672	5 (6.2%)	2 (25.0%)	0.119
MYC	4 (4.9%)	1 (14.3%)	0.343	4 (5.3%)	1 (7.1%)	0.584	3 (4.5%)	2 (8.7%)	0.601	3 (3.7%)	2 (25.0%)	0.062
APC	5 (6.1%)	0 (0%)	1.000	4 (5.3%)	1 (7.1%)	0.584	4 (6.1%)	1 (4.3%)	1.000	3 (3.7%)	2 (25.0%)	0.062
TET2	3 (3.7%)	2 (28.6%)	0.048	3 (4.0%)	2 (14.3%)	0.174	2 (3.0%)	3 (13.0%)	0.106	4 (4.9%)	1 (12.5%)	0.383

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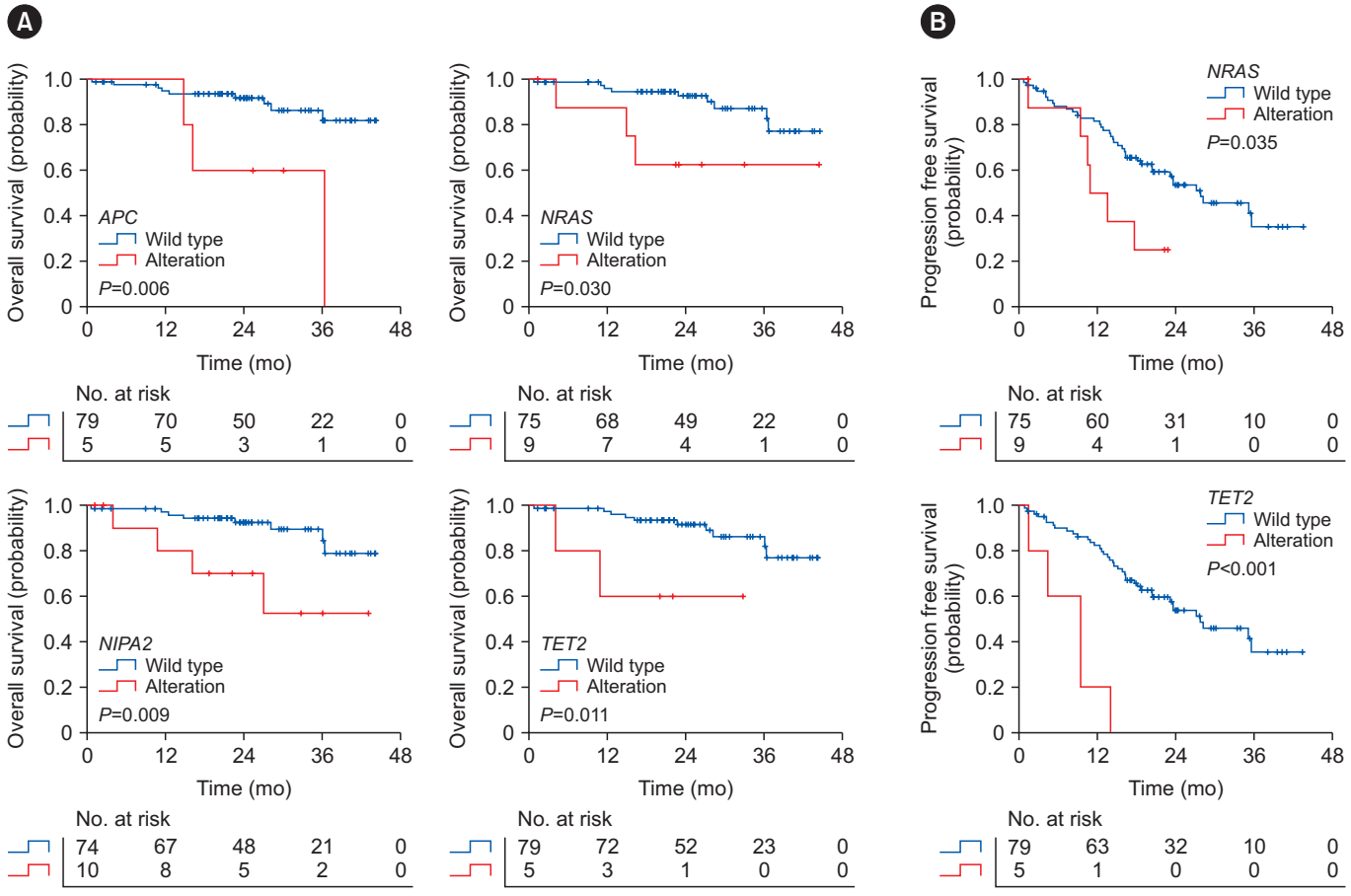

Fig. 2. ctDNA mutations significantly associated with survival outcomes. (A) *APC*, *NRAS*, *NIPA2*, and *TET2* mutations associated with overall survival. (B) *NRAS* and *TET2* mutations associated with progression-free survival.

were also significantly associated with poor OS in univariate analysis (Supplemental Data Table S8). However, multivariate analysis, including candidate genes and clinical variables that were significant in the univariate analysis, demonstrated that none were independent prognostic factors for OS (Table 3).

Univariate analysis revealed that patients with *NRAS* (P=0.035) and *TET2* (P<0.001) alterations showed significantly poorer PFS than that of those without *NRAS* and *TET2* alterations (Fig. 2B and Supplemental Data Table S8). Additionally, five clinical variables—transplant ineligibility (P<0.001), older age (P=0.004), high-risk cytogenetic profile (P=0.01), fewer treatment regimens (P=0.026), and lambda chain-type (P=0.037)—were significantly associated with poorer PFS (Supplemental Data Table S8). Multivariate analysis showed that the presence of *TET2* alterations [hazard ratio (HR)=7.11; 95% CI, 1.92–26.24; P=0.003] and three clinical variables [transplant ineligibility (HR=4.001; 95% CI, 2.117–25; P=0.001), lambda chain-type (HR=2.07; 95% CI, 1.1–3.9; P=0.025), and high-risk cytogenetic profile (HR=2.65; 95% CI, 1.14–5.7; P=0.023)] were independent prognostic factors for poor PFS (Table 3).

Monitoring genetic alteration profiles in ctDNA during treatment

We analyzed the genetic alteration profiles of serial ctDNA samples (N=20) from seven patients with MM to evaluate the clinical utility of liquid biopsies during therapy. In two patients whose PFS was >12 months from the first-line treatment (MM510 and MM429), the baseline levels of variant allele frequency (VAF) of detected mutations decreased over 20 months (Fig. 3A). No newly detected mutations were observed during first-line therapy in these patients. They exhibited favorable treatment responses to the second-line therapy, with MM510 achieving a very good partial response (VGPR) and MM429 achieving a complete response (CR) following second-line treatment.

In contrast, five patients with PFS ≤ 12 months showed increasing VAFs until relapse (Fig. 3B). Newly acquired mutations

Table 3. Multivariate analysis for overall and progression-free survival

Variables	HR	95% CI	*P*
Overall survival			
NIPA2			
Wild type	1.00	0.45–9.32	0.354
Alteration	2.05		
APC			
Wild type	1.00	0.44–10.88	0.339
Alteration	2.19		
NRAS			
Wild type	1.00	0.46–25.75	0.229
Alteration	3.44		
TET2			
Wild type	1.00	0.17–12.16	0.737
Alteration	1.44		
Lactate dehydrogenase			
Normal range	1.00	0.79–10.93	0.107
≥Upper limit of normal	2.94		
Transplant eligibility			
Eligible	1.00	0.95–18.45	0.059
Ineligible	4.19		
Progression-free survival			
NRAS			
Wild type	1.00	0.59–4.82	0.327
Alteration	1.69		
TET2			
Wild type	1.00	1.92–26.24	0.003
Alteration	7.11		
Transplant eligibility			
Eligible	1.00	2.13–25.00	0.001
Ineligible	4.00		
Light chain-type			
Kappa	1.00	1.10–3.90	0.025
Lambda	2.07		
Cytogenetic profile			
Standard risk	1.00	1.14–5.70	0.023
High risk	2.65		
Age			
<65 yrs	1.00	0.22–4.44	0.601
≥65 yrs	1.37		
Treatment			
Quadruplet	1.00		
Triplet	2.16	0.86–5.43	0.102
Doublet	9.09	0.94–99.90	0.056

Abbreviations: HR, hazard ratio; CI, confidence interval.

were detected in four patients (80%) during the course of therapy, indicating that the acquisition of new genetic alterations during treatment is relatively common in patients with poor PFS. For instance, in MM564, the newly acquired *KRAS* p.G12V (VAF=40.0%) and p.A11P (VAF=39.3%) after achieving CR to bortezomib-melphalan-prednisone (VMP) might be related to relapse observed 2 months after completing first-line therapy. Similarly, MM347 was initially treated with lenalidomide-dexamethasone (Rd). Although this patient achieved VGPR, MM progressed within 9 months of the initiation of Rd, and extramedullary MM occurred 5 months after Rd termination. During this period, *AKT1* mutations disappeared, while *EGFR* p.V786M (VAF=51.8%) and *SPOP* p.S298S (VAF=48.4%) mutations were newly acquired in ctDNA at the time of the first relapse, suggesting clonal evolution from BM or extramedullary sites.

Notably, MM233 received frontline treatment with VMP, followed by second-line Rd for the first relapse. Before initiating third-line treatment, the VAF of the *TP53* p.T125T mutation increased more than three-fold compared with baseline at diagnosis. Subsequently, the patient received carfilzomib-dexamethasone (Kd); however, the additional acquisition of the *RAC1* p.D38N mutation and an increased VAF of the *GNAS* mutation resulted in disease progression with extramedullary MM within 4 months of Kd initiation.

Fourth-line daratumumab monotherapy was administered, resulting in PR, but early disease progression occurred 4 months after daratumumab initiation. Consistent with the observed clinical progression, the VAFs of all three mutations decreased during the first 3 months of daratumumab treatment but increased again at the fourth relapse.

DISCUSSION

The advent of NGS and improved accessibility to these platforms has advanced the understanding of mutational characteristics in ctDNA from patients with MGUS and SMM, as well as explored their evolution into symptomatic MM. Our data support three major conclusions: first, the number of mutations detected in ctDNA increases with disease progression from MGUS to SMM/MM; second, MM exhibits a higher frequency of shared mutations between BM and ctDNA compared with that in precursor diseases, with frequent RAS/RAF and *TP53* mutations; and third, serial ctDNA profiling is feasible as a minimally invasive method in patients with MM.

ctDNA burden correlates with disease status across MGUS, SMM, NDMM, post-treatment MM, and MM at relapse [13, 24].

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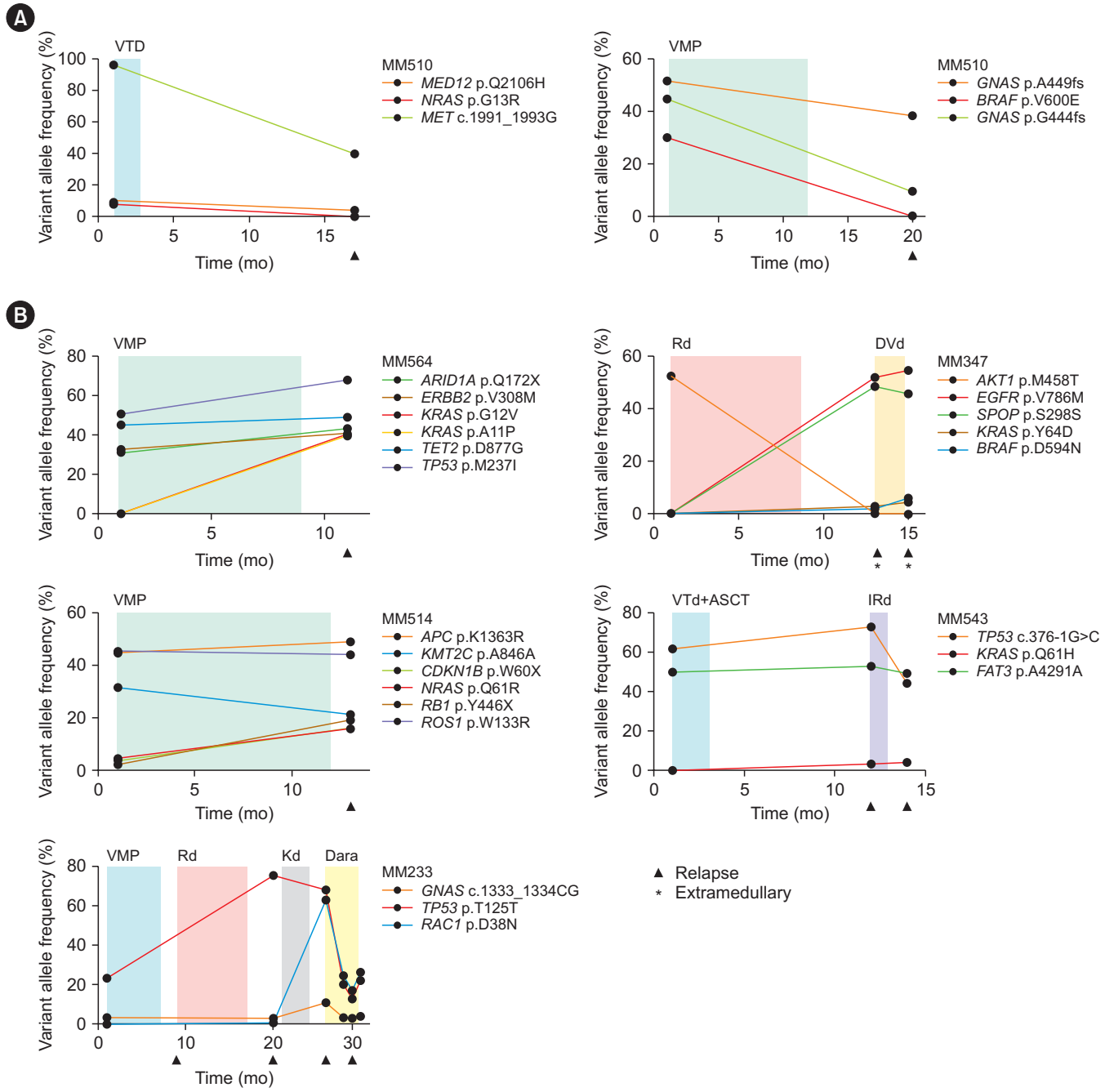

Fig. 3. Sequential monitoring of mutant clones in a subset of seven patients with MM. (A) MM510 and MM429 had a PFS of more than 12 months, representing a favorable outcome for first-line treatment. The PFS for MM510 and MM429 following first-line treatment was 14 and 13 months, respectively. (B) MM564, MM347, MM514, MM543, and MM233, with a PFS of less than 12 months (10, 11, 9, 11, and 9 months, respectively), had severely adverse first-line treatment outcomes.

Abbreviations: MM, multiple myeloma; PFS, progression-free survival; VTD, bortezomib-thalidomide-dexamethasone; VMP, bortezomib-melphalan-prednisone; Rd, lenalidomide-dexamethasone; VTd+ASCT, bortezomib-thalidomide-dexamethasone+autologous stem cell transplantation.

Consistent with these findings, we observed an average of 1.0 mutations in ctDNA from MGUS and 1.9 mutations in SMM/MM. Additionally, a high concordance rate of mutations (68.9%) between BM and ctDNA was observed in MM, consistent with a reported 70.9% concordance in patients with MM [25]. However, we did not find any significant differences in VAF among disease

groups. RAS/RAF and *TP53* mutations were exclusively detected in MM genomes but not in precursor diseases. The Myeloma XI trial reported that up to 50% of $CD138^+$ sorted BM samples from patients with NDMM harbored mutations in genes involved in the RAS signaling cascade, including *KRAS*, *NRAS*, and *BRAF* [26]. *TP53* dysregulation also plays a pivotal role in the pathogenesis of various cancers, including MM. Collectively, mutations detected in ctDNA from precursor diseases are, both quantitatively and qualitatively, compared with ctDNA in MM and do not sufficiently represent mutations in malignant PCs within the BM. The acquisition of additional driver mutations in RAS/RAF or *TP53* may serve as ctDNA markers to identify the progression from precursor diseases to symptomatic MM.

Interestingly, recurrent *NIPA2* mutations were detected in patients with MM. Although *NIPA2* has previously been reported as recurrently mutated in prostate cancer [27], to the best of our knowledge, no study has demonstrated a direct link between *NIPA2* mutations and the pathogenesis of MM. *NIPA2* is involved in mitophagy regulation [28], and recent research has highlighted the involvement of mitophagy-related genes in MM pathogenesis [29, 30]. The potential connection between *NIPA2* and MM pathogenesis remains an intriguing avenue for future investigation.

Targeted NGS has identified RAS mutations as a common feature in patients with extramedullary myeloma [31]. *TP53* mutations have been associated with paramedullary myeloma, a condition indicative of advanced bone lytic disease independent of extramedullary myeloma. These findings provide additional evidence supporting our results [32, 33]. To the best of our knowledge, our study is the first to identify statistically significant associations between ctDNA mutations and clinical presentations in a relatively large real-world cohort.

Several studies have actively explored the potential of ctDNA profiles as prognostic biomarkers for NDMM [25, 34, 35]; however, limited evidence exists for its utility alongside established clinical prognostic variables in large cohorts. We investigated the prognostic significance of ctDNA profiles in conjunction with clinical variables in a real-world MM cohort. Mutations in *TET2* were correlated with adverse effects on the 2-yr PFS rate, persisting even after adjusting for other clinical variables, such as age, transplant eligibility, cytogenetic risk, and treatment intensity. This finding offers compelling evidence for the utility of ctDNA as a prognostic biomarker in MM.

Among individuals undergoing serial ctDNA monitoring, two with sustained long-term responses exhibited reduced ctDNA VAFs and lacked driver mutations, specifically RAS/RAF mutations. Conversely, all five patients progressing to relapsed/refractory MM (RRMM) within a short period showed increased VAFs in RAS/RAF. In three cases of RRMM in which *TP53* trajectory data were available (MM564, MM543, and MM233), a similar trajectory pattern of increasing *TP53* mutation VAFs was observed with disease progression. A recent study reported an increased frequency of RAS/RAF and *TP53* mutations in RRMM compared with those in NDMM [25], highlighting their association with advanced disease status. Additionally, the acquisition of mutations beyond RAS/RAF and *TP53* is a well-documented characteristic of RRMM [36, 37]. Our data further support the notion that changes in these mutations serve as potential biomarkers for predicting a deep response and favorable response duration.

This study has some limitations. First, continuous ctDNA profile monitoring from MGUS to SMM to MM was not performed in the same patient. Second, despite confirming the prognostic significance of *TET2* mutation in the MM cohort, we could not distinguish between ctDNA and clonal hematopoiesis of indeterminate potential. Further prospective trials involving ctDNA profiling together with leucocyte sequencing in a larger MM cohort will provide clinically applicable biomarkers related to disease development and/or prognosis. Third, the increase in the total ctDNA mutation number, which indicates the overall mutation burden, is considered a biomarker for MM [14, 38]. However, the specific mutations required for standardization in this context remain unknown [38]. Similarly, we noted a highly heterogeneous appearance of mutations beyond RAS/RAF and *TP53* in the ctDNA trajectory data. The development of personalized ctDNA assays, such as NGS panels or droplet digital PCR covering individual mutational profiles, is required to overcome this heterogeneity.

In summary, our findings suggest that profiling genetic alterations in ctDNA using a minimally invasive approach during the precursor disease stage is feasible for predicting the development of MM and its specific clinical presentation. ctDNA profiling may enhance conventional risk stratification in NDMM and serve as a dynamic tool for identifying significant genetic events during treatment. Ultimately, ctDNA assessment may provide valuable insights into the optimal timing for precise assessments, including BM examinations and PET/CT, to assist in confirming MM progression and the necessity for treatment.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/

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AUTHOR CONTRIBUTIONS

Park SS, Chung YJ, Jung SH, and Min CK conceived and designed the study; Park SS, Lim JY, Lee JY, and Min CK participated in the clinical sample acquisition; Kim NY, Yun S, Chung YJ, and Jung SH participated in the next-generation sequencing (NGS) data generation and conducted the bioinformatics analysis; Park SS, Kim NY, Jung SH, and Min CK wrote the manuscript.

CONFLICTS OF INTEREST

None declared.

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