***KRAS*, *NRAS*, and *BRAF* mutations in plasma cell myeloma at a single Korean institute**

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Background

Plasma cell myeloma (PCM) is a genetically heterogeneous disease. The genetic spectrum of PCM has been expanded to mutations such as *KRAS*, *NRAS*, and *BRAF* genes in the RAS-RAF-MAPK pathway. In this study, we have evaluated the frequency of these mutations and their significance, including baseline characteristics and clinical outcomes.

Methods

We explored 50 patients who were newly diagnosed with PCM between 2009 and 2012 at a single Korean institute. Clinical and laboratory parameters were gathered through careful review of medical records. Mutation analysis was carried out using DNA from the bone marrow at the time of diagnosis. Pyrosequencing was performed to detect *KRAS* G12V, *KRAS* G13D, and *NRAS* G61R. *BRAF* V600E was analyzed by allele-specific real-time PCR. Comparison of clinical and laboratory parameters was carried out according to those mutations.

Results

We identified 14 patients (28%) with activating mutations in the RAS-RAF-MAPK pathway (RAS/RAF mutations): *KRAS* (N=3), *NRAS* (N=4), *BRAF* (N=7), and both *KRAS* and *BRAF* (N=1). RAS/RAF mutations were more frequently observed in patients with complex karyotypes and showed poorer progression free survival (PFS). Specifically, the *BRAF* V600E mutation had a significantly negative impact on median PFS.

Conclusion

We first showed the frequency of RAS/RAF mutations in Korean patients with PCM. Screening of these mutations could be considered as a routine clinical test at the time of diagnosis and follow-up due to their influence on clinical outcome, as well as its potential as a therapeutic target.

Key Words *KRAS*, *NRAS*, *BRAF*, Plasma cell myeloma

INTRODUCTION

Plasma cell myeloma (PCM) is a multifocal neoplastic proliferation of plasma cells in the bone marrow (BM) [1]. Karyotype abnormalities are the main drivers of PCM, including hyperdiploidy characterized by trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, and rearrangements involving the immunoglobulin heavy locus (*IGH*) gene translocations [2].

Recent studies have expanded the genetic spectrum of PCM regarding genetic mutations. The RAS pathway is the most frequently mutated pathway in PCM, with about 20% of newly diagnosed patients with PCM having driver mutations in *KRAS* or *NRAS* [3, 4]. Mutations occur at codons 12 and 61 in *KRAS* and *NRAS*, respectively, preventing GTP hydrolysis and keeping RAS in its active state, subsequently activating the mitogen-activated protein kinase (MAPK) pathway [5]. In addition, about 5% (4% to 12%) of patients with PCM harbored the *BRAF* mutation at diagnosis, mostly

on amino acid V600 [3, 4, 6-10]. *BRAF* V600E causes constitutive activation of the RAS pathway, presumably leading to increased cell growth and preventing apoptosis [11]. It has been known that activating mutations in the RAS-RAF-MAPK pathway are more frequently observed in relapsed and refractory PCM and are associated with worse prognosis, shorter patient survival, and tumor progression; however, a majority of studies have been performed before the era of novel therapeutic agents [12-15].

In this study, we analyzed the *KRAS*, *NRAS*, and *BRAF* genes to evaluate the prevalence of mutations in these genes in patients from a single Korean institute. We also have

studied their associations with clinical characteristics and karyotypes, and evaluated their influence on clinical outcomes.

MATERIALS AND METHODS

Patients

We explored 50 patients who were newly diagnosed with PCM between 2009 and 2012 at Seoul St. Mary's Hospital. We selected available BM samples of inpatients who were previously performed with a BM study and were treated for PCM. We analyzed the data in April 2020. This study

Table 1. Baseline characteristics of the patients.

Characteristics	Total (N=50)
Age years, median (range)	66 (31-82)
Gender, male, N (%)	27 (54%)
Type of myeloma, N (%)	
Ig G	25 (50%)
Ig A	10 (20%)
Ig M	1 (2%)
Ig D	3 (6%)
Light chain disease	11 (22%)
Clonality of Light chain, N (%)	
kappa	33 (66%)
Lambda	17 (34%)
Extramedullary disease	
Yes, N (%)	8 (16%)
No, N (%)	42 (84%)
Lactate dehydrogenase	
> Upper limit of normal	19 (38%)
Normal	31 (62%)
Median renal function (creatinine clearance) before transplant, mL/min, (range)	58.8 (5.73-110.8)
> 60, N (%)	24 (48%)
≥ 30 to < 60, N (%)	16 (32%)
< 30, N (%)	10 (20%)
ISS stage at diagnosis	
I, N (%)	6 (12%)
II, N (%)	16 (32%)
III, N (%)	26 (52%)
Unknown, N (%)	2 (4%)
Frontline treatment	
Bortezomib-melphalan-prednisolone with transplant	11 (22%)
Bortezomib-melphalan-prednisolone without transplant	36 (72%)
Others with transplant	3 (6%)
Eligibility of autologous stem cell transplantation	
Eligible, N (%)	14 (28%)
Not-eligible, N (%)	36 (72%)
Best response of frontline treatment	
CR or better	23 (46%)
VGPR	13 (26%)
PR	13 (26%)
SD	1 (2%)
Median PFS of frontline treatment, months, median (95% CI)	23.5 (16.5-25.6)
Median OS, months, median (95% CI)	105.7 (63.7-not available)

Abbreviations: CI, confidence interval; CR, complete response; OS, Overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease; VGPR, very good partial response.

was performed according to the Declaration of Helsinki and approval for this study was obtained from the Institutional Review Board of Seoul St. Mary's Hospital, The Catholic University of Korea (KC12SISE0594).

KRAS and NRAS mutation analysis by pyrosequencing

Genomic DNA from BM aspirates were isolated using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). Pyrosequencing was carried out using a PCR primer mix for *KRAS* exon 2 (codon 12, 13) and *NRAS* exon 3 (codon 61) (Supplementary Table 1). Each PCR mix contained forward and reverse primers (1 µL), 10× PCR buffer (2 µL), dNTP (0.2 µL), water (19.65 µL), Hotstar Taq Polymerase (0.15 µL), and 2 µL of genomic DNA for a total volume of 25 µL. PCR was done on a GeneAmp PCR system 9700 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with an initial activation step at 95°C for 15 min, 45 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, followed by a final extension cycle at 72°C for 15 min. Pyrosequencing was performed with an 80 µL final volume, containing 40 µL of biotinylated PCR product with high purity water, 37 µL of PyroMark Binding Buffer, and 3 µL of streptavidin beads (GE Healthcare, Uppsala, Sweden). Pyrosequencing was performed using a PyroMark Q96 ID instrument according to the manufacturer's instructions (Biotage, Uppsala, Sweden).

BRAFV600E detection by AS-PCR

We performed additional *BRAF* V600E detection tests using a Real-Q *BRAF* V600E detection kit (BioSewoom Inc., Seoul, Korea) on an Applied Biosystems 7500 Real-time PCR (Thermo Fisher Scientific) according to the manufacturer's

instructions. Briefly, PCR was performed in a 25 µL reaction volume containing 10 µL DNA, 12.5 µL 2× PCR reaction mix, and 2.5 µL *BRAF* probe and primer mixture. Reactions were performed for 40 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 58°C for 45 sec. The assay was repeated at least two times.

Definitions and Statistics

Comparisons of clinical and laboratory parameters at diagnosis between patient subgroups were done with the Mann-Whitney test or Fisher's exact test, respectively. Stages were classified according to the International Staging System for multiple myeloma [16]. Treatment response was evaluated according to the International Myeloma Working Group (IMWG) criteria [17]. Events for progression-free survival (PFS) were indicated as the first progression after front-line treatment or any cause of death. Overall survival (OS) was indicated as the time from initiation of frontline treatment to death (from any cause) or the date of the last follow-up. OS and PFS were determined using the Kaplan-Meier method and compared using a log-rank test. Variables with $P < 0.1$ in univariate analyses were entered into multivariate models using Cox proportional hazards regressions with a backward stepwise model selection. P -values < 0.05 were considered significant. All statistical analyses were conducted using R.3.1.1 statistical software (<http://cran.r-project.org/>).

RESULTS

Clinical characteristics of patients

The patient demographics, clinical, and laboratory characteristics are summarized in Table 1. The median age of pa-

Fig. 1. Overview of the genetic abnormalities identified in 50 plasma cell myeloma patients.

tients was 66 years (range, 31–82). The most frequent type of myeloma was IgG (54%, 27/50), followed by IgA (20%), light chain (18%), IgD (6%), and IgM (2%). Karyotype analyses and interphase FISH were performed using diagnostic BM aspirates with the same methods used in a previous study [18]. Results were classified and described according to the 2016 International System for Human Cytogenetic Nomenclature (ISCN) guidelines [19]. Of the 50 patients, 31 (62%) harbored abnormal karyotypes. Hyperdiploidy (≥ 47 chromosomes) and hypodiploidy (≤ 45 chromosomes) were observed in 18 (36%) and 6 patients (12%), respectively. Sixteen patients presented with *IGH* gene rearrangements including t(11;14) (N=9), t(8;14) (N=1), and other *IGH* gene rearrangements (N=6). Fourteen patients (28%) showed 1q gain and one patient (2%) had a 17p deletion. Complex karyotype, defined as ≥ 3 chromosome abnormalities, was identified in 23 patients (46%). Regarding the selection of treatment, we had intention-to-treat with autologous stem cell transplantation following frontline chemotherapy for 14 patients (28%), whereas 36 patients (72%) classified as transplant-ineligible received chemotherapy consisting of bortezomib-melphalan-prednisolone for their frontline treatment.

Activating mutations in RAS-RAF-MAPK pathway

RAS mutations were detected in 16% patients (8/50), including four *KRAS* mutations and four *NRAS* mutations. *BRAF* mutations were detected in seven patients (14%). *KRAS* and *NRAS* mutations were mutually exclusive. A patient with both *KRAS* G13D and *BRAF* mutations was found (Fig. 1). Thus, fourteen patients showed any *KRAS*, *NRAS*, and/or *BRAF* (RAS/RAF) mutations. RAS/RAF mutations were more commonly identified in patients with abnormal karyotype [odds ratio (OR), 5.368; 95% CI, 1.048–27.502; $P=0.050$] and complex karyotype (OR, 4.423; 95% CI, 1.153–16.964; $P=0.031$). Other parameters included age, sex, type of monoclonal proteins, hemoglobin, creatinine, total protein, albumin, LDH, and $\beta 2$ -microglobulin. The stage according to the International Staging System and frontline treatment type were distributed equally or non-significantly different according to RAS/RAF mutations.

In the total cohort, there was an overall response rate of 98% (49 of 50 patients) through frontline treatment: 23 patients (46%) with complete response, 13 patients (26%) with very good partial response, and 13 patients (26%) with partial response. The remaining patient showed stable disease despite 6 cycles of treatment with bortezomib-melphalan-prednisolone, finishing her frontline treatment early due to unacceptable peripheral neuropathy. RAS/RAF mutations did not provide a significant impact on either complete response rate or the achievement of partial response of any degree (Table 2).

With a respective median PFS of 23.5 months (95% CI, 16.5–25.6) and median OS of 105.7 months (95% CI, 63.7–not estimable) (Supplementary Fig. 1), RAS/RAF mutations were significantly associated with poor median PFS [24.0 mo (95% CI, 16.5–49.3) vs. 18.2 mo (3.6–24.2), $P=0.015$, Fig. 2A]. There

was no statistical difference in median OS according to the presence of RAS/RAF mutations (85.2 mo vs. not estimable, Fig. 2B). In the subgroup analysis for PFS, *BRAF* V600E mutations had a significantly negative impact on median PFS [18.2 mo (95% CI, 1.8–24.2) for patients with *BRAF* V600E mutation (N=7) vs. 23.9 mo (95% CI, 16.5–31.2 mo) for patients with wild-type *BRAF* (N=43), $P=0.04$, Fig. 2C]. Patients with RAS mutations, presented in either *KRAS* and *NRAS* (N=8), showed a statistical trend of poor median PFS compared to those without RAS mutations (N=42) [16.3 mo (95% CI, 0.4–26) vs. 23.9 mo (95% CI, 17.6–29), $P=0.081$, Fig. 2D]. Multivariable analysis showed that RAS/RAF mutations were independent factors associated with poor PFS (hazard ratio=2.28, 95% CI, 1.15–4.5, $P=0.018$). However, the factor of *BRAF* V600E mutation lost their statistical significance in the multivariable analysis (Table 3, Supplementary Table 2).

DISCUSSION

In this study, we identified 14 patients with PCM and RAS/RAF mutations (28%). *KRAS* and *NRAS* mutations were detected in 16% of patients, and *BRAF* V600E was in 14%. The prevalence was similar with the results from previous studies except for *BRAF* V600E, which was slightly higher than others [4, 6–9, 20]. This may be explained by the higher

Table 2. Characteristics of patients with any *KRAS*, *NRAS*, and/or *BRAF* mutations.

Characteristics	RAS/RAF(+) (N=14)	RAS/RAF(-) (N=36)
Age years, median (range)	69 (64–82)	66 (32–80)
Gender, male, N (%)	9 (64%)	18 (50%)
Cytogenetics		
Abnormal	12	19
Complex (≥ 3)	10	13
ISS stage at diagnosis		
I, N (%)	0 (0%)	6 (17%)
II, N (%)	5 (36%)	11 (31%)
III, N (%)	9 (64%)	17 (47%)
Unknown, N (%)		2 (6%)
Frontline treatment		
Bortezomib-melphalan-prednisolone with transplant	2 (14%)	9 (25%)
Bortezomib-melphalan-prednisolone without transplant	12 (86%)	24 (67%)
Others with transplant	0 (0%)	3 (8%)
Best response of frontline treatment		
CR or better	5 (36%)	18 (50%)
VGPR	4 (29%)	9 (25%)
PR	5 (36%)	8 (22%)
SD	0 (0%)	1 (3%)

Abbreviations: CR, complete response; PR, partial response; RAS/RAF(+), presence of any *KRAS*, *NRAS* and/or *BRAF* mutation; RAS/RAF(-), absence of *KRAS*, *NRAS* or *BRAF* mutations; SD, stable disease; VGPR, very good partial response.

Fig. 2. Clinical outcomes of patients with any *KRAS*, *NRAS*, and/or *BRAF* mutation [RAS/RAF(+)] or without mutations [RAS/RAF(-)] (**A**, **B**). Comparison between subgroups; *BRAF* V600E (+) vs. *BRAF* V600E (-) (**C**) and RAS (+) vs. RAS (-) (**D**).

sensitivity of AS-PCR in detecting *BRAF* V600E compared to other molecular techniques, including pyrosequencing [21]. We also found that RAS mutations were mutually exclusive, but not with *BRAF* V600E [22]. Interestingly, RAS/RAF mutations were associated with abnormal karyotype and complex karyotype. These results were in line with previous findings that RAS mutations can cause a transition from the pre-malignant monoclonal gammopathy of undetermined significance to PCM [23, 24]. However, RAS mutations did not correlate with clinical stage, such as ISS [25].

From a clinical perspective, the prognostic significance of RAS/RAF mutations in PCM is controversial. The application of novel therapeutic agents has changed the influence of these mutations. One study showed that RAS mutations appeared to be significantly associated with a favorable outcome [26]. Another study showed that functional activation

of the RAS pathway was observed in 75% of patients with relapsed/refractory PCM and about half had RAS/RAF mutations [27]. In this study, we demonstrated that RAS/RAF mutations were associated with poor outcomes, such as a shorter PFS. Aside from RAS/RAF mutations, a subgroup with *BRAF* V600E mutations also provided poor PFS compared to the wild-type RAS/RAF. Because the activation of the MAPK pathway via RAS/RAF mutations was concordant with the gene expression profile data of the same patients, inhibition of this signaling would be effective in this subgroup of patients [7, 27, 28]. Of them, *BRAF* has received considerable attention as a result of the success of targeted malignant melanoma therapy [29]. The genomic and transcriptional mutant allele burdens of *BRAF* were highly concordant in patients with *BRAF*-mutant PCM [9]. These findings further strengthen the hypothesis that patients with *BRAF* V600E mutations may benefit from new

Table 3. Univariable and multivariable analysis for progression-free survival.

Variables (N=50)	N	Univariate analysis		Multivariable analysis	
		Median PFS, mo (95% CI)	P	Hazard ratio (95% CI)	P
Patient age (yr)			0.837		-
< 66	19	23.9 (15.4–51.3)		-	
≥ 66	31	18.9 (12.6–29)		-	
Sex			0.496		-
Male	27	23.9 (15.4–31.4)		-	
Female	23	18.9 (12.6–24.9)		-	
Type of myeloma			0.605		-
IgG	25	24.2 (15.4–49.3)		-	
Non-IgG	25	18.9 (12.6–24.9)		-	
Type of light chain			0.649		-
Kappa	33	18.9 (15.4–25.5)		-	
Lambda	17	24.6 (8.5–51.3)		-	
Lactate dehydrogenase			0.373		-
> Upper limit of normal	31	23.9 (16.3–31.4)		-	
Normal	19	22.2 (8.5–26)		-	
ISS stage at diagnosis			0.826		-
I or II	22	24.2 (18.2–31.4)		-	
III	26	16.5 (8.8–51.3)		-	
Unknown	2			-	
Cytogenetic status			0.536		-
Standard risk	26	23.9 (16.3–31.2)		-	
High risk	13	23.5 (8.5–51.3)		-	
Unknown	11			-	
Extramedullary disease			0.925		-
Present	8	25.1 (6–52.8)		-	
None	42	22.2 (14.8–25.6)		-	
Transplant eligibility			0.601		-
No	36	18.9 (12.6–24.9)		-	
Yes	14	26.5 (15.4–51.3)		-	
<i>BRAF</i> V600E mutation			0.04		0.623
No	43	23.9 (16.5–31.2)		1	
Yes	7	18.2 (1.8–24.2)		1.32 (0.44–3.98)	
RAS/RAF mutation			0.015		0.018
No	36	24.0 (16.5–49.3)		1	
Yes	14	18.2 (3.6–24.2)		2.28 (1.15–4.5)	

Abbreviations: CI, confidence interval; PFS, progression free survival; RAS/RAF, any of *KRAS*, *NRAS* and/or *BRAF* mutations.

targeted treatments with *BRAF* inhibitors, such as vemurafenib [30] and dabrafenib [31]. A recent study suggested that the development of mutation-specific *KRAS* inhibitors could be of great value in patients with *KRAS*-mutant PCM [32, 33]. Moreover, results of the correlation between RAS/RAF mutations and complex karyotypes suggested the therapeutic benefit of checkpoint inhibitors in this group [34].

Our study has several limitations. This is a retrospective study using selected BM samples of inpatients considered to be in a more severe condition with a high disease burden. Thus, the proportion of karyotype abnormalities and ISS stage II were relatively higher compared to a previous study in Asian patients [35]. There is also a possibility that the prevalence of *KRAS*, *NRAS*, and *BRAF* mutations have been overestimated due to the same reason. Next, the number

of enrolled samples was small and their baseline characteristics, including frontline treatment, were heterogeneous. Our results should be further validated with a large number of cases before implementation in the clinic.

Consequently, this study first showed the frequency of RAS/RAF mutations in Korean patients with PCM. Screening of these mutations could be considered as a routine clinical test at the time of diagnosis and follow-up due to their influence on clinical outcomes and their potential as a therapeutic target.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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Supplementary Table 1. Primers used for pyrosequencing.

Mutation	Primer
<i>KRAS</i> G12V	F-ATAAGGCCTGCTGAAAATGACTG
	R-(biontin)-AGAATGGTCCTGCACCAGTAATA
	S-CTTGTGGTAGTTGGAGC
<i>KRAS</i> G13D	F-GGCCTGCTGAAAATGACTGA
	R-(biontin)-AGAATGGTCCTGCACCAGTAAT
	S-TGTGGTAGTTGGAGCTG
<i>NRAS</i> Q61R	F-TTTGTTGGACATACTGGATACAGC
	R-(biontin)-TTCCCTAGTGTGGTAACCTCATT
	S-GGACATACTGGATACAGCT

Supplementary Fig. 1. Progression free survival after autologous stem cell transplantation in this study (N=50).

Supplementary Table 2. Clinical outcome after frontline treatment according to mutations.

	Number	PFS*	95% CI	P-value	OS*	95% CI	P-value
RAS/RAF							0.243
Yes	14 (28%)	18.2	7.5–29.0	0.015	NE	NE–NE	
No	36 (72%)	23.9	21.8–26.0		85.2	56.6–113.8	
<i>KRAS</i> G12V				<0.001			0.798
Yes	2 (4%)	0.4	0.4–NE		NE	NE–NE	
No	48 (96%)	23.7	16.5–26.9		105.7	63.7–NE	
<i>KRAS</i> G13D				0.771			0.17
Yes	2 (4%)	10	1.8–NE		4.6	4.6–NE	
No	48 (96%)	23.9	16.5–26		105.7	67.9–NE	
<i>NRAS</i> Q61R				0.603			0.907
Yes	4 (8%)	21.2	6–NE		NE	13.2–NE	
No	46 (92%)	23.5	16.5–25.6		105.7	63.7–NE	
<i>BRAF</i> V600E				0.04			0.273
Yes	7 (14%)	18.2	1.8–24.2		NE	4.6–NE	
No	43 (86%)	23.9	16.5–31.2		85.2	46.7–NE	

*Median, months; PFS, progression free survival; OS, overall survival; CI, confidence interval; RAS/RAF, any of *KRAS*, *NRAS* and/or *BRAF* mutations; NE, not estimated.