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Integration of clinical outcomes and molecular features in extramedullary disease in multiple myeloma

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Multiple myeloma (MM) remains incurable despite novel therapeutics. A major contributor to the development of relapsed/refractory and resistant MM is extraosseous extramedullary disease (EMD), whose molecular biology is still not fully understood. We analyzed 528 MM patients who presented to our institution between 2014 and 2021 and who had undergone molecular testing. We defined EMD as organ plasmacytoma distinct from bones and evaluated patients for the development of EMD with the goal of defining their molecular characteristics. Here, we show that *RAS/BRAF* mutations are likely essential for the development of EMD. Our results also indicate that the underlying reason for the negative outcomes in patients with poor prognostic factors such as duplication 1q and deletion 17p is largely due to the development of EMD. However, the presence of *TP53* mutation remains a poor prognostic factor regardless of EMD development. Furthermore, mutation sites of *TP53* were different between EMD versus non-EMD patients, with gain-of-function mutations enriched in patients with EMD. Our data highlights distinct molecular abnormalities in patients with EMD and provides potential mechanistic insights for novel therapeutic targets for the future.

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INTRODUCTION

Multiple myeloma (MM) is a bone marrow (BM)-based, multifocal neoplastic proliferation of plasma cells and Extramedullary disease (EMD) is defined by plasma cell neoplasms that arise in tissues distinct from bone [1]. Treatment of MM continues to evolve, resulting in dramatically improved patient outcomes in recent years. Despite these advances, cure remains elusive [2], with EMD playing a major role in the development of relapsed/refractory MM (RRMM) [3, 4] resistant to novel therapies. Typically, EMD is seen in advanced MM, but rarely patients can present with EMD at diagnosis. The prevalence of EMD is approximately 10–15% in all RRMM patients, with only 0.5–6.4 of cases presenting at diagnosis [5–9]. EMD is associated with poor prognosis and is clinically distinct from MM without EMD [3, 10, 11]. Previous reports suggest that EMD can arise in any organ, with the site of origin varying from patient to patient [11, 12]. Although EMD has been defined by plasmacytomas distinct from bone, there are studies and trials which include para-skeletal plasmacytomas as EMD, while others do not [12–15]. The mechanisms involved in the pathogenesis of EMD remain unknown and unclear diagnostic criteria may hinder the understanding of it. Previous studies have reported risk factors for EMD, with some suggesting the potential role of *RAS/BRAF* mutations in the intramedullary to extramedullary transition in a limited number of patients [16, 17]. Patients with *RAS/BRAF* mutations showed a higher likelihood of developing EMD compared to patients without this mutation [16–19]. Prevalence of *RAS/BRAF* mutations increases in RRMM patients [20]. Mutations in *TP53*, deletion 17p (del (17p)), and duplication 1q (dup (1q)) are well-known poor prognostic factors, and previous reports have suggested that these risk factors are enriched in EMD patients [16, 21–23]. However, the consequent impact of these abnormalities on EMD remains unknown [20]. Thus, defining the molecular characteristics of EMD is critical to a better understanding of EMD biology and will help with advancing treatment of these patients. In the present retrospective study, we report the landscape of molecular features and clinical outcomes of MM patients with EMD from a cohort of 528 MM patients who had undergone molecular testing at a single center.

METHODS

Patient cohort

Five hundred twenty-eight MM patients who presented to Massachusetts General Hospital between 2014 and 2021 were evaluated if they had molecular profiling completed on at least one sample (BM and/or EMD and/or bone plasmacytoma). All patients voluntarily provided informed consent approved by the Institutional Review Board for molecular testing. Clinical data was retrospectively collected from electronic medical records. Histological analysis, fluorescence in situ hybridization (FISH), and mutational analysis were conducted on BM aspirates, BM biopsies, and EMD tumor specimens when available.

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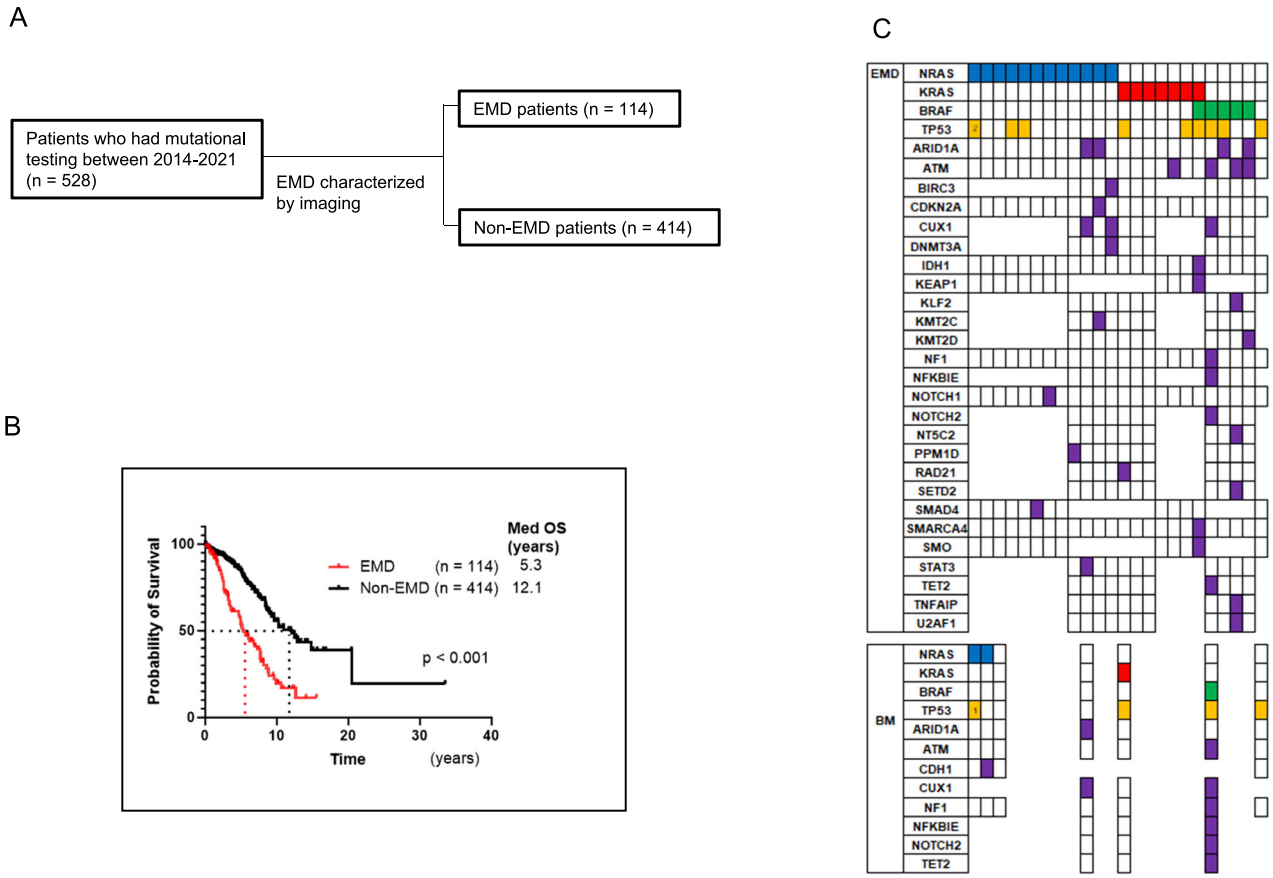

Fig. 1 *RAS/BRAF* mutations are enriched in extramedullary disease (EMD). A Study overview of myeloma patients with mutational testing and EMD development characterized by imaging. **B** Overall survival (OS) of EMD patients ($n = 114$) versus non-EMD patients ($n = 414$) demonstrated a median OS of 5.3 years vs 12.1 years ($p < 0.001$). **C** Mutational profile of 24 EMD and 7 paired bone marrow (BM) samples. Each column represents a patient sample, and the different rows highlight the mutated genes: 11 patients harbored an *NRAS* mutation, 7 had a *KRAS* mutation and 5 patients had a *BRAF* mutation with 1 patient harboring both *BRAF* and *KRAS* mutations.

Diagnosis of EMD

We defined EMD by the presence of plasmacytomas in organs and soft tissue distinct from bones, based on imaging data. The diagnosis of EMD was determined by imaging (CT, PET CT, or MRI) as part of clinical care (Fig. 1A). In some cases, EMD was confirmed by biopsy. Plasma cell leukemia was not included.

Mutational analysis

Nucleic acid extraction was performed using Promega Maxwell columns from BM and/or EMD samples obtained from MM patients. Heme panel which is a Multiplexed mutational analysis was conducted with primers designed to cover 91 (until 2018) or 111 (after 2019) genes (Supplementary Table 1), using Anchored Multiplex PCR for single nucleotide variant and insertion/deletion in genomic DNA using ArcherDx platform and Illumina NextSeq. The threshold of tumor cell frequency in BM aspirates and biopsies was determined as 9% based on previous laboratory cutoffs. If samples had a mutation at any time, we defined them as positive for that mutation.

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was performed at Mayo Clinic laboratories. The probes are listed in Supplementary Table 2. We followed Mayo Clinic laboratories' interpretation regarding the positivity.

Statistical analysis

Median survival rates for the whole group, and by mutation status, were calculated using the Kaplan-Meier method, while the log-rank test was used to compare survival. In the descriptive analysis, *p*-values were calculated using Fisher's exact test. The Kolmogorov-Smirnov test was used to compare the distributions of mutations. All analyses were conducted using Prism software, and a p-value ≤ 0.05 was considered statistically significant.

RESULTS

Mutational testing reveals *RAS/BRAF* mutations are likely essential for the development of EMD

Five hundred and twenty-eight patients who underwent mutation analysis at our institution were included in this study. All patients were included in the OS study. Data from 528 patients, with a median follow-up of 3.8 years is presented here (Table 1). Overall, 114 patients (21.6%) were classified as having EMD as plasmacytomas distinct from bones by imaging, while 414 patients (78.4%) did not show any evidence of EMD (Fig.1B). Patients with EMD had poorer prognosis compared to those without EMD; median OS was 5.3 years for 114 EMD patients vs 12.1 years for 414 non-EMD patients ($p < 0.001$) (Fig. 1B). The median time of developing EMD was 3.2 years, and once patients developed EMD, median survival was only 10.1 months (Data not shown). No significant differences were found between EMD and non-EMD patients regarding MM type, gender, and International Staging System (ISS) stage (Table 1). However, age at diagnosis was significant with younger patients developing EMD (mean age = 59.6) compared to non-EMD patients (mean age = 65.2), ($p < 0.001$). Age stratification showed that the EMD incidence rate decreases with age at MM diagnosis, especially after the age of 50 years (Supplementary Fig. 1A). To compensate for the influence of the duration of follow-up, we analyzed the data by cumulative incidence curve. The data revealed that MM diagnosis at the age of 50 and under was at a

Table 1. Patient Demographics.

	EMD patients	Non-EMD patients	
Number	114	414	
Follow-up (years)			
Range	0–15.5	0–33.5	
(Median)	3.5	3.9	
Age at Diagnosis			
Range	28–93	29–86	$p < 0.001$
(Median)	60	65	
Sex			
Male	72 (63.2%)	230 (55.6%)	$P = 0.17$
Female	42 (36.8%)	184 (44.4%)	
Myeloma type			
IgG	53 (46.5%)	244 (58.9%)	
IgA	26 (22.8%)	77 (18.6%)	
IgD	1 (0.9%)	4 (1.0%)	
Light Chain	30 (26.3%)	84 (20.3%)	
Non-secretory	3 (2.6%)	5 (1.2%)	
ISS			
1	38 (33.3%)	149 (36.0%)	
2	23 (20.2%)	102 (24.6%)	
3	34 (29.8%)	115 (27.8%)	
Unknown	19 (16.7%)	48 (11.6%)	

significantly higher risk of EMD development ($p = 0.027$) (Supplementary Fig. 1B). Treatment details are now included in supplementary information (Supplementary Table 3) as no firm conclusions could be drawn due to patient heterogeneity.

Among 114 EMD patients, 64 had biopsies of EMD tissue, of whom 24 underwent mutational testing. Twenty-three of 24 samples (95.8%) harbored at least one of the *RAS/BRAF* mutations (*NRAS, KRAS*, and *BRAF*); 12 had *NRAS* mutations, 6 had *KRAS* mutations, and 4 had *BRAF* mutations, and 1 had both *KRAS* and *BRAF* mutations (Fig. 1C). For BM, samples with less than 9% tumor cells were excluded from mutation analysis evaluation as mentioned. As a result, there were 7 patients with paired BM samples. Interestingly, two EMD-*NRAS*-positive patients did not have *NRAS* mutations in their BM (Fig. 1C). The tumor cell ratios in BM of those two patients were 70% and 75%, and the sequencing coverages were 267x and 183x, respectively, which is sufficient to eliminate false negatives. For both patients, EMD samples were collected prior to BM samples. One patient received chemotherapy between those two-time points, and the other patient had focal radiation therapy at the EMD site. Additionally, one of the patients had two *TP53* mutations in the EMD site but only had one in the BM. These mutational discrepancies may suggest the heterogeneity of tumor cells throughout the body and indicate that *RAS/BRAF*-mutated cells predispose to the development of EMD.

The prognosis of patients with BM-*RAS/BRAF* is largely defined by EMD development

Since *RAS/BRAF* mutations were enriched in EMD samples, we compared the incidence of EMD and OS of BM-*NRAS*, *KRAS*, *BRAF* single-positive patients and non-*RAS/BRAF* patients. There were 374 patients whose BM samples had more than 9% of tumor cells (198 patients had samples taken at diagnosis and 45 patients had samples taken at multiple time points). One hundred and forty-nine of 374 patients (39.8%) harbored BM-*RAS/BRAF* mutations; 45 single-*NRAS*, 65 single-*KRAS*, 18 single-*BRAF*, and 21 had more than 1 mutation present throughout their clinical course (Fig. 2A). The incidence rates of EMD were significantly higher in patients with BM-*NRAS* and with more than 1 mutation than non-*RAS/BRAF* patients (Fig. 2B). Generally, the incidence of EMD increases with recurrences, and the majority (54/114, 47.3%) of patients developed EMD after three or more recurrences in our cohort (Data not shown). Therefore, we examined the percentage of BM-*RAS/BRAF* mutations in relation to disease progression. Similarly, the percentage of patients with BM-*RAS/BRAF* mutation gradually increased with each relapse, and the highest rate was observed at the time of 3 or more recurrences in EMD patients (61.1%) (Supplementary Fig. 2). Even considering the percentage of *RAS/BRAF* mutations is associated with relapses, the percentage of *RAS/BRAF* mutations in EMD samples (95.1%) was significantly higher than that in BM of EMD patients with three or more recurrences (61.1%) ($p = 0.004$). This may suggest the existence of clonal heterogeneity and the evolutional processes between BM and EMD. We also analyzed the impact of EMD on OS by BM mutation status. Among 45 BM-*NRAS*, 65 BM-*KRAS* patients, and 225 non-BM-*RAS/BRAF* patients, EMD patients had poorer OS than non-EMD patients (Fig. 2C–E). No statistically significant difference was found in 18 patients with BM-*BRAF* patients and 21 patients with more than 1 mutation by the development of EMD, but this may be as a consequence of limited patient numbers (Data not shown). Among non-EMD patients, neither BM-*KRAS* nor *NRAS* had a negative impact on prognosis. These results indicate that the development of EMD has a greater impact on patient's prognosis than the BM mutation status itself.

Poor prognosis of patients with dup (1q) and del (17p) is conferred by the development of EMD

Since the presence of EMD impacts patients' prognosis, we analyzed molecular abnormalities other than *RAS/BRAF* mutations in EMD and non-EMD patients. We had three EMD samples analyzed for FISH with the only common abnormality being dup (1q), suggesting a possible underlying contribution of this molecular abnormality to EMD development. In addition to dup (1q), we analyzed del (17p), and *TP53* mutation status in bone marrow samples. Among the 362 patients whose BM was analyzed for dup (1q), 160 patients (44.2%) were positive (Fig. 3A). As previously reported, patients with dup (1q) had a poorer prognosis than those without it (Median OS 7.4 years vs 9.6 years, $p = 0.023$) (Fig. 3B). Patients with dup (1q) also had a higher incidence of EMD compared to those without it (27.5% vs 12.9%, $p < 0.001$, Odds ratio 2.57) (Fig. 3C). Next, we classified patients into four groups: EMD with and without dup (1q), and non-EMD with and without dup 1q, in order to examine the association between high-risk features, EMD development, and OS. Surprisingly, the presence of dup (1q) did not differentially affect OS in either EMD or non-EMD groups (Fig. 3D). *TP53* is on chromosome 17 and both del (17p) and *TP53* mutation are reported as risk factors on MM. Regarding del (17p), 83 patients (20.4%) were positive among the 407 patients whose BM was analyzed (Fig. 3E). Similarly, patients with del (17p) also had poorer prognosis compared with those without it (Median OS 7.7 years vs 8.9 years, $p = 0.024$) (Fig. 3F) and had a higher incidence of EMD (31.3% vs 16.4%, $p = 0.003$, Odds ratio 2.33) (Fig. 3G). Interestingly, the presence of del (17p) did not differentially affect OS in EMD versus non-EMD patients (Fig. 3H). This suggests that poor prognosis of del (17p) is largely conferred by the development of EMD. With respect to *TP53* mutation, it was present in 52 out of 374 patients (13.9%) (Fig.3I). Similar to the dup (1q) and del (17p) data, patients had a poorer prognosis compared to patients with *TP53* wild-type (wt) (Median OS 5.1 years vs 9.0

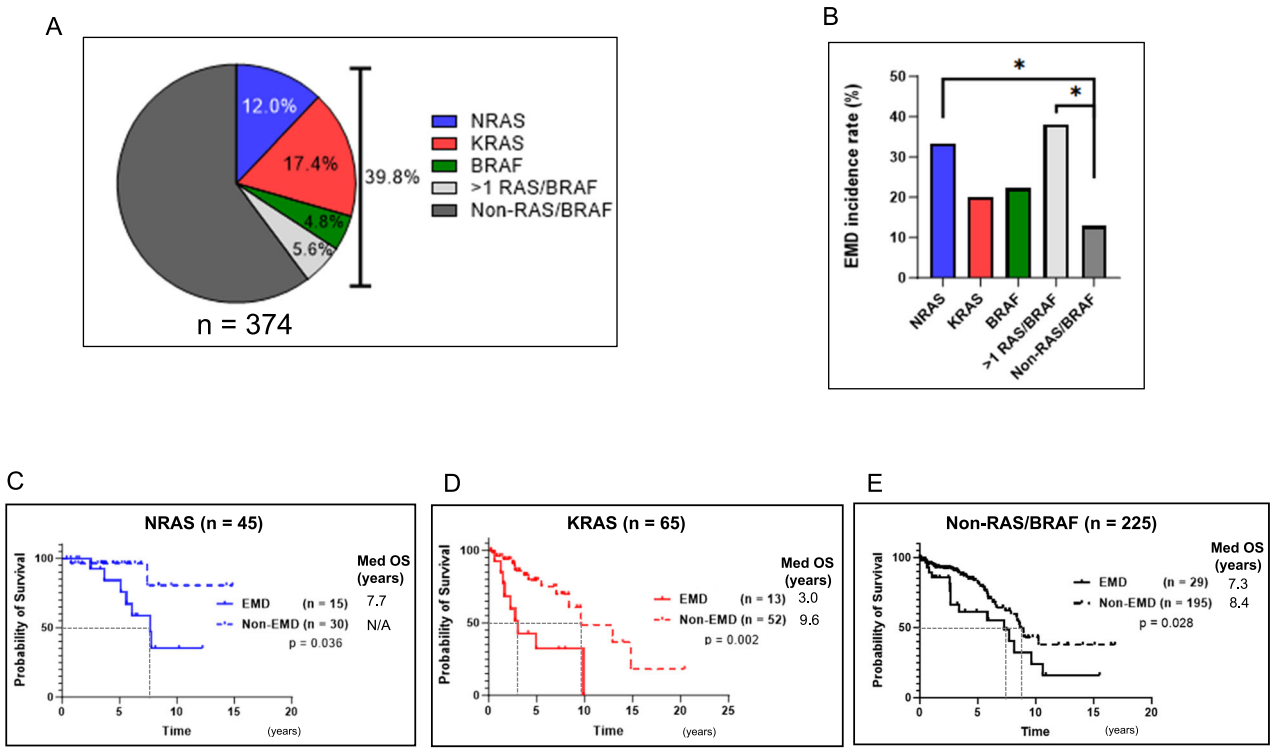

Fig. 2 Impact of BM-*RAS/BRAF* mutations on the development of EMD and overall survival (OS). A Bone marrow mutational status of 374 in patients who met the criteria for plasma cell cut-off of 9%: single-*NRAS* (12.0%), single-*KRAS* (17.4%), single-*BRAF* (4.8%), and >1 mutation (5.6%)). **B** EMD incidence rate by BM mutational status ($n = 374$: single-*NRAS* (33.3%), single-*KRAS* (20.0%), single-*BRAF* (22.2%), >1 mutation (38.1%) and non-*RAS/BRAF* (12.9%)) ($p < 0.05$). **C** OS of 45 BM-*NRAS* patients with EMD (Median OS: 7.7 years vs Not reached) ($p = 0.036$). **D** OS of 65 BM-*KRAS* patients with EMD (Median OS: 3.0 years vs 9.6 years) ($p = 0.002$). **E** OS of 225 Non-BM-*RAS/BRAF* patients with EMD (Median OS: 7.3 years vs 8.7 years) ($p = 0.028$).

years, $p < 0.0001$) (Fig. 3J) and these patients had a higher incidence of EMD as well (32.7% vs 16.1%, $p = 0.003$, Odds ratio 2.33) (Fig. 3K). However, unlike the other poor prognostic factors, there was a statistical difference between *TP53* mut and wt even after stratification by EMD development (Fig. 3L). Additionally, there was no statistically significant difference in OS between EMD (Median OS 3.4 years) and non-EMD (Median OS 5.5 years) if the patients had *TP53* mutation ($p = 0.07$). Although the number is limited, these data may indicate that the reason why dup (1q) and del (17p) present a poor prognosis may be largely due to their propensity to develop EMD. On the other hand, *TP53* mutations have a negative impact on OS regardless of EMD development.

The site of *TP53* mutation, but not *RAS/BRAF*, likely affects EMD development

We next sought to understand the influence of *RAS/BRAF* and *TP53* mutation sites on EMD occurrence. All mutations from BM and EMD samples were plotted (Fig. 4A–D). Our results showed that most of the mutations were seen in recurrent mutation sites and no difference was found between EMD and non-EMD patients among *RAS/BRAF* mutation sites (Fig. 4A–C). In contrast, there were differences in *TP53* mutation sites between EMD and non-EMD patients. Non-EMD patients showed mutations distributed throughout the gene, while EMD patients had more mutations in the C-terminus of the *TP53* gene (Fig. 4D) ($p = 0.009$). One patient showed two *TP53* mutations in both C- and N-terminus in the EMD sample but only an N-terminal *TP53* mutation in a subsequent BM sample. Notably, six of 17 EMD patients had *TP53* mutations accumulated in the sites of aa175, 248, and 273 which are reported as gain-of-function mutation sites in other cancers, while only 2 were found in 35 non-EMD patients. (35.3%: 5.7%, $p = 0.011$). This suggests that *TP53* mutations have different molecular effects depending on their sites and gain-of-function may contribute to the development of EMD.

DISCUSSION

Several studies have conducted mutational analyses in MM patient samples and demonstrated significant heterogeneity [24–27]. The clinical course of MM patients also varies widely across patients, which underscores the use of an individualized treatment approach. In recent years, there has been an unmet need to elucidate the molecular mechanisms underlying the development of EMD, one of the most refractory and intractable conditions of MM even with the availability of novel T cell redirected therapies. Myeloma typically occurs in older people and its incidence in patients below the age of 50 is low. In the past, there has been a multi-center retrospective study which showed that patients younger than 50 showed favorable factors with regard to ISS stage, as well as a fair prognosis [28]. However, according to our data, there was no clear correlation between ISS staging and EMD development. Our cumulative incidence data revealed that patients who were diagnosed with MM under the age of 50 were at a higher risk for EMD development even in the era of new drugs. Going forward, larger prospective studies will be needed to confirm our preliminary findings.

Numerous past studies have reported clinical data on EMD; however, sample sizes were often limited and the definition of EMD has not been clear or consistent [5–8]. With the definition of EMD as organ plasmacytoma distinct from bones, our data shows a strong relationship between EMD and *RAS/BRAF* mutations observed in MM patients. It is known that *RAS/BRAF* mutations are usually observed in advanced-stage MM rather

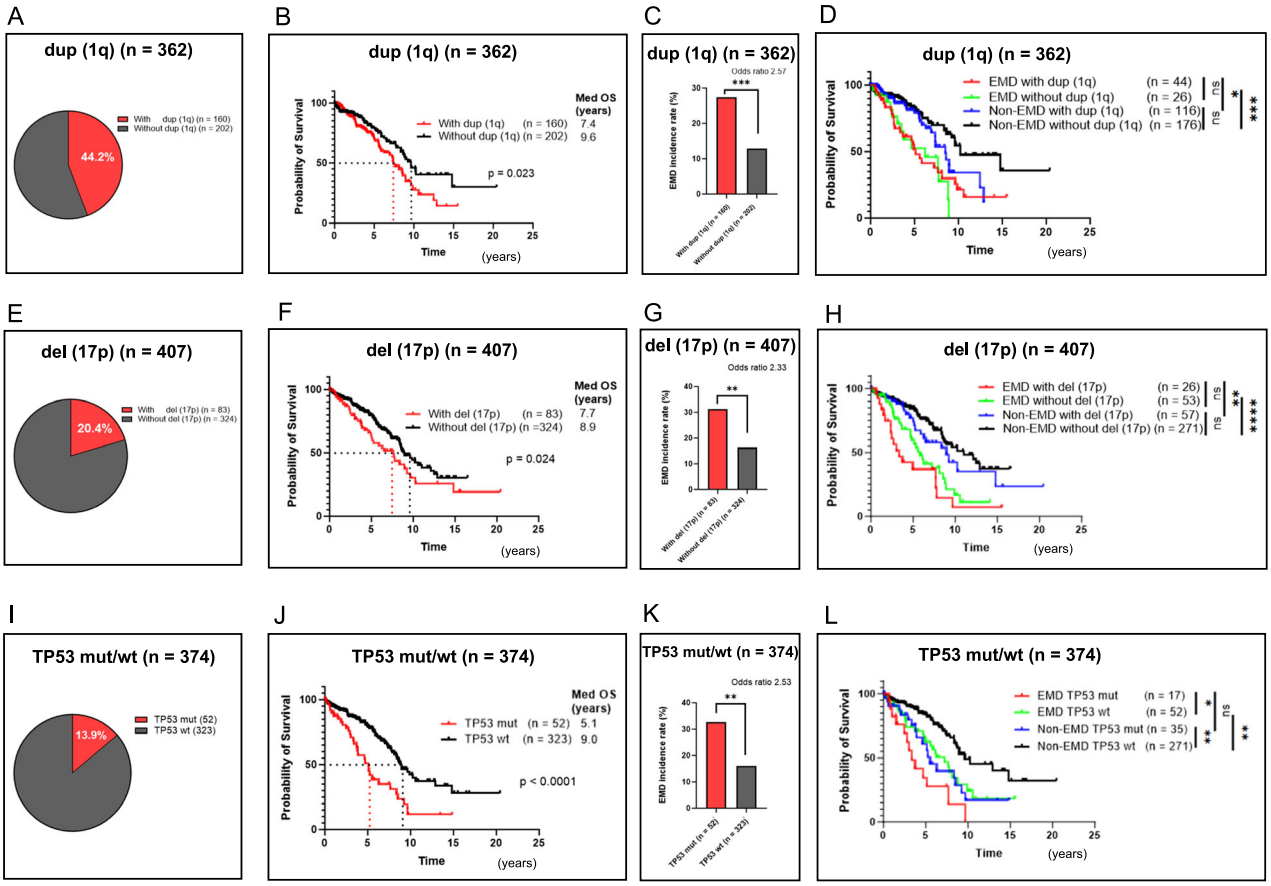

Fig. 3 Poor outcomes are noted in EMD patients with or without duplication 1q (dup (1q)) and deletion 17p (del (17p)). *TP53* mutation, however, portends a poor outcome regardless of the development of EMD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant. **A** Percentages of patients with and without dup (1q) ($n = 362$, 44.2% vs 55.8%). **B** OS of patients with and without dup (1q) (Median OS 7.4 years vs 9.6 years, $p = 0.023$). **C** EMD incidence rates of patients with and without dup (1q) (27.5% vs 12.9%, $p < 0.001$). **D** OS of EMD and non-EMD patients with and without dup (1q) (Median OS 5.1 years (red), 6.2 years (green), 8.5 years (blue), 10.2 years (black)). **E** Percentages of patients with and without del (17p) ($n = 407$, 20.4% vs 79.6%). **F** OS of patients with and without del (17p) (Median OS 7.7 years vs 8.9 years, $p = 0.024$). **G** EMD incidence rates of patients with and without del (17p) (31.3% vs 16.4%, $p = 0.003$). **H** OS of EMD and non-EMD patients with and without del (17p) (Median OS 3.4 years (red), 5.8 years (green), 9.0 years (blue), 11.0 years (black)). **I** Percentages of patients with *TP53* mutation (mut) and *TP53* wild-type (wt) ($n = 374$, 13.9% vs 86.1%). **J** OS of patients with *TP53* mut and *TP53* wt (Median OS 5.1 years vs 9.0 years, $p < 0.0001$). **K** EMD incidence rates of patients with *TP53* mut and wt (32.7% vs 16.1%, p = 0.007). **L** OS of EMD and non-EMD patients with *TP53* mut and *TP53* wt (Median OS 3.4 years (red), 5.5 years (green), 7.3 years (blue), 9.6 years (black)).

than MGUS, SMM, or newly diagnosed MM, and previous data indicate that *RAS/BRAF* mutations are acquired throughout the clinical course with disease progression as a consequence of clonal evolution [29]. In solid cancers, commonly occurring missense mutations in three members of *RAS* family genes result in their constitutive activation, and importantly, these mutations serve as predictors of poor patient survival [30, 31]. This is largely due to the association of *RAS/BRAF* mutations with the presence of metastases at diagnosis and with inherent or acquired resistance to treatments [32–34]. Our data indicates that EMD incidence is higher in BM-*RAS/BRAF* patients than non-BM-*RAS/BRAF* patients and the development of EMD is a poor prognostic factor regardless of the BM mutational status. Having a *RAS/BRAF* mutation alone does not result in a uniform clinical phenotype, nor does it directly determine prognosis. In our cohort, EMD patients with BM-*KRAS* had the worst outcome, with an OS of 3.0 years. Several studies have reported that the prognosis of patients who had *KRAS* mutations is worse than that of non-*RAS/BRAF* patients [35–37]. The poorer OS among EMD patients may be a contributing factor to the unfavorable prognosis of BM-*KRAS* patients in previous studies.

Whole genome sequencing studies have revealed that the genetic landscape of metastasis in solid cancers is distinct from the original site and is thought to be an evolutionary process [38, 39]. It has been reported that multiple myeloma patients have a mixture of clones in their BM and the dominance of these clones changes throughout the clinical course, leading to refractory disease [40]. Interestingly, in our cohort, two EMD-*NRAS*-positive patients did not have *NRAS* mutations in their BM. Also, one of those EMD samples had two *TP53* mutations, one of which was not detectable in BM. Of note, both BM samples were acquired after EMD samples, and one patient only had radiotherapy without systemic therapy between the two biopsies. Previous studies have shown similar results, but the authors concluded that detection limitations may have occurred owing to the low tumor burden in the BM [16]. However, BM samples collected in our study met the 9% tumor cell frequency threshold, which is sufficient to detect mutational burden. Although *RAS/BRAF* mutations are likely essential to the development of EMD and incidence of EMD is enriched in BM-*RAS/BRAF* patients, there was no difference in the site of *RAS/BRAF* mutation between EMD and non-EMD patients. It is also possible that patients who were negative for *RAS/BRAF* mutations in BM may have had mutated cells at an undetectable level which was not a dominant clone. These data may suggest a temporal and spatial heterogeneity of MM cells in patients.

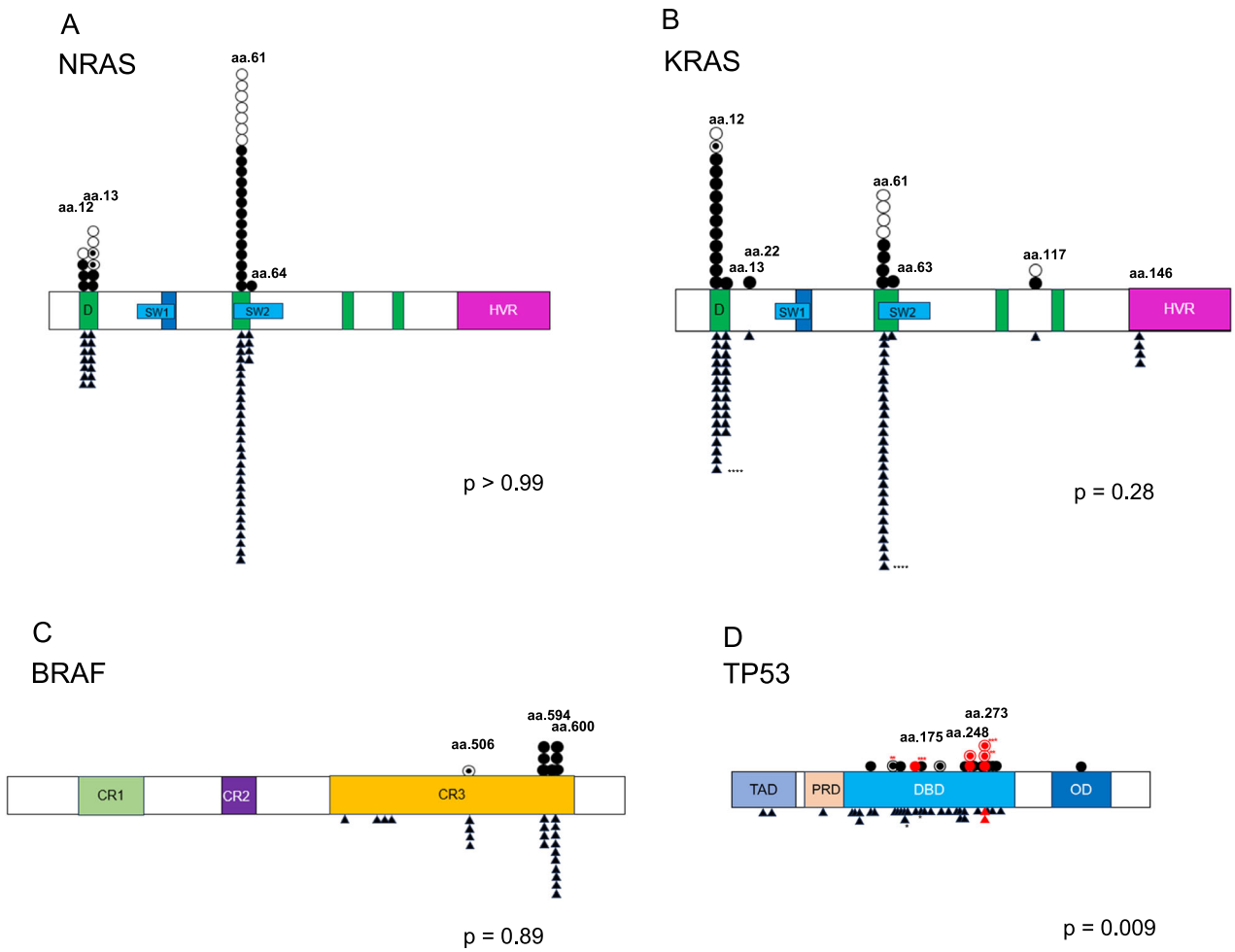

Fig. 4 Mutation sites differ with respect to *TP53*, but not *RAS/BRAF*, in EMD versus non-EMD patients. Individual data points of mutations from EMD patients (above the gene bar) and non-EMD patients (below the gene bar) are represented(○EMD tissue of EMD patients, ●BM of EMD patients, ◉Both BM and EMD tissue of EMD patients, ▲BM of non-EMD patients). The bar represents D (DNA binding lesion); SW (switch region); HVR, (hypervariable region); CR, (conserved region); TAD, (transactivation domain); PRD, (proline rich domain); DBD, (DNA binding domain); OD, (oligomerization domain). One non-EMD patient (*) and three EMD patients had 2 mutations in their samples (**, ***, and ****, respectively). Red symbols represent gain-of-function mutations. **A** Plots demonstrating *NRAS* mutation sites ($p > 0.99$). **B** Plots demonstrating *KRAS* mutation sites ($p = 0.28$). **C** Plots demonstrating *BRAF* mutation sites ($p = 0.89$). **D** Plots demonstrating *TP53* mutation sites ($p = 0.009$).

Although *RAS/BRAF* mutations are likely essential for the development of EMD, not all BM-*RAS/BRAF* patients develop EMD suggesting that the mutation alone is not sufficient to develop EMD. It is well known that dup (1q), del (17p), and *TP53* mutations are poor prognostic factors, and these are enriched in EMD patients which we confirmed in our cohort. Surprisingly, our detailed analyses revealed that EMD is a poor prognostic factor even in patients with dup (1q) and del (17p). In addition, there was no statistical difference in OS with or without the presence of these risk factors in both EMD and non-EMD patient groups. This indicates that the manner in which these are poor prognostic factors is largely due to the propensity to the development of EMD. *TP53* is one of the most significant tumor suppressor genes, and its mutation is often observed in a variety of cancers [41]. It consists of four domains, with most mutations contained in the DNA binding domain [42]. Some studies have shown the distinct functions of *TP53* N-terminus and C-terminus, with the C-terminus controlling site-specific DNA binding and structural changes within the central DNA binding domain. It is reported that the *TP53* gene has not only loss-of-function but also gain-of-function mutations [43]. Those gain-of-function mutations are known to promote cancer progression and metastasis in xenograft models and are associated with poor clinical outcomes in patients [44–47].

In the context of myeloma, it has been previously reported that mutations of *TP53* are associated with poor clinical outcomes, as they are linked with more aggressive and advanced forms of the disease, but the exact molecular role of *TP53* mutations in MM patients is unclear, including the mechanism of loss-of-function and gain-of-function mutations [47]. Interestingly, we found significant differences in the site of *TP53* mutations between EMD and non-EMD patients; non-EMD patients had mutations distributed throughout the gene, while EMD patients showed more mutations near the C-terminus, and accumulated in the sites of aa175, 248, and 273. These specific mutations are known as gain-of-function mutations. Currently, the function of *TP53* mutations in MM is poorly understood and no risk classification based on the site of *TP53* mutation exists. Our data indicates that the site of *TP53* mutations has a molecularly important impact on the development of EMD and that specific mutations may help to elucidate the underlying mechanisms of EMD. It also suggests that not all *TP53* mutations should be considered equal and that depending on mutation site the prognostic significance may vary. To our knowledge, this is the first report suggesting the relevance between EMD and *TP53* gain-of-function mutations. This analysis encompasses both newly diagnosed and RRMM; therefore, a more extensive and homogeneous prospective study in the future

would be desirable. This was also why no specific conclusions could be drawn on impact of treatment on our current cohort.

In summary, EMD sites may have different mutational landscapes from the BM in MM which suggests that *RAS/BRAF* mutations may play a critical role in the development of EMD, coupled with other associated molecular abnormalities, especially dup (1q) and del (17p), and site-specific *TP53* mutations. By integrating clinical outcomes and molecular features, we have revealed the clinical impact of EMD on MM patients and its association with the influences of known risk factors. These results will enable us to provide more accurate EMD risk stratification and provide insights for novel approaches in the treatment of MM.

DATA AVAILABILITY

The data that support the findings of this study are available upon request from the corresponding author. The data is not publicly available due to privacy and ethical restrictions.

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AUTHOR CONTRIBUTIONS

RN-M and NSR were responsible for the conceptualization and acquisition of clinical information. VN performed the mutational analysis. RN-M and NH analyzed the data. TF, RS, FR, KO, CP, KF, FR, and DST provided helpful discussion. ARB, DC, AJY, and NSR contributed to the sample collection and patient treatments. RN-M, R-HS, and NSR wrote the manuscript and all the authors reviewed the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Review Board of Massachusetts General Hospital (15-190). All methods were performed in accordance with the Declaration of Helsinki, the relevant guidelines, and regulations. Informed consent was obtained from all individual participants in this analysis.

ADDITIONAL INFORMATION

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