The genetic landscape of extramedullary plasmacytoma: a comparative analysis with extramedullary disease of multiple myeloma

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Abstract

Extramedullary (or extraosseous) plasmacytoma (EMP) is a localized plasma cell neoplasm lacking apparent bone marrow involvement and other features of multiple myeloma (MM). The prognosis is usually favorable and progression to MM is infrequent. The genetic landscape of EMP remains largely unknown. Importantly, EMP must be distinguished from extramedullary disease of MM (EMD), which typically exhibits an aggressive behavior. We performed a comprehensive molecular analysis of EMP (N=24) in comparison to EMD (N=24) using fluorescence *in situ* hybridization, copy number profiling, and targeted next-generation sequencing. Overall, EMP shared several genetic features with MM, but exhibited markedly lower complexity than EMD. EMP revealed a mean of 4.3 copy number changes *versus* 19.3 in EMD (P<0.001). IGH breaks were detected in six EMP and ten EMD, and hyperdiploidy in ten and 14 cases, respectively. Gains of 1q21 occurred in nine EMP and 15 EMD (P=0.045). Likewise, EMP harbored fewer mutations (median 2.0 *vs*. 3.0; P<0.01), yet was enriched for *TRAF3* mutations, detected in six cases, which were absent in EMD (P=0.022). In contrast, EMD more frequently harbored mutations associated with advanced MM, including *NRAS/KRAS* (P<0.01), *TP53*, *TENT5C*, *ARID1A*, and *ATM*. In conclusion, we demonstrate that EMP shows a significantly less complex and, in part, distinct genetic profile compared to EMD. This may aid in differentiating the two entities in diagnostically challenging cases and could help to identify EMP with an increased risk of progression.

Introduction

Multiple myeloma (MM) is a systemic plasma cell neoplasm defined by bone marrow infiltration in combination with myeloma-associated organ damage and/or specific myeloma-defining biomarkers (summarized by the SLiM-CRAB criteria).[1] MM follows a variable clinical course, but characteristically evolves in a stepwise manner, starting from its indolent precursor monoclonal gammopathy of undetermined significance (MGUS) and ultimately leading to biologically advanced manifestations such as extramedullary disease (EMD), which frequently occurs in relapsed disease, typically exhibits an aggressive behavior, and carries a poor prognosis.[2-4] The genetic events initiating this process are either (i) trisomies of three or more of the odd-numbered chromosomes 3, 5, 7, 9, 11, 15, 19, 21, a constellation known as hyperdiploidy, or (ii) rearrangements of the immunoglobulin heavy chain (IGH) locus involving *NSD2* or members of the *CCND* and *MAF* gene families.[5] Secondary events driving disease progression are heterogeneous and include loss of 13q14, aberrations of chromosome 1, alterations of *MYC* and *TP53*, as well as mutations affecting the NF-κB and MAPK/ERK pathways.[6,7] Several alterations, among them gains of *CKS1B* (1q21.3–q22) and deletions of *CDKN2C* (1p32.3) and *TP53* (17p13.1), have been associated with an unfavorable prognosis and are enriched in patients with EMD.[3,8,9]

Importantly, EMD must be distinguished from solitary extramedullary (or extraosseous) plasmacytoma (EMP). EMP is a localized plasma cell neoplasm that most commonly arises in the upper aerodigestive tract and lacks overt bone marrow infiltration and other defining features of MM.[5,10,11] In contrast to EMD, EMP generally shows a favorable prognosis and can be effectively managed with local therapy.[11,12] Still, a subset of patients (10-30%) progresses to MM, and although some adverse features have been proposed, such as sinonasal localization and minimal bone marrow involvement detected by flow cytometry, predicting an unfavorable course continues to be a subject of research.[12-14] Previous studies have shown that EMP – while morphologically and immunophenotypically often indistinguishable from EMD – tends to display more mature cytology, lower proliferative activity, and less frequent aberrant expression of CD56 and cyclin D1.[15-18] Genetically, EMP appears to harbor hyperdiploidy and recurrent IGH translocations at a frequency comparable to that in MM, with the exception of *CCND1* rearrangements, which seem to be virtually absent.[15,17,18] The mutational profile and the prevalence of secondary alterations, including those deemed high-risk in MM do, however, remain largely unknown.[15]

Here, we conducted the first comprehensive molecular analysis of EMP in comparison with EMD, focusing on secondary alterations and employing fluorescence *in situ* hybridization (FISH), copy-number profiling, and targeted next-generation sequencing (NGS), complemented by immunohistochemical analyses, to elucidate the biological basis of the distinct behavior of EMP and possibly take a first step toward its molecular risk stratification.

Methods

Sample selection

Cases were identified by searching the archives of the Institute of Pathology of Tuebingen University Hospital, Germany, and the Institute of Medical Genetics and Pathology of Basel University Hospital, Switzerland, for formalin-fixed and paraffin-embedded (FFPE) tissue samples from patients diagnosed with EMP or EMD. Cases were reviewed by experienced hematopathologists (FF, Tuebingen; AT, Basel) and diagnosed in accordance with the International Consensus Classification of Myeloid and Lymphoid Neoplasms and the 5th edition of the World Health Organization Classification of Haematolymphoid Tumours.[5,10] In one EMD case, multiple samples were analyzed (*Online Supplementary Material*). The study was approved by the Ethics Committee of the University of Tuebingen (approval number 462/2022BO2).

Immunohistochemistry and fluorescence *in situ* hybridization

Samples were stained for MUM1, CD138, cyclin D1, immunoglobulin (IG) κ and λ light chains, MIB-1, p53, MYC, and Epstein-Barr virus (EBV)-encoded RNA (in EMP cases only), and analyzed by FISH for IGH (14q32.33) rearrangements, gains of *CKS1B* (1q21.3–q22), losses of *CDKN2C* (1p32.3) and *TP53* (17p13.1), as well as gains of 5p15.2–p15.3, 9q22.3–q31, and 15q22.3 as a surrogate for hyperdiploidy. For additional information, including further characterization of IGH translocations in EMP, see the *Online Supplementary Material*.

DNA extraction and copy-number analysis

DNA was extracted from paraffin sections using the Maxwell RSC Instrument (Promega, Mannheim, Germany). Samples with sufficient material (15 EMP and 10 EMD) were hybridized on the OncoScan FFPE Array platform (Thermo Fisher Scientific, Waltham, MA, USA). Copy-number alterations (CNA), i.e., gains and losses, as well as copy number neutral loss of heterozygosity (CNN-LOH) regions, were assessed and inspected using Nexus Copy Number software (version 9.0; BioDiscovery, Hawthorne, CA, USA). Further details, particularly regarding integration with FISH results, are provided in the *Online Supplementary Material*.

Targeted next-generation sequencing

Targeted NGS was performed on the Ion GeneStudio S5 Prime System (Thermo Fisher Scientific), employing two Ion AmpliSeq custom panels (Thermo Fisher Scientific) covering 89 genes frequently altered in MM and B-cell lymphoma (*Online Supplementary Tables S1* and *S2*). Library preparation, sequencing, and data analysis are described in the *Online Supplementary Material*.

Statistical analysis

Statistical analyses were performed using R (version 4.4.3; R Foundation for Statistical Computing, Vienna, Austria). Comparisons were conducted using non-parametric tests (Mann-Whitney U and Fisher exact tests), and correlations were assessed using Spearman correlation. All tests were two-sided, with $P<0.05$ considered statistically significant. Most genetic comparisons were exploratory and not adjusted for multiple testing, and *P* values should be interpreted accordingly. The comparison regarding enrichment of copy number regions was performed using the Fisher exact test, considering a minimum of five altered cases for adjusted *P* value calculations.

Results

Patients' characteristics and clinical features

Forty-eight cases were included, comprising 24 EMP and 24 EMD. Among the EMP cases, the median age at diagnosis was 63 years (range, 18-93), with a male predominance (male:female = 3:1), and the upper aerodigestive tract being the most common site of manifestation (Table 1; *Online Supplementary Figure S1*). None of the EMP patients showed evidence of MM based on clinical assessment, imaging, and

a trephine bone marrow biopsy (available in 21/24 cases). In addition, no clonal plasma cells were detected by flow cytometry in the bone marrow of any of the 13 patients with available data, and only 4/21 patients with available data showed a low-level paraprotein. Most patients were treated with surgery combined with radiotherapy or radiotherapy alone and achieved complete remission. Five patients experienced a locoregional relapse, with follow-up times ranging between 3 and 194 months. Only one patient (case 11) showed progression to MM and died from the disease. In the EMD group, the median age at disease onset was 66 years (range, 47-86) with a more balanced gender distribu-

Table 1. Clinical characteristics of the cohort with extramedullary plasmacytoma.

Case	Sex	Age, years	Site	BM	Treatment	Follow-up,⁺ months	PP	Additional information
1	M	18	Cervical LN	–	S + RT	29 – CR	–	History of classical Hodgkin lymphoma
2	F	70	Epipharynx	–*	RT	16 – CR	–	–
3	F	71	Epipharynx	–*	S + RT	24 – DOC	+	Relapse; primary EMP 50 months previously (larynx; S + RT); EMP in CR at death
4	F	46	Epipharynx	–*	S + RT	102 – CR	–	–
5	F	47	Epipharynx	–	S + RT	64 – CR	–	–
6	M	64	Paranasal sinuses	NA	NA	NA	NA	–
7	M	64	Paranasal sinuses	–*	RT	143 – CR	–	Relapse 68 months later (paranasal sinuses; VRD followed by RD; RD discontinued due to toxicity)
8	M	55	Cervical LN	–*	S + RT	120 – CR	–	–
9	F	55	Larynx	–	S + RT	176 – CR	–	–
10	M	73	Epipharynx	–*	RT	141 – CR	–	Second relapse; primary EMP 15 months previously (larynx; S + RT); first relapse 5 months previously (epipharynx; S)
11	M	84	Oropharynx + cervical LN	–*	S + RT	7 – DOD	–	Progression to MM 6 months later; no BM infiltration or PP at EMP diagnosis
12	M	50	Oropharynx	–	S + RT	194 – CR	–	–
13	F	59	Orbital soft tissue	–	RT	38 – AWR	–	Relapse 38 months later (lacrimal gland; therapy not yet initiated at last follow-up)
14	M	74	Nasal cavity	–	RT	5 – CR	+	–
15	M	69	Palatine tonsil + cervical LN	–	S + RT	8 – CR	–	History of primary myelofibrosis
16	M	93	Paranasal sinuses	NA	W & W	3 – AWD	NA	BM biopsy forgone (palliative setting); history of chronic lymphocytic leukemia
17	M	41	Nasal cavity	–*	S + RT	41 – CR	–	Relapse; primary EMP 48 months previously (oropharynx; S)
18	M	74	Palatine tonsil + cervical LN	–*	RT	135 – DOC	+	EMP in CR at death
19	M	81	Epipharynx	–*	RT	NA	–	–
20	M	67	Oropharynx	–*	S	96 – SD	–	–
21	M	62	Soft tissue of the thigh	–*	RT	31 – CR	+	–
22	M	45	Cervical LN	–	NA	7 – AWUS	NA	–
23	M	55	Lung + mediastinal LN	–*	CT + ASCT	29 – CR	–	Treated with VRD followed by HDM/ASCT
24	M	59	Epipharynx	NA	RT	12 – CR	–	BM biopsy forgone (no clinical evidence of MM)

A dash (–) indicates absence of the respective finding when used as a standalone entry. "BM" refers to the presence of bone marrow involvement as assessed by trephine biopsy. Cases marked with an asterisk (*) had available bone marrow flow cytometry data, none of which demonstrated clonal plasma cells. ⁺Follow-up was calculated from the manifestation/lesion included in the cohort. BM: bone marrow; PP: paraprotein; M: male; LN: lymph node(s); S: surgery; RT: radiation therapy; CR: complete remission; F: female; DOC: dead of other causes; EMP: extramedullary plasmacytoma; NA: information not available; VRD: bortezomib, lenalidomide, dexamethasone; RD: lenalidomide, dexamethasone; DOD: dead of disease; MM: multiple myeloma; AWR: alive with recurrence; W & W: watch and wait; AWD: alive with disease; SD: stable disease; AWUS: alive with unclear status; HDM/ASCT: high-dose melphalan followed by autologous stem cell transplantation; CT: chemotherapy.

tion (male:female = 1.18:1). EMD most commonly involved soft tissues at various sites, with 11/24 cases developing in patients with previously diagnosed MM, while in the remaining cases, EMD represented the first manifestation of systemic disease. Only 2/16 EMD patients with available data achieved complete remission (*Online Supplementary Table S3*; *Online Supplementary Figure S2*).

Morphology and immunophenotype

The EMP displayed typical morphology, characterized by monotonous sheets of plasma cells with variable degrees of atypia, but predominantly mature cytology (Figure 1). Immature cytology, defined by prominent nucleoli, an increased nucleus-to-cytoplasm ratio, open/blastoid nuclear chromatin and/or anaplasia, was observed in only 2/24 EMP compared to 6/24 EMD. Of note, one of the EMP with immature cytology (case 16) was the only EBV-positive sample in our cohort (Table 2). By immunohistochemistry, EMP demonstrated significantly lower proliferation rates (EMP *vs*. EMD: median 19.8% *vs*. 42.2%; *P*<0.001), lower MYC expression (*P*=0.001), as well as less frequent CD56 expression (8/24 *vs*. 16/24; *P*=0.043). Likewise, cyclin D1 positivity was rare in EMP compared to EMD (2/24 *vs*. 9/24; *P*=0.039). Notably, one EMP (case 23) showed strong and homogeneous expression of cyclin D1, typically only observed in MM, with a rearrangement of *CCND1*, which was confirmed by FISH. This was also the only EMP demonstrating aberrant, albeit focal, strong p53 staining (compared to 4/22 EMD showing aberrant staining) and the only EMP initially treated with chemotherapy due to an unusual presentation with involvement of the lung and mediastinal lymph nodes, despite never showing bone marrow infiltration. No clear trend toward higher proliferation or other features was observed in cases that experienced a relapse.

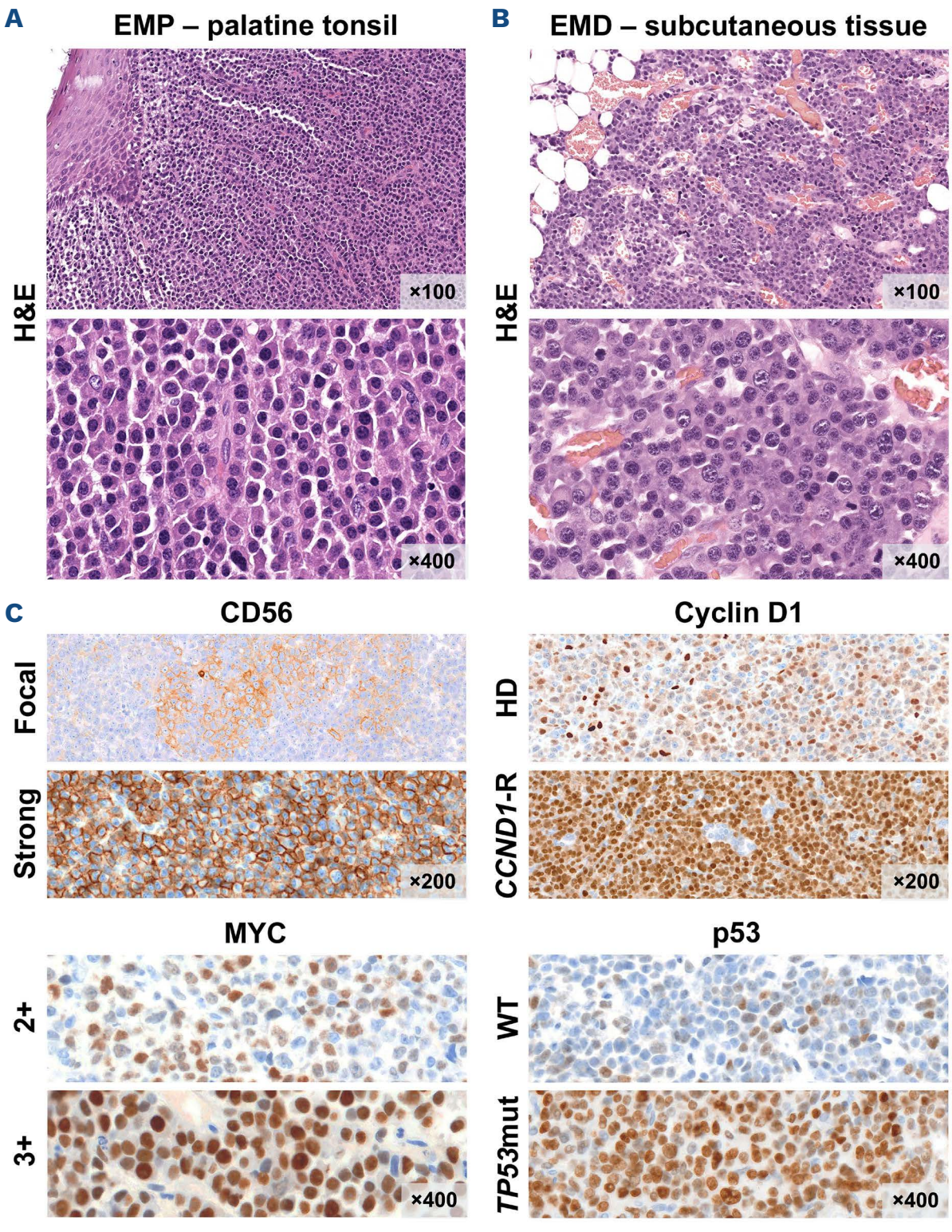


Figure 1. Morphology and immunophenotype of extramedullary plasmacytoma and extramedullary disease of multiple myeloma. (A) Hematoxylin and eosin (H&E) stain of an extramedullary plasmacytoma (EMP; case 15) infiltrating the palatine tonsil, with sheets of plasma cells beneath the squamous epithelium. The tumor cells demonstrate mature cytology with eccentric nuclei, ample cytoplasm, and inconspicuous nucleoli. Note the scattered binucleated forms (original magnifications ×100 and ×400). (B) H&E stain of extramedullary disease of multiple myeloma (EMD; case 33) involving the abdominal subcutaneous tissue. The tumor cells display immature cytology with an increased nucleus-to-cytoplasm ratio, prominent nucleoli, and frequent mitotic figures (×100 and ×400). (C) Immunohistochemical findings illustrating characteristic expression patterns. Upper left: focal aberrant CD56 expression in the EMD of case 28 (top) and strong aberrant CD56 expression in the EMD of case 43 (bottom; ×200). Upper right: heterogeneous aberrant cyclin D1 expression in the EMD of case 43 (top), associated with hyperdiploidy (HD), and strong, homogeneous cyclin D1 expression in the EMP of case 23 (bottom) due to an underlying *CCND1* rearrangement (*CCND1*-R) (×200). Bottom left: MYC expression in two EMD samples scored as 2 (top; case 43) and 3 (bottom; case 46) (×400). Bottom right: weak and heterogeneous p53 expression in the EMD of case 42 (top), corresponding to a *TP53* wild-type (WT) staining pattern, and strong p53 overexpression in the EMP of case 23 (bottom), consistent with a *TP53*-mutant (*TP53*mut) pattern (×400).

Fluorescence *in situ* hybridization and copy-number profile

An IGH break, indicative of a translocation involving the IGH locus, was detected by FISH in 6/24 EMP and 10/22 EMD. Apart from case 23, no EMP showed an IGH translocation involving any of the tested partner loci (*CCND1*, *NSD2*, *MAF*, *MAFB*). By combining FISH and copy-number analyses, hyperdiploidy was demonstrated in 10/24 EMP and 14/20 EMD. Regarding secondary alterations, losses of *CDKN2C* (1p32.3) were detected at similar frequencies in EMP (3/24) and EMD (4/22), although homozygous deletions were exclusively observed in EMD (2/22). In contrast, gains of *CKS1B* (1q21.3–q22) were less frequent in EMP than in EMD (9/24 *vs*. 15/22; *P*=0.045), with amplifications (>3 copies by FISH) detected in 4/24 EMP and 7/22 EMD, respectively. In 6/9 EMP, compared to only 4/15 EMD, the *CKS1B* gain occurred concomitantly with a gain of *CDKN2C*, most likely reflecting trisomy 1. Losses of *TP53* (17p13.1) were also less frequent in EMP (5/24) than in EMD (8/22). In two EMP and one EMD, del(17p) occurred in the context of a chromosome 17 gain, resulting in a relative 17p loss with retention of two *TP53* copies.

Based exclusively on OncoScan-derived copy-number data, available for 15 EMP and 10 EMD, EMP exhibited significantly lower genomic complexity, with a mean of 4.3 CNA (range, 0-14) and 0.5 CNN-LOH events per case, in contrast to 19.3 CNA (range, 7-43) and 3.0 CNN-LOH events per case in EMD (both *P*<0.001) (Figure 2). Notably, the highest genomic complexity among EMP samples, including a polyploid genome, was observed in case 11, which progressed to MM, whereas no clear trend toward increased CNA burden or specific alterations was seen in cases that experienced a

Table 2. Immunohistochemical findings.

Group	Case	CD56	Cyclin D1	MYC score[$]	p53*	Ki67, %
EMP	1	–	–	1	WT	57.69
	2	+	–	0	WT	5.39
	3	–	–	0	WT	6.71
	4	–	–	1	WT	15.83
	5	–	–	1	WT	22.21
	6	–	–	1	WT	5.29
	7	–	–	0	WT	2.88
	8	–	–	1	WT	40.44
	9	–	–	0	WT	4.17
	10	–	–	1	WT	1.64
	11	–	+ (h)	1	WT	27.42
	12	+	–	0	WT	3.66
	13	+	–	0	WT	25.08
	14	–	–	2	WT	72.06
	15	–	–	1	WT	20.53
	16	–	–	2	WT	63.82
	17	–	–	1	WT	28.38
	18	–	–	0	WT	0.07
	19	–	–	1	WT	19.9
	20	+	–	0	WT	21.69
	21	+	–	0	WT	19.67
	22	+	–	0	WT	4.78
	23	+	+	0	+ (f)	7.42
	24	+	–	1	WT	61.66
EMD	25	+ (f)	–	NA	NA	NA
	26	+	+ (h)	1	WT	41.05
	27	+	–	1	WT	56.9
	28	+ (f)	–	3	WT	76.83
	29	+	–	1	–	85.99
	30	–	+	0	WT	12.39
	31	–	–	3	+	39.97
	32	+	–	1	WT	42.22
	33	–	–	2	WT	71.65
	34	+	+	1	WT	35.69
	35	+	–	2	WT	36.66
	36	–	–	2	WT	77.46
	37	+	+ (h)	2	WT	32.99
	38	+	+ (h)	2	WT	23.55
	39	+	+ (h)	1	WT	7.88
	40	+	+ (h)	2	WT	28.13
	41	–	–	3	+	65.19
	42	–	–	1	WT	65.6
	43	+	+ (h)	2	WT	71.69
	44	–	–	0	WT	6.51
	45	–	+	0	WT	8.21
	46	+	–	3	WT	74.41
	47	+	–	NA	NA	50[#]
	48	+	–	1	–	61.4

[$]MYC was scored as 0 (no expression), 1 (<30% positive cells), 2 (30-70% positive cells), and 3 (>70% positive cells). *Concerning p53, "(+)" indicates aberrant overexpression, whereas "(–)" designates complete loss of expression. [#]Manual evaluation. EMP: extramedullary plasmacytoma; WT: wild-type expression pattern; (h): heterogeneous positivity; (f): focal positivity; EMD: extramedullary disease of multiple myeloma; NA: not available.

local EMP relapse (*Online Supplementary Tables S4* and *S5*). The most frequent CNA in EMP were gains of 9q21.11–q34.3 and losses of 8p23.3–p21.2 (each in 4/15 samples), followed by deletions of 1p33–p13.2 (harboring *CDKN2C*) and gains of 1q21.1–q44 (involving *CKS1B*; each in 3/15 samples). In comparison, loss of 13q14.11–q31.2 (including *RB1* and *DIS3*), as well as gains of 5p, 15q11.1–q21.3, 19p, 6p, and 3q23–q26.1, were significantly enriched in EMD.

A comparison of EMP with copy-number data from two previously published MM cohorts revealed significantly lower complexity in EMP (mean CNA 4.3 *vs*. 12.9; $P<0.001$) as well as a trend toward fewer CNN-LOH events (mean 0.5 *vs*. 1.3; $P=0.2$) (*Online Supplementary Figure S5*).[6,19] MM samples were comparatively enriched for gains at 1q21.1–q22, 7q31.1–q32.1, 11q, and entire chromosomes 3, 5, 9, 15, 19, and 21. Further details are available in the *Online Supplementary Material*.

Mutational analysis

Targeted NGS of EMP revealed a significant variability in the number of mutations per case (median 2.0 mutations; mean 2.41; range, 0-12) and a broad distribution of affected genes. *TRAF3* was the most commonly mutated gene in EMP, affected in 6/24 (25%) samples. Notably, in three samples (cases 3, 11, and 24), the variant allele frequency (VAF) exceeded 50%, with another sample (case 19) showing three distinct *TRAF3* mutations – findings indicative of a biallelic or hemizygous event (Figure 3). Accordingly, a heterozygous loss and LOH of the chromosomal 14q32.32 region, which encompasses *TRAF3*, were identified in the

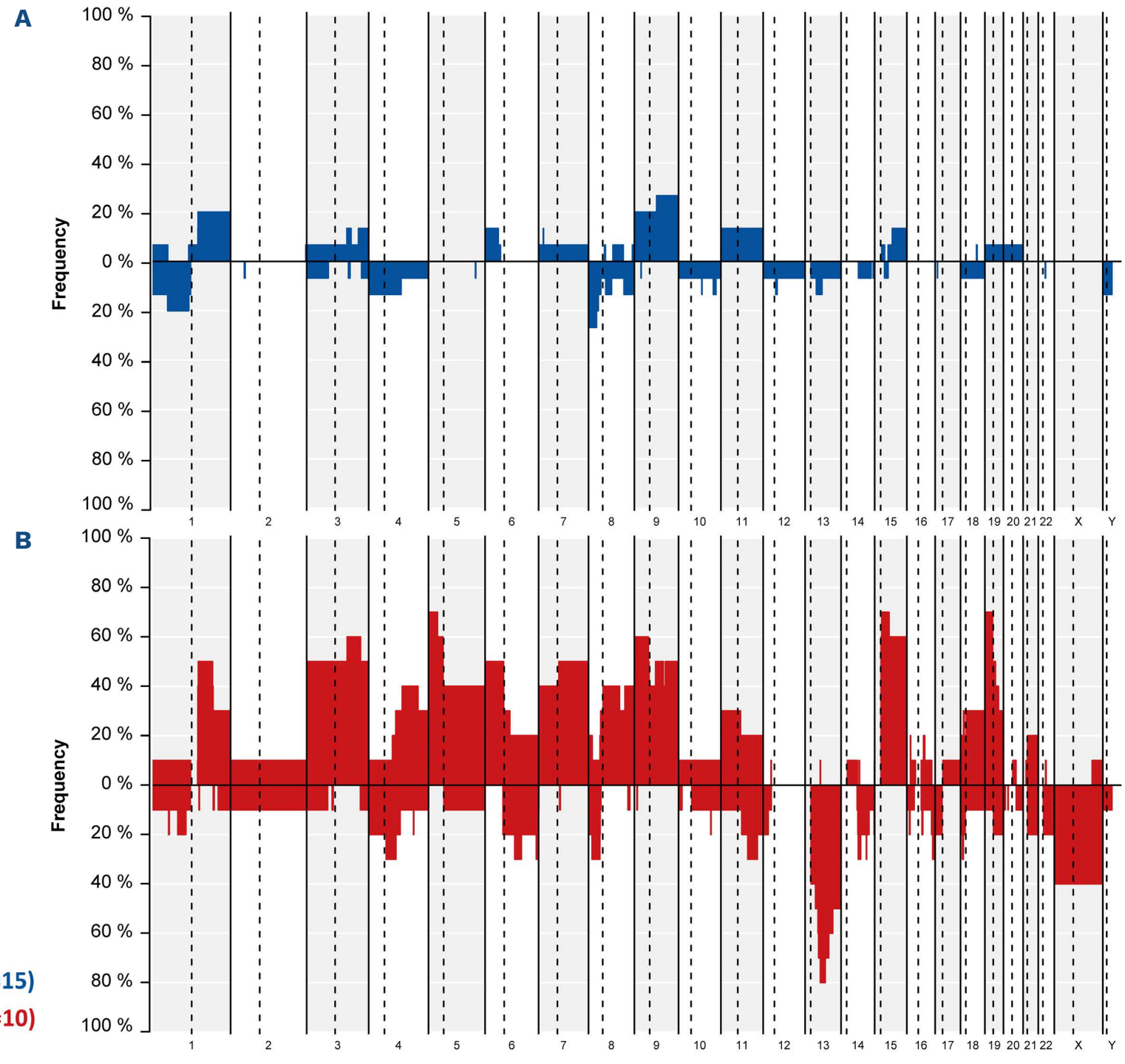


Figure 2. Frequency plots of copy number alterations in extramedullary plasmacytoma and extramedullary disease of multiple myeloma. The frequency of copy number alterations, i.e., gains and losses, is shown on the Y-axis, with the X-axis depicting chromosomes 1 through Y, from the p- to the q-arm. (A, B) Alterations in extramedullary plasmacytoma samples (EMP) are shown in blue in the top panel (A), whereas those in extramedullary disease (EMD) of multiple myeloma samples are shown in red in the bottom panel (B).

CNA analysis of cases 3 and 11, respectively (*Online Supplementary Table S4*). Other genes recurrently mutated in EMP included *BRAF* (4/24 EMP, all p.V600E), *HIST1H1E* (3/24), as well as *NRAS*, *KMT2D*, *PCLO*, *PLCG2*, *POT1*, *NFKBIA*, and *PIM1* (each in 2/24 samples). The number of mutations was positively correlated with the CNA burden (Spearman ρ=0.54, *P*=0.04). Accordingly, case 11, which progressed to MM, demonstrated above-average complexity (7 mutations). The number of mutations was also positively correlated with the proliferation rate in EMP (Spearman ρ=0.42, *P*=0.04). No consistent association between relapse and mutational complexity was observed.

EMD showed a higher number of mutations per case (median 3.0; mean 3.63; range, 1-8) compared to EMP (*P*=0.008). In EMD, the most frequently mutated gene was *NRAS* (11/24 samples), followed by *PCLO* (6/24), *KRAS* and *TP53* (each in 5/24), *KMT2D*, *ATM*, *ARID1A*, and *TENT5C* (each in 4/24), as well as *BRAF* and *ROBO2* (each in 3/24). Of note, there was a trend toward higher mutational complexity in EMD arising as a late event in patients with previously diagnosed MM (mean 4.18 mutations; range, 1-8), compared to EMD representing the primary manifestation (mean 3.15; range, 1-5), with the latter being on average still more complex than EMP.

In comparison with EMP, EMD samples were significantly enriched for *RAS* alterations (3/24 *vs*. 16/24; *P*<0.001), with *NRAS* mutations detected in 2/24 EMP and 11/24 EMD samples (*P*<0.01), and *KRAS* mutated in 1/24 EMP and 5/24 EMD. In contrast, *BRAF* mutations occurred at a similar frequency in both groups (4/24 EMP *vs*. 3/24 EMD). *TP53* was also more frequently mutated in EMD (5/24) than in EMP (1/24). Notably, in all *TP53*-mutated cases with available FISH data (i.e., all except case 25), a concurrent del(17p) was detected, consistent with biallelic inactivation. In line with this, all mutated EMD samples, including case 25, demonstrated a *TP53* VAF exceeding 50% and exhibited an aberrant p53 staining pattern. In contrast, the only *TP53*-mutated EMP (case 23) showed a VAF of 22%, suggesting a subclonal event, as supported by the only focal overexpression of p53. Additional genes more frequently altered in EMD included *PCLO* (2/24 EMP *vs*. 6/24 EMD), *TENT5C* and *ATM* (each 0/24 *vs*. 4/24), *ARID1A* (1/24 *vs*. 4/24), as well as *ROBO2* (0/24 *vs*. 3/24). In contrast, *TRAF3* mutations were not detected in EMD (6/24 EMP *vs*. 0/24 EMD; *P*=0.022), making it the only gene significantly enriched in EMP.

Discussion

This study provides the first comprehensive molecular characterization of EMP, integrating CNA, FISH, and mutational data, in addition to histopathological and immunophenotypic features. We demonstrate that EMP shares several genetic features with MM, but exhibits significantly lower genomic complexity and a partially distinct mutational profile compared to EMD, which usually occurs in advanced MM. These findings support the concept that most EMP represent early, localized plasma cell neoplasms, provide a genetic basis for their more indolent behavior, and may help to identify EMP cases with an increased risk of progression.

The clinical characteristics of our EMP cohort, including the predilection for the upper respiratory tract and the favorable response to localized therapy with few relapses and only one case progressing to MM, are consistent with the literature and stand in stark contrast to the aggressive behavior observed in EMD.[12,20] Likewise, the less common occurrence of immature morphology, the lower proliferation rate, the less frequent aberrant expression of CD56, and the infrequent overexpression of p53 and cyclin D1 in EMP are in agreement with published data.[16] In concordance with findings by Billecke *et al.*, MYC expression was also significantly less frequent in EMP.[21] Of note, MGUS is typically negative for MYC as well, and higher MYC expression has been associated with shorter survival in MM.[22] Intriguingly, the two EMP with MYC expression in >30% of cells (cases 14 and 16) were the only ones displaying immature morphology and demonstrated high proliferation, which might suggest more aggressive behavior. However, the relatively short follow-up period in both patients precludes a firm conclusion. In line with previous studies from our group and others, only 2/24 EMP, compared to 9/24 EMD, showed cyclin D1 expression. Surprisingly, one EMP (case 23) showed strong and homogeneous expression of cyclin D1 due to an underlying *CCND1* rearrangement – a finding that, to our knowledge, has only been reported once in an EMP patient who subsequently developed MM.[15,23] Our case displayed an unusual clinical course with involvement of the lung and mediastinal lymph nodes, ultimately requiring chemotherapy and autologous stem cell transplantation. Notably, however, the patient did not fulfill the criteria for MM at any point, despite thorough clinical evaluation. The second cyclin D1-positive EMP, which progressed to MM, showed only weak and heterogeneous staining, typically associated with additional copies of *CCND1* or hyperdiploidy, which was confirmed by FISH.[18,24,25] Importantly, this staining pattern has previously been associated with aggressive behavior and development of MM in EMP.[18] Therefore, in keeping with previous reports, our data support that expression of cyclin D1 in a putative EMP should prompt an intensive search for an underlying systemic plasma cell neoplasm and might indicate aggressive behavior.[16]

MM and its precursors can be divided into two genetic groups based on their primary genetic alterations: those with hyperdiploidy and those harboring recurrent IGH translocations.[5] The frequency of hyperdiploidy and IGH translocations in our EMP cohort was comparable to previously published data.[15] In comparison to EMD, there was a larger proportion of EMP samples that lacked both

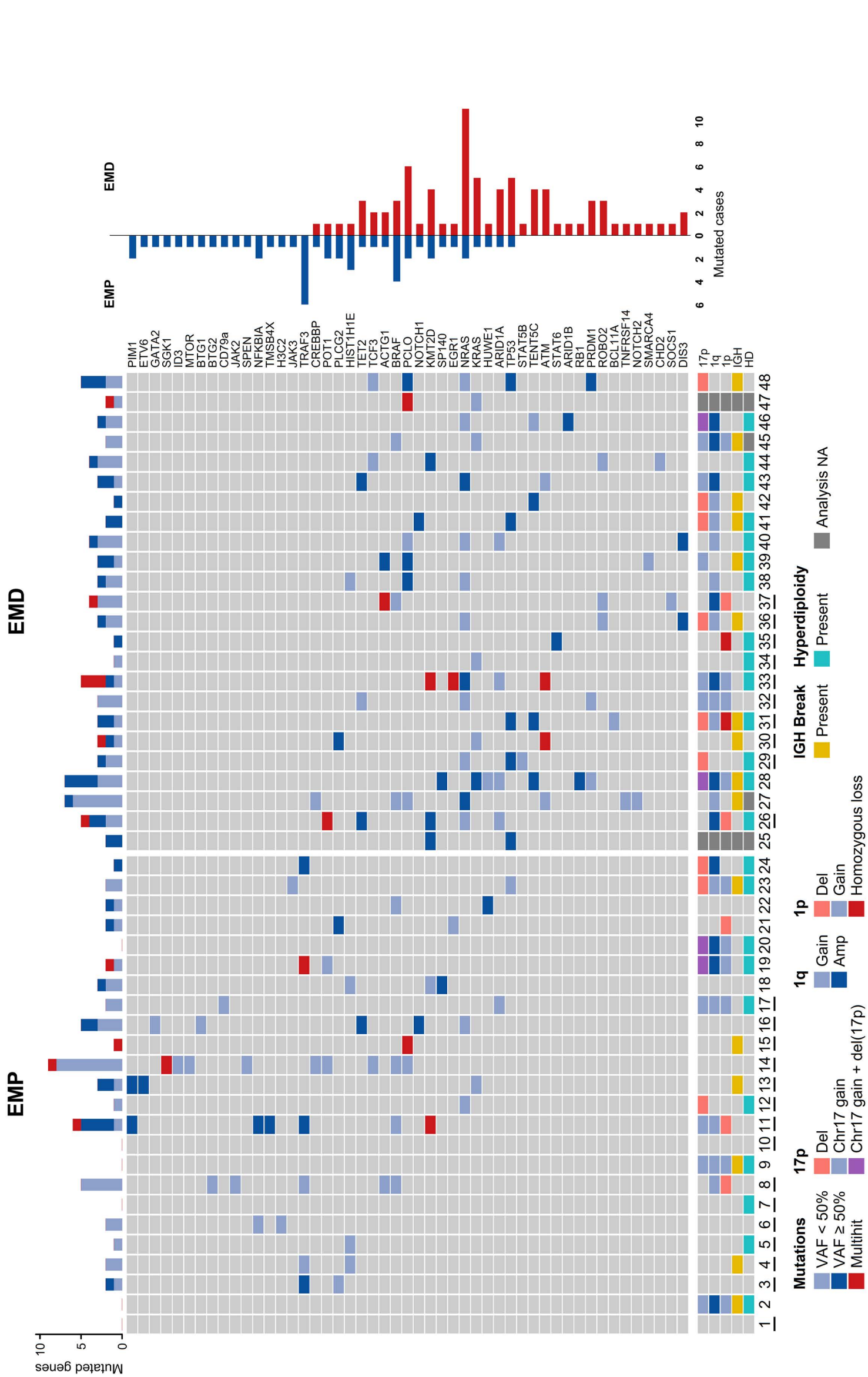


Figure 3. Genetic landscape of extramedullary plasmacytoma and extramedullary disease of multiple myeloma. Each column represents an individual case and each row corresponds to a specific gene or cytogenetic alteration. The top bar plot indicates the number of mutated genes in each case: those with a variant allele frequency (VAF) <50% in light blue, VAF ≥50% in dark blue, and those with more than one mutation in red. The right bar plot displays the number of cases of extramedullary plasmacytoma (blue) and extramedullary disease of multiple myeloma (red) cases harboring mutations in the respective gene. In underscored cases, chromosomal alterations reflect an integrated interpretation of fluorescence *in situ* hybridization and OncoScan analysis. "Chr17 gain + del(17p)" indicates a chromosomal gain accompanied by a relative *TP53* loss in cases 19, 20, and 46, whereas case 28 demonstrated a gain of chromosome 17 with an absolute *TP53* loss, i.e., with only one allele remaining. EMP: extramedullary plasmacytoma; EMD: extramedullary disease of multiple myeloma; del: deletion; amp: amplification; NA: not available.

alterations (46% *vs*. 9%), which is in line with the overall lower genomic complexity and resembles observations made in MGUS.[26] Of note, a subset of IGH translocations in EMD, particularly in cases also harboring hyperdiploidy, may represent secondary events, such as rearrangements involving *MYC*, which arose during disease progression.[3]

The CNA profile of EMP, although less complex overall, was reminiscent of MM: gains of 9q and losses of 8p, the most common CNA next to alterations of chromosome 1, are frequently found in MM and its precursors, with the former most often occurring in the context of trisomy 9.[6,27] Consistent with its more aggressive course, EMD not only carried a higher CNA burden but was also enriched for alterations that are significantly more frequent in MM than in MGUS, such as gains of 19p and 6p, suggesting that these events occur during disease progression.[6] Surprisingly, del(13q) was also enriched in EMD and detected in only 2/15 EMP (13%), which contrasts with previous studies reporting deletions of 13q in up to 40% of cases.[15,17] This discrepancy may, in part, be due to sensitivity differences between array-based methods and FISH.[28] Alternatively, the apparently higher frequency of 13q loss in FISH-based studies may be attributable to nuclear sectioning artifacts.[15]

Two secondary alterations, loss of *CDKN2C* and gain of *CKS1B*, were detected in EMP at frequencies similar to those observed in MM.[29,30] Both alterations have also been observed in MGUS, supporting their potential role as relatively early clonal events in plasma cell neoplasms.[26] Nevertheless, they have more commonly been associated with aggressive behavior and extramedullary spread in MM.[30] This was also reflected in our cohort, given the significant enrichment of 1q gains in the EMD samples, as well as the more frequent occurrence of 1q amplifications in the latter, which has been linked to a particularly poor prognosis.[30] Likewise, biallelic inactivation of *CDKN2C*, also shown to define a subset of ultra-high-risk myeloma, was only found in EMD.[31] Notably, six of nine EMP with a 1q gain, including three cases with amplification, showed concurrent gains of 1p, most likely as a result of gains of the entire chromosome. Although chromosome 1 gains are recognized as one mechanism of 1q gain, it remains unclear whether this pattern conveys the same poor prognosis.[32,33] Loss of 17p13.1 (*TP53*), an alteration detected in around 8% of MM and associated with an unfavorable clinical course, was also surprisingly common in EMP, although, again, less frequent than in EMD.[7] Importantly, in two out of five EMP with a del(17p), compared to only one out of eight EMD, the deletion occurred in the context of a chromosome 17 gain, which resulted in a relative 17p loss with two *TP53* copies remaining. Patients with myelomas harboring this constellation show survival comparable to those with a typical del(17p).[34] The clinical implications of these presumed high-risk alterations and higher genomic complexity in EMP remain to be elucidated, as no clear association with more aggressive behavior or relapse was observed in our cohort. Of note, however, the case that progressed to MM exhibited the highest CNA complexity among all EMP.

The mutational analysis of EMP, to our knowledge the first dataset published in adults, revealed several recurrently mutated genes, including *TRAF3*, *BRAF*, *NRAS*, *PCLO*, and *NFKBIA*, which have also been implicated in the pathogenesis of MM.[7,35] Intriguingly, despite the overall lower mutational burden, mutations of *TRAF3* were detected in 25% of EMP and were significantly enriched compared to our EMD cohort. Inactivation of the negative regulator *TRAF3* is a well-recognized mechanism of NF-κB activation in MM, resulting from mutations and/or deletions involving 14q32, which have been reported in approximately 10-15% of cases.[7,36-38] In four of six *TRAF3*-mutated EMP, biallelic inactivation was suspected and confirmed in two cases by the detection of a concurrent deletion and LOH of the 14q32 region, respectively. Biallelic *TRAF3* inactivation has been described in MM, although more commonly in the relapse setting, where it does not appear to confer a poor prognosis.[39] In fact, *TRAF3* mutations have been linked to a favorable outcome in MM, which is consistent with their absence in our EMD cohort and the rarity of such mutations in prior reports addressing EMD.[3,9,30] Notably, *NFKBIA*, which is also involved in the NF-κB pathway, was mutated exclusively in EMP as well.[36]

In turn, EMD was significantly enriched for RAS mutations (*NRAS* and *KRAS*), which are part of the MAPK/ERK pathway and among the most commonly mutated genes in MM.[7] In line with our findings, these mutations have been reported to be overrepresented in patients with EMD and associated with poor prognosis in MM, although they have also occasionally been detected at the MGUS stage.[9,26,40,41] Interestingly, *BRAF* mutations, despite their similar association with unfavorable outcome and involvement in the MAPK pathway, were detected at comparable frequencies in both groups, with all mutations in EMP being the canonical p.V600E.[41-43] Several additional genes more commonly mutated in the EMD group have been associated with EMD and/or poor survival in MM, including *ARID1A*, *ATM*, *TENT5C*, *DIS3*, *ROBO2*, and, in particular, *TP53*.[9,30,42,44] Of note, all *TP53*-mutated EMD demonstrated a concurrent del(17p), consistent with biallelic inactivation, which is a hallmark of ultra-high-risk MM with a dire prognosis.[30] Surprisingly, this molecular constellation was also observed in one EMP (case 23), which – although the *TP53* mutation was only subclonal – likely contributed to its unusual clinical course. Nevertheless, future studies on EMP are needed to clarify the prognostic impact of individual mutations and their overall number, the latter being associated with increased proliferative activity and CNA burden but not generally higher in relapsing cases. Interestingly, in contrast to our study, a recent investigation of a small pediatric EMP cohort did not identify MM-type alterations, pointing to potential biological differences in this rare EMP subtype.[45] Of note,

case 1, the only sample lacking detectable aberrations in our series, arose in an 18-year-old male.

Importantly, EMP occasionally needs to be separated from entities other than EMD. Case 16, characterized by EBV positivity, immature cytology, MYC expression, and high proliferation, needed to be distinguished from plasmablastic lymphoma. However, as EBV-positive EMP is well described and frequently exhibits features similar to our case, and given that none of the criteria for plasmablastic lymphoma recently proposed by Zhou *et al*. were met, a diagnosis of EMP was rendered.[46,47] Intriguingly, case 14, notably the second EMP with immature cytology and high MYC expression, harbored an unusual and complex mutational profile, including mutations of *TCF3* and *ID3*, which are commonly detected in Burkitt lymphoma.[48] Despite this, the case demonstrated clear-cut morphological and immunophenotypic features of EMP and lacked a *MYC* translocation.

Established prognostic parameters are sparse in EMP and so far, no genetic risk factors have been defined. However, it is tempting to speculate that, in line with recent data for solitary plasmacytoma of bone, high-risk genetics might indicate an increased risk of and shorter time to progression in EMP as well.[49] Of note, both cases (11 and 23) that showed progression or more extensive disease had genetic features associated with higher risk in MM. Ultimately, the genetic profile might enable a separation into progressive and stable forms of local plasma cell neoplasms.

Given the modest cohort size and the reliance on archival FFPE tissue, which likely reduced sensitivity for detecting subclonal alterations in a subset of samples, our findings warrant confirmation in larger cohorts. These future studies should also more extensively characterize IGH translocations in EMP, as the translocation partner was only identified in a single case. Even so, this study provides the first comprehensive analysis of genetic alterations in EMP and offers novel insights into its biology, further highlighting the differences from EMD.

Disclosures

No conflicts of interest to disclose.

Contributions

FF and AV conceived and designed the study, selected and reviewed the cases, supervised the experimental work and data analysis, and wrote the manuscript. A-LW was involved in case selection and performed the next-generation sequencing experiments. FO, AS, and IB supervised the experimental work and analyzed next-generation sequencing data. JS-V and IS performed the copy-number analysis and helped to write the manuscript. AC performed the ASCAT analyses and contributed to data interpretation. BM performed FISH analysis. EB provided bioinformatic support and performed statistical analyses. AT, MSD, and MN contributed cases and provided clinical information. All authors revised the manuscript.

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Data-sharing statement

Sequencing data are deposited in the European Nucleotide Archive (PRJEB102117). Copy number data are deposited in ArrayExpress (E-MTAB-16327).

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