IGHV segment utilization in immunoglobulin gene rearrangement differentiates patients with anti-myelin-associated glycoprotein neuropathy from others immunoglobulin M-gammopathies

Demyelinating neuropathy associated with antibodies against myelin-associated glycoprotein (anti-MAG) is a rare acquired immune-mediated neuropathy.[1,2] It predominantly consists of sensory deficiency in the lower limbs. Frequently, an invalidating tremor, sensory ataxia, and painful paresthesia are present. Anti-MAG neuropathy is associated with IgM monoclonal protein produced by an underlying B-cell lymphoproliferative disorder. This latter is most often an immunoglobulin M-monoclonal gammopathy of undetermined significance (IgM-MGUS) or a Waldenström's macroglobulinemia (WM). The management of anti-MAG neuropathy remains a challenge, and no predictive factor has been described to identify patients with IgM monoclonal gammopathy at risk to develop anti-MAG neuropathy. The description of the mutational profile of the *MYD88, CXCR4,* and *TP53* genes have radically changed the diagnosis and prognostic evaluation of IgM monoclonal gammopathies.[3-5] Acting as a trigger for nuclear-factor κB (NFκB) signaling, $MYD88^{L265P}$ is the most prevalent mutation in WM and IgM-MGUS.[6,7] Somatic mutations in the C-terminal domain of CXCR4 are frequently described in WM and associated with a more aggressive disease.[4] Moreover, the *MYD88/CXCR4* status is predictive of the response to B-cell receptor (BCR)-inhibitor treatment in WM.[8] The frequency of these mutations is currently unknown in anti-MAG neuropathy and could be relevant for the management of patients. Moreover, analysis of the immunoglobulin heavy chain variable (*IGHV*) sequence of clonal tumor B cells provides information on cell origin and antigen dependence.[9] The *IGHV* gene encodes the complementary-determining region 3 (CDR3) that most closely interacts with the antigen. Thus, analysis of the *IGHV* sequence from clonal B cells in patients with monoclonal gammopathy could identify a subset of patients with a biased *IGHV* segment utilization, prone to developing a demyelinating neuropathy. The aim of the study herein was to analyze the mutational profile of the *MYD88, CXCR4*, and *TP53* genes along with the *IGHV* sequence in anti-MAG neuropathy *via* high-throughput sequencing (HTS).

Table 1. Baseline characteristics of anti-MAG, WM and IgM-MGUS.

Characteristics	Anti-MAG group (n=26)	WM control group (n=24)	IgM-MGUS control group (n=22)
Male sex - n (%)	18 (69.2%)	14 (58.3%)	14 (63.6%)
Median age (IQR) – year	61 (58-71)	75 (60 - 78)	68 (63-79)
Median anti-MAG titers (IQR) – BTU	43666 (21206->70 000)	–	–
Symptoms - n (%)			
Paresthesia / Dysesthesia	21 (80.8%)	–	–
Sensory deficit	20 (76.9%)	–	–
Motor deficit	6 (23.1%)	–	–
Ataxia	11 (42.3%)	–	–
Pain	7 (26.9%)	–	–
Median monoclonal protein (IQR) - g/L	6.4 (2.9-18.7)	12.3 (2.8-25.8)	2.6 (1.0-7.9)
Light chain isotype[†] - n (%)			
κ	21 (80.8 %)	20 (83.3%)	18 (81.8%)
λ	6 (23.1%)	4 (17.4%)	4 (18.2%)
Median hemoglobin level (IQR) - g/dL	14.0 (13.1-14.6)	11.5 (9.5-13.3)	13.4 (11.0-14.5)
Median platelet count (IQR) – 10^9/L	232 (209-283)	188 (116-272)	239 (209-292)
Median neutrophil count (IQR) - 10^9/L	3.5 (2.6-4.5)	3.9 (2.6-5.0)	4.0 (3.1-4.9)
Median lymphocyte count (IQR) - 10^9/L	1.7 (1.5-2.2)	1.6 (1.2-2.9)	1.6 (1.0-2.0)
Median bone marrow infiltrate (IQR) -%	1 (<1-12)	37.5 (13.8-80)	1 (<1-2)
Somatic mutations – n (%)			
$MYD88^{L265P}$	19 (73.1%)	23 (95.8%)	13 (59%)
CXCR4	3 (11.5%)	12 (50.0%)	3 (13.6%)
TP53	1 (3.8%)	2 (8.3%)	0 (0.0%)
Detection of clonality with HTS			
Positive patients– n (%)	17 (65.4%)	19 (79.2%)	11 (50%)
IGHV segment utilization			
VH4-34 – n (% of patients)	6 (23.1%)	1 (4.2%)	0 (0.0%)
VH4-34 – n (% of clonotypes)	7 (24.1%)	1 (2.3%)	0 (0.0%)

[†]One patient in the anti-MAG group has biclonal IgM κ and λ gammopathy. [‡]Bone marrow infiltrate was quantified by flow cytometry. MAG: myelin-associated glycoprotein; WM: Waldenström macroglobulinemia; IgM-MGUS: immunoglobulin M monoclonal gammopathy of undetermined significance; IQR: interquartile range; BTU: Buhlmann titer units; HTS: high-throughput sequencing.

In the study herein, approved by our institutional ethics committee, we analyzed and compared the genomic profile of 26 anti-MAG neuropathy patients with 46 cases of IgM monoclonal gammopathies without neurologic symptoms or the detection of anti-MAG antibodies (24 WM and 22 IgM-MGUS). All patients underwent bone marrow (BM) investigations in our lab. Genomic DNA was extracted from BM mononuclear cells using a Qiagen DNA extraction kit (Qiagen, Valencia, CA, USA). Target enrichment was performed using the Access Array System (Fluidigm, San Francisco, CA, USA) from 50 ng of DNA. Subsequently, purified libraries were sequenced with MiSeq (Illumina, San Diego, CA, USA). Somatic mutations were defined as frameshift, stop-gain or missense variants not reported as a polymorphism, with a variant allele frequency (VAF)

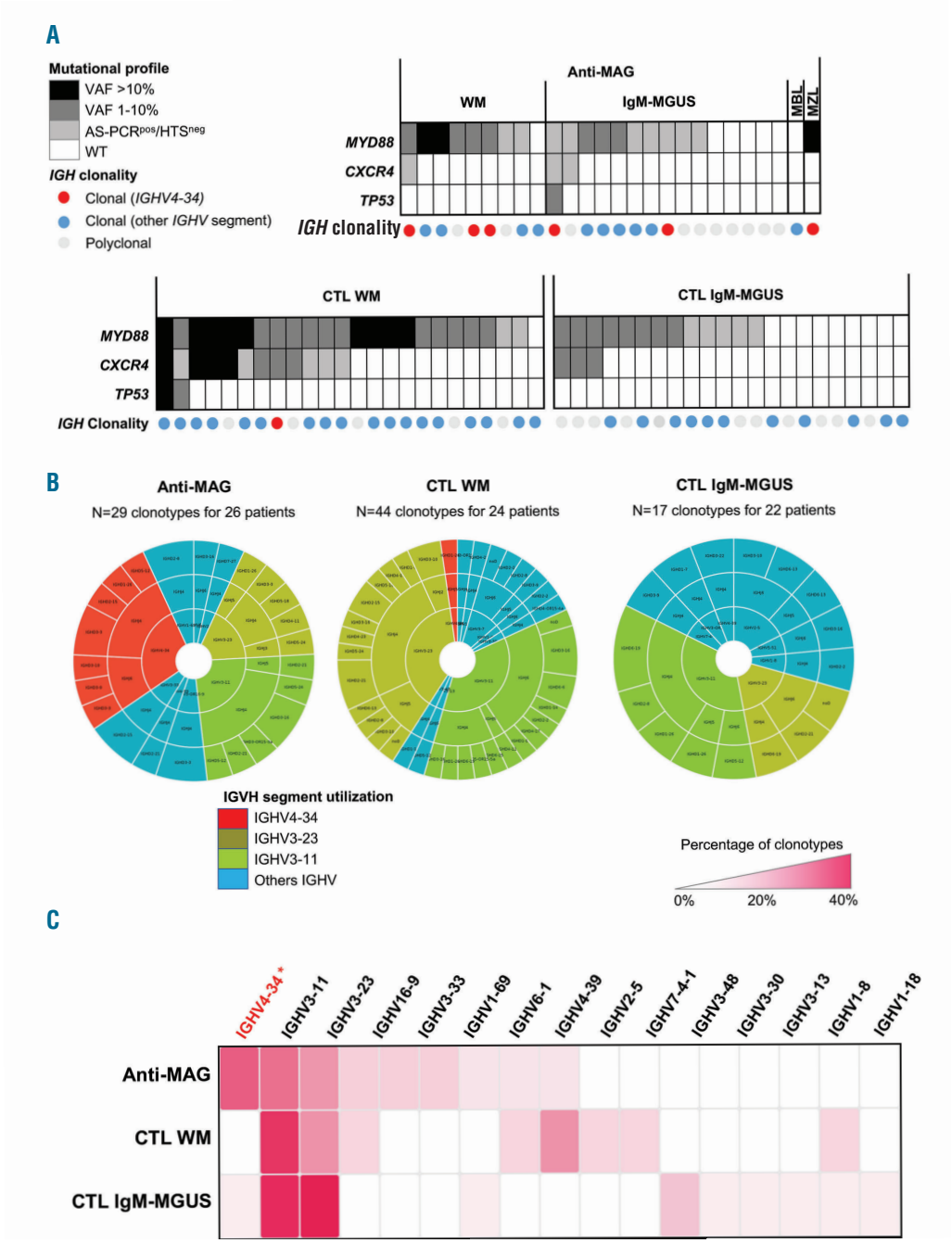

Figure 1. Genomic profile of anti-MAG neuropathy. (A) Comparison of *MYD88, CXCR4*, and *TP53* gene mutational status in the anti-MAG, WM and IgM-MGUS control groups. All patients were tested with both HTS and AS-PCR for $MYD88^{L265P}$ and $CXCR4^{S338X}$. Below, results of clonality assessment with *IGH* gene rearrangement sequencing revealed an over-representation of *VH4-34* segments (red circles) in the anti-MAG group (P=0.008). (B) Sunburst representation of the utilization of VDJ segments in the dominant clonotypes identified in the anti-MAG, WM and IgM-MGUS groups (C) Heatmap showing the *IGHV* segment utilization frequency (% of dominant clonotypes) in anti-MAG samples, as compared to the WM and IgM-MGUS control groups. *indicates a significant *VH4-34* over-representation in anti-MAG clonotypes *vs.* WM (P=0.002) and IgM-MGUS clonotypes (P=0.019). CTL: control; MAG: myelin associated glycoprotein; WT: wild-type; WM: Waldenström macroglobulinemia; IgM-MGUS: monoclonal gammopathy of undetermined significance; VAF: variant allele frequency; AS-PCR: allele specific-polymerase chain reaction; HTS: high-throughput sequencing.

higher than 2%. In parallel, *MYD88*^{L265P} and *CXCR4*^{S338X} mutations were screened by allele specific – polymerase chain reaction (AS-PCR), with a sensitivity of 0.1%.[10,11] The *IGH* gene was sequenced by HTS using a two-step PCR protocol adapted from Biomed-2 recommendations from 100 ng of genomic DNA.[12] Libraries were sequenced on an Illumina MiSeq platform and analysis were performed using Vidjil software.[13] A clonotype was defined as a dominant sequence showing a frequency of at least 1% of total reads, well separated from the polyclonal background reads. Patients' characteristics are reported in Table 1.

In the anti-MAG group, all patients (N=26) presented an IgM monoclonal gammopathy, high anti-MAG antibodies titers, and clinical and electrophysiological evidence of demyelinating neuropathy.[2] Anti-MAG titers ranged from 3,620 to >70,000 Buhlmann titer units (BTU). For 24 out of 26 patients (92.3%), anti-MAG was >7,000 BTU, including nine strongly positive (>70,000 BTU) patients (34.6%). The underlying lymphoproliferative disorders were 15 IgM-MGUS, nine WM, one splenic marginal zone lymphoma (SMZL), and one monoclonal B-cell lymphocytosis (MBL) with a Matutes score of 5. *MYD88*^{L265P} was detected in 19 subjects (73.1%) (Figure 1A). Among them, nine were identified by AS-PCR only and ten by both HTS and AS-PCR. *MYD88*^{L265P} was found in ten IgM-MGUS (66.7%), in eight WM (88.8%) and in the SMZL. Three patients (11.5%) were mutated for *CXCR4* in the anti-MAG group and one harbored a *TP53* mutation (3.8%) (Figure 1A and *Online Supplementary Table S1*).

In the control WM group, we detected *MYD88*^{L265P} mutations for 23 patients (95.8%). Truncating mutations of *CXCR4* genes were detected for 12 patients (50.0%): seven patients with HTS and five AS-PCR positive cases. The *TP53* gene was mutated in two WM patients (8.3%). In the control IgM-MGUS group, we evidenced a *MYD88*^{L265P} mutation for 13 patients (59.1%), including five detected by AS-PCR only. Three patients harbored a CXCR4 variant (13.6%), while no TP53 mutation was detected in this group (Figure 1A and *Online Supplementary Table S1*).

Our study demonstrates that the *MYD88*^{L265P} mutation is highly prevalent in a cohort of anti-MAG patients. The MYD88 mutational rate in anti-MAG neuropathy is closely related to the underlying B-cell disorder. Indeed, the prevalence observed is comparable to our internal control groups and previous larger cohorts of WM or IgM-MGUS without anti-MAG.[3,6,7] With regard to HTS, no CXCR4 mutation was detected for anti-MAG patients, while 13.6% of IgM-MGUS and 29.1% of WM were mutated. *CXCR4*^{S338X} represents 70% of CXCR4 mutations detected by HTS, and a third of variants consist of frameshift mutations. Combining HTS with AS-PCR for the most common variant *CXCR4*^{S338X}, we showed that 10% of anti-MAG patients exhibited a mutation in *CXCR4*, independently of the associated B-cell malignancy, while up to 50% of patients were mutated in our WM group. This difference is partly explained by a relatively lower BM infiltrate for anti-MAG patients with WM compared with our control WM group (means of 22.6 *vs.* 44.6%, respectively, not significant). Consequently, the *CXCR4* mutational rate in the anti-MAG group might be slightly underestimated by the lack of detection of frameshift variants by AS-PCR for lower infiltrated samples.

MYD88 mutations are early events in the pathogenesis of monoclonal gammopathy, promoting the emergence of a B-cell clone responsible for IgM paraprotein production.[3,11] *CXCR4* mutations have been shown to occur later in the lymphomagenesis, explaining the progression from IgM-MGUS to WM.[11] Herein, the similar prevalence of *MYD88* mutations between anti-MAG and control and the low prevalence of *CXCR4* mutations in the anti-MAG group suggest that these mutations do not influence the occurrence of anti-MAG neuropathy during this oncogenic process. However, since the *MYD88*^{L265P}/*CXCR4*^{WT} genomic profile has been shown to predict a better efficiency of ibrutinib in WM, the high frequency of this genomic profile in our anti-MAG cohort suggests the potential interest of BCR inhibitors for the treatment of anti-MAG patients.[8]

In the second part of this study, we compared the *IGH* locus sequences from anti-MAG patients with our control WM and IgM-MGUS patients *via* HTS (Figure 1B,C, and *Online Supplementary Table S1*). In the anti-MAG group, 29 clonotypes were identified for 16 patients: seven patients with WM (77.7%), seven with IgM-MGUS (46.7%), and the 2 remaining patients with SMZL and MBL (Figure 1A). In comparison, we detected 44 different clonotypes from 19 patients in the WM control group (79.2%), and 17 clonotypes from 11 patients in the IgM-MGUS control group (50.0%). The frequency of the detection of clonotypes suggests that our anti-MAG group is comparable with our control groups. As recently reported for WM, we evidenced a high frequency of *VH3* segment usage, particularly *VH3-23* and *VH3-11* in our three groups, suggesting a pathogenic link between anti-MAG, IgM-MGUS, and WM (Figure 1B).[9] Interestingly, the *VH4-34* segment was more frequently used in dominant clonotypes from anti-MAG patients independently of the underlying B-cell proliferation. Indeed, six anti-MAG patients (three MW, two IgM-MGUS, and one SMZL) (23.1%) exhibited at least one *VH4-34* clonotype, whereas only one patient among the 46 control IgM gammopathies presented one *VH4-34* clonotype (2.2%) (P=0.008) (Figure 1B). The *VH4-34* segment was used significantly more in anti-MAG clonotypes (24.1%) compared with the control WM group (2.3%) (P=0.002) and with the control IgM-MGUS group clonotypes (0.0%) (P=0.019) (Figure 1C). *VH4-34* is a human heavy chain family that has germline-encoded polyreactivity towards multiple self-antigens.[14] The *VH4-34* segment has been identified as a diagnostic marker of variant hairy cell leukemia. Its over-representation has been found in other demyelinating processes, such as multiple sclerosis and other autoimmune diseases.[14,15] Therefore, our results suggest the potential interest of detecting *VH4-34* segment utilization in the *IGH* loci of patients with IgM monoclonal gammopathy, hence allowing for an earlier identification of patients with a higher risk of developing an anti-MAG neuropathy. For these patients, treatment might be initiated before the installation of irreversible demyelinating lesions. However, a prospective study evaluating the occurrence of anti-MAG neuropathy in a large cohort of IgM gammopathies according to their *IGH* sequence might be useful in order to exclude a potential bias based on the small size of our sample.

In conclusion, the results herein demonstrate that *MYD88*^{L265P}/*CXCR4*^{WT} is the most frequent genomic profile in anti-MAG patients, supporting the initiation of clinical trials with BCR inhibitors in anti-MAG patients. *VH4-34* segment utilization in immunoglobulin gene recombination could predict a higher risk of developing an anti-MAG neuropathy in patients with an IgM monoclonal gammopathy. Earlier identification of high-risk patients and genomic adapted therapy could improve the

management of anti-MAG neuropathy, however, further studies are needed in order to address the use of these markers in daily practice.

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