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RESEARCH ARTICLE

Clonal immunoglobulin λ light-chain gene rearrangements detected by next generation sequencing in POEMS syndrome

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Polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes (POEMS) syndrome is a rare plasma cell dyscrasia characterized by polyneuropathy, organomegaly, endocrinopathy, extravascular fluid overload, M protein, and a myriad of skin changes. The pathogenesis is poorly understood, but monoclonal plasma cells are λ-restricted and these immunoglobulin λ light chain variable (IGLV) region genes are derived from only two germlines, either *IGLV1-44* or *1-40*. Here we analyzed the clonal *IGLV* gene rearrangements of genomic DNA samples of bone marrow mononuclear cells using next-generation sequencing (NGS) to understand the clonal composition of *IGLV* genes in patients with POEMS syndrome (n = 30). The dominant *IGLV* gene rearrangement of POEMS syndrome-specific germline sequences were significantly increased in 11 POEMS patients (36.7%; *IGLV1-44*: n = 9, *IGLV1-40*: n = 2). In some cases, *IGLV* gene rearrangement clone was not detected as significant increase but was detected using cDNA samples by heteroduplex (HD) analysis and Sanger sequencing, suggesting that the quite small number of monoclonal plasma cells may produce large quantity of mRNA of monoclonal proteins. However, significant increase of dominant clone sizes was not directly linked to the initial disease status. On the other hand, in cases with significantly increased dominant clones, they decreased and increased accompanying with disease remission and relapse. These data demonstrate that monoclonal plasma cells are related to the pathogenesis of POEMS syndrome.

1 | INTRODUCTION

Polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes (POEMS) syndrome is a rare paraneoplastic disorder that occurs secondary to a plasma cell dyscrasia. It is characterized by polyneuropathy, organomegaly, endocrinopathy, λ-type M protein, and a myriad of skin changes, such as hypertrichosis, skin thickening, cherry angiomata, and hyperpigmentation.[1] Other important features of the disorder include sclerotic bone lesions, extravascular fluid overload causing pleural effusions and/or ascites, papilledema, thrombocytosis, plasmacytomas, clubbing, pulmonary hypertension, multicentric Castleman disease, elevated levels of vascular endothelial growth factor (VEGF), and fatigue.[2]

Until recently, treatment for POEMS syndrome was largely unsuccessful, and the prognosis was extremely poor.[3] Treatments for POEMS syndrome have focused on the underlying plasma cell disorder, and have had some success. Autologous peripheral blood stem cell transplantation (ASCT) after high-dose melphalan-based chemotherapy and novel agents used to treat multiple myeloma such as proteasome inhibitors and immunomodulatory drugs (IMiDs), have significantly improved the prognosis for patients with POEMS syndrome.[2,4–11]

Nevertheless, the pathogenesis of POEMS syndrome and the role of monoclonal plasma cells in the disease remain unclear. Plasma cells represent only 5% of the total nuclear cells in the bone marrow of patients with POEMS syndrome.[12] This tumor burden is lower to that in patients with multiple myeloma (MM). VEGF strongly promotes neovascularization and vasopermeability,[13] and VEGF overproduction triggers symptoms of POEMS syndrome, such as angiomata, pleural effusion/ascites, edema, polyneuropathy, and organomegaly.[14–18] It is presumed that VEGF is produced from or released by monoclonal plasma cells or platelets in POEMS syndrome, but this has not been confirmed. In addition, M protein is essentially a λ light chain and the immunoglobulin λ light chain variable region (*IGLV*) genes are highly restricted to two germline sequences, *IGLV1-40* and *IGLV1-44* in patients with POEMS syndrome beyond races.[19–21] However, those findings were identified using classical polymerase chain reaction (PCR)-based methods.

Next-generation sequencing (NGS) has been applied to many lymphoid malignancies, including MM, to monitor disease activity and assess the minimal residual disease (MRD).[22] NGS-based multiplex sequencing of the immunoglobulin heavy chain (IgH) in plasma cells from MM patients may have the potential to achieve a higher level of sensitivity (up to 1×10^6) with an improved quantifiable range NGS enables the analysis of genetic diversity and clonogenic heterogeneity, which can contribute to our current understanding of disease biology and relapse kinetics in patients with hematological malignancies.[23–25]

Here, we analyzed clonal *IGLV* gene rearrangements using an NGS-based approach to understand *IGLV* clonal composition in patients with POEMS syndrome. The goal of our study was to clarify the association between monoclonal plasma cells and POEMS syndrome pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Patients and preparation of BM samples

Thirty patients whose condition fulfilled the criteria[2] and were diagnosed with POEMS syndrome between November 2006 and July 2016 at Chiba University Hospital were included in the study. Six patients with MM with λ-type monoclonal light chains were also analyzed. Nine negative control patients had either immune thrombocytopenia (ITP) or malignant lymphoma without bone marrow invasion. In total, 77 samples were analyzed by NGS including 30 samples at the diagnosis, 32 samples after induction therapy and post ASCT, and 15 control samples: 6 MM samples and 9 negative control samples.

Bone marrow mononuclear cells (BMMNCs) were isolated from the bone marrow of each patient using a Ficoll Paque Plus kit (GE Healthcare, Boston, Massachusetts) and preserved at −80°C. Genomic DNA was extracted from the preserved BMMNCs of patients with POEMS syndrome using a Wizard genomic DNA purification kit (Promega, Madison, WI USA) or Qiagen All Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Detailed clinical data were collected retrospectively from each patient.

All subjects provided written informed consent to participate in the study, in accordance with the Declaration of Helsinki. This study was approved by the ethics committee of Chiba University Graduate School of Medicine (accession #639 [492]).

2.2 | Next-generation sequencing library construction and IGLV gene sequencing

We amplified *IGLV1*, *IGLV2*, and *IGLV3* from the *IGLV* gene family. Several studies have shown that *IGL* gene representation is biased in normal and malignant B cells; over 90% of the *IGLV* genes expressed by normal B cells are *IGLV1*, *IGLV2*, or *IGLV3*, which comprise 60% of the functional genes expressed by these cells.[26–29]

The *IGLV* genes were amplified by polymerase chain reaction (PCR) using a 5′ primer for the *IGLV1/2* framework 3 (FR3) region (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGATTCTCTGGCT CCAAGTCTGGC-3′), *IGLV3* FR3 region (5′-TCGTCGGCAGCGT CAGA TGTGTATAAGAGACAGGGATCCCTGAGCGATTCTCTGG-3′) and 3′ consensus primers for the *IGLJ1/2/3* joining regions (5′-GTCTC GTGGGCTCGGAGATGTGTATAAGA GACAGCTAGGACGGTGAGC TTGGTCCC-3′).[30] These primers are the combinations of primers targeting the immunoglobulin λ light chain locus and adopter sequences.

First stage PCR was carried out using FastStart Taq DNA polymerase, (Roche Diagnostics, Mannheim, Germany) for 29 cycles. The amplified product was then used as the template for the second PCR reaction, which was carried out for 10 cycles. Sample-specific barcodes were attached in the second PCR step. Small PCR fragments were removed using the AMPure PCR purification system (Agencourt, Beverly, Massachusetts) according to the manufacturer's instructions. Multiple samples were pooled, and paired-end 2×250 base pair sequencing reactions were carried out using an Illumina MiSeq sequencer (Illumina, La Jolla, California).

2.3 | Sequence data analysis

All sequencing reads were analyzed using the Immunocenetics (IMGT) database and the IMGT/HighV-Quest web-based analysis tool (http://imgt.cines.fr, IMGT, European Bioinformatics Institute, Montpellier, France). *IGLV* and *IGLJ* segments were identified based on the most closely matched human germline sequences. In addition, IMGT/HighV-Quest identifies clonotypes that were characterized by a unique V-J rearrangement, conserved complementarity determining region 3 (CDR3) anchors, and a unique CDR3-IMGT amino acid (AA) junction sequence.[31]

First, we excluded inappropriate sequences; particularly sequences that included stop codons, pseudogenes, and sequences that were nonfunctional due to insertions/deletions. Sequences with low V region identity (< 85%) were also excluded, as they indicated potential nucleotide insertions and/or deletions. Subsequently, we categorized each sequence by its closest matched germline sequences. Sequences that were not specific to a unique germline sequence, or not specific to *IGLV1/2* or *IGLV3*, were categorized as "unclassified". Sequences identified as "*IGLV1-36*" or "*IGLV1-44*" were categorized as "*IGLV1-36/44*" as it was not possible to distinguish between *IGLV1-36* and *IGLV1-44* due to sequence homology in the PCR target regions. Clonotypes were defined as sharing the same *IGLV* germline sequence, CDR3 length, and CDR3 AA sequence.[32,33] In each germline sequence category, the number of sequences with the same clonotype and their frequency out of sequences from all the categories were calculated. Raw data files were processed with Python (ver2.7) scripts to remove inappropriate sequences, categorize the sequences, and calculate the frequencies.

In evaluating the results, we defined "dominant clone" as the most frequently detected clonotype in each corresponding germline category. Specific cut-off is not determined to decide dominant clone. The percentage number, which demonstrates "clone size" for each clonotype, is the number that accounts for the proportion of all germline sequences. We statistically evaluated the significant increase of dominant clone size using χ^2-test, comparing dominant clone size of each POEMS and MM samples with the mean of negative controls.

2.4 | Amplification of monoclonal IGLV gene and sequencing by heteroduplex analysis

As a control, we amplified monoclonal *IGLV* gene and sequenced by Sanger method using heteroduplex (HD) analysis, as previously described.[19] Total RNA was extracted from the preserved BMMNCs at the diagnosis using Qiagen All Prep DNA/RNA Mini Kit, and single-stranded cDNA was synthesized using Takara Prime Script RT reagent Kit (TakaraBio, Shiga, Japan). The V-J region of the *IGL* gene was amplified by reverse-transcribed PCR using 5′ degenerate primers for the *IGLV1/2/3* consensus leader lesion (5′-ATGGCCKGSWYY-SYTCTCCTC-3′) and 3′ primers matching the consensus upstream part of the *IGLC* exon (5′-CTCCCGGGTAGAGAAGTCA CT-3′). Subsequently, we used HD analysis in which PCR products are denatured at 95°C for 5 minutes and subsequently renatured at 4°C for 1 hour to induce homoduplex or heteroduplex formation. Annealing of the PCR product gives duplexes with one or more mismatched bases, and mismatching causes the double helix to take on a conformation, which retards its mobility during electrophoresis. Homoduplex bands on the polyacrylamide gel were excised and PCR fragments were eluted from the gel slice using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL, Duren, Germany) and subjected to cycle sequencing. Sequence data were analyzed using IMGT database.

2.5 | Statistical analysis

Comparisons between controls and samples were performed by χ^2 test and student *t*-test. Categorical variables were compared using Fisher's exact test. Continuous variables were compared using the Wilcoxon rank-sum test. All descriptive statistics were expressed as medians with their ranges. All *P* values were 2-tailed, and $P < 0.05$ was considered statistically significant. All data analyses were performed using Microsoft Excel version 14.6.2 (Microsoft Corporation, Redmond, Washington), JMP 12 version 12.1.0 (SAS institute Japan, Tokyo, Japan), GraphPad PRISM version 7 (GraphPad Software, San Diego, California), and R software (www.r-project.org/).

3 | RESULTS

3.1 | Clinical and laboratory features of patients

Thirty patients with POEMS syndrome with λ-type M protein (57.0 ± 12.6 years old, range 34-79 years), six patients with λ-type MM, and nine negative controls (five with ITP, four with malignant lymphoma with no bone marrow invasion; 48.0 ± 15.3 years old, range 18-66 years, $P = 0.39$) were included in the study. The clinical characteristics of the patients with POEMS syndrome are summarized in Table 1.

3.2 | The clonal IGLV gene rearrangements restricted to specific germline sequences can be detected by NGS in patients with POEMS syndrome

We first analyzed the germline sequence of the *IGLV* genes in control, MM, and POEMS samples (Figure 1). Compared to the control samples, there was a dominant germline sequence in all MM samples (#1: *IGLV3-21*, #2: *IGLV1-36/44*, #3: *IGLV3-1*, #4: *IGLV1-47*, #5: *IGLV3-19*, #6: *IGLV2-23*, respectively) (Figure 1A). Interestingly, the *IGLV* germline sequence varied in each POEMS syndrome sample (Figure 1B). Some showed a specific dominant germline sequence; for example, *IGLV1-36* or *IGLV1-44* usage was dominant in eight POEMS syndrome samples, accounting for more than 30% of all germline sequence usage. One sample showed more than 80% usage of *IGLV1-40*. These data support our previous work, which demonstrated the restriction of germline sequence usage to *IGLV1-44* or *IGLV1-40*.[19] However, other samples showed minimal or no differences from controls, indicating that the clonal expansion of plasma cells is generally lower in patients with POEMS syndrome than MM.

Next, we analyzed the clone size of *IGLV* gene rearrangements in each germline sequence group. In all MM patients as positive control, significantly increased dominant clone sizes were confirmed in one germline for each patient (#1: *IGLV3-21*, #2: *IGLV1-36/44*, #3:

TABLE 1 Patient characteristics

Variables			Patients (n = 30)	%
Gender	Male	…	15	50
	Female	…	15	50
Median age at diagnosis	…	…	57.0	Range, 34–79
M protein	Heavy chain	IgA	22	73.3
		IgA + IgG	1	3.3
		IgG	6	20.0
		None (BJP)	1	3.3
	Light chain	λ	30	100
		κ	0	0
Symptoms at diagnosis	Polyneuropathy	…	30	100
	Organomegaly	…	25	83.3
	Endocrinopathy	…	17	56.7
	Skin change	…	29	96.7
	Bone lesion	…	14	46.7
	Castleman's disease	…	2	6.7
	Pulmonary hypertension	…	1	3.3
Median % of plasma cells in BM at diagnosis (%)	…	…	3.50	Range, 0.3-10.5
Median serum VEGF at diagnosis (pg/mL)	…	…	5190	Range, 1150-31 700

Abbreviations: BJP, Bence Jones protein; BM, bone marrow; VEGF, vascular endothelial growth factor.

IGLV3-1, #4: *IGLV1-47*, #5: *IGLV3-19*, #6: *IGLV2-23*, respectively; all $P < 0.001$). In patients with POEMS syndrome, the clonal IGLV gene rearrangements were significantly increased in 11 out of 30 patients with POEMS syndrome (36.7%; *IGLV1-36/44*: n = 9, *IGLV1-40*: n = 2, $P < 0.001$) (Figure 2A). Unfortunately, our method was not able to distinguish between *IGLV1-36* and *IGLV1-44* sequences; however, the present data is in good agreement with our previous report that clonal *IGLV* gene rearrangements in POEMS patients come from *IGLV1-44* or *IGLV1-40* germline sequences.

3.3 | The confirmation of clonal IGLV gene rearrangements by heteroduplex analysis

Our NGS results have shown that some of the patients have very small monoclonal plasma cell clone in their bone marrow. On the other hand, the detection of serum monoclonal protein is one of the most important factors for diagnosis of POEMS syndrome. Thus, we hypothesized that even small monoclonal plasma cell clones have large impact on the pathogenesis of POEMS syndrome.

Based on this hypothesis, to confirm the presence of clonal *IGLV* gene rearrangement, we examined HD analysis in 28 patients with POEMS syndrome in whom enough amount of cDNA was extracted from the preserved BMMNCs. Among them, homoduplex bands were recognized in 25 patients, including 15 patients with no significant increase of clonal *IGLV* gene rearrangement in NGS analysis (Supporting information Table S1 and Supporting information Figure S1). The *IGLV* gene rearrangement sequences were detected by Sanger sequencing in 22 patients as shown previously (*IGLV1-40*, n = 5:22.7%, *IGLV1-44*, n = 17:77.3%, Supporting information Table S1 and Supporting information Figure S2).[19] All samples with significant increase of *IGLV1-36/44* by NGS were confirmed to have *IGLV1-44* as the most dominant clonotype by HD analysis and Sanger sequencing. In 13 patients among them, the clonotypes detected by Sanger sequencing were homologous with the most dominant clonotype in NGS analysis. Notably, in three patients among these 13 cases, the most dominant clonotypes, which were detected as clonal *IGLV* gene rearrangements in HD analysis were not detected as "significantly increased clonal *IGLV* gene rearrangement" in NGS analysis. Although Li et al. reported the relationship between clinical features and *IGLV* genes,[20] there was no significant association between the *IGLV* gene usage and clinical features of POEMS syndrome in our study.

3.4 | The clone size of IGLV gene rearrangements is not directly linked to initial disease status in patients with POEMS syndrome

Specific clonal *IGLV* gene rearrangements could not be detected in some patients. The clinical characteristics of patients with and without significantly increased dominant clonal *IGLV* gene rearrangements were shown in Table 2. The only factor that exerted a significant influence was age. Patients with significantly increased dominant clonal *IGLV* gene rearrangements were significantly younger ($P = 0.03$). There were no significant differences in gender, serum VEGF level, or plasma cell content in the BM at diagnosis and it's κ/λ restriction by in situ hybridization (Table 2). Clinical presentations and immunoglobulin heavy chain type did not show any differences (data not shown). These results indicate that the clone size of plasma cells in the BM is not directly correlated with initial disease activity in patients with POEMS syndrome.

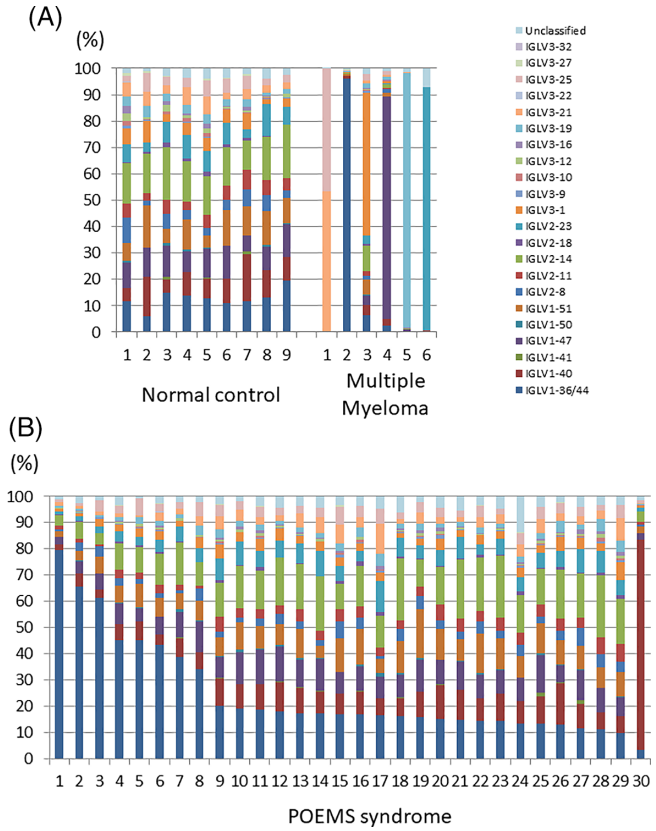

FIGURE 1 *IGLV* germline sequence analysis of control, multiple myeloma, and newly diagnosed polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes (POEMS) syndrome patients. A, Control samples from patients without λ-type M protein (n = 9) and samples from multiple myeloma (n = 6). A dominant *IGLV* germline frequency was detected (#1: *IGLV3–21*, #2: *IGLV1–36/44*, #3: *IGLV3–1*, #4: *IGLV1–47*, #5: *IGLV3–19*, #6: *IGLV2–23*). B, *IGLV* sequence analysis of patients with POEMS syndrome (n = 30) in descending order of *IGLV1–36/44*. In POEMS patients #1-7, *IGLV1–36/44* was predominant. POEMS patient #30 exhibited more than 70% usage of *IGLV1–40* [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | The clone size of IGLV gene rearrangements correlates with disease courses in most patients with POEMS syndrome

Next we analyzed the *IGLV* gene rearrangements in 12 patients during the clinical course of POEMS syndrome and compared with immunofixation electrophoresis (IFE) results and serum VEGF levels (Figure 2B). Ten patients obtained hematological complete response with negative IFE after or at the time of ASCT. In three patients with clinical relapse (#3, #5, #25), IFE remained positive or became positive at the time of relapse. These results suggested that IFE levels had good correlation with serum VEGF levels and clinical course. In patient #1, #3, #5, #6, and #30, the frequency of the most dominant gene rearrangement clonotype in NGS correlated to serum VEGF levels and IFE results. As seen in POEMS patients #2, #4, and #24, the clonotype frequency and serum VEGF levels or IFE results were not correlated, although the dominant clone and serum VEGF levels decreased following disease remission. Among three patients who relapsed, POEMS patient #3 exhibited only VEGF relapse at 6 months after ASCT without clinical relapse. In agreement with this observation, the clonotype frequency also slightly increased in this patient at this point; subsequently 1 year after ASCT, this patient clinically relapsed with clonotype frequency significantly increasing. In POEMS patient #25, we did not detect a significant increase in clonotype frequency at diagnosis. However, the specific *IGLV1-40* clonotype increased at the time of relapse. Interestingly, this clonotype was already the most dominant clonotype at diagnosis, although it was not significantly increased at diagnosis. The frequencies of the most dominant clonotype in POEMS patients #12, #16, and #18 were low at first, and could not be detected during all courses. To be added, all patients who had repeated NGS after treatment whose clones decreased, their monoclonal proteins in their serum also turned out to be negative. These results suggest that POEMS-associated *IGLV* gene rearrangement clones can exist at very low levels that are impossible to detect in some cases, but they can still play a role in the pathogenesis of POEMS syndrome.

Additionally, comparing the homoduplex band of samples in clinical courses, the bands detected at diagnosis became faint at the time of remission and reappeared at the time of relapse (Figure 2C). These results also suggest the association between the state of POEMS syndrome and the *IGLV* gene rearrangements.

4 | DISCUSSION

POEMS syndrome was recognized as a paraneoplastic syndrome due to the underlying plasma cell disorder, but the pathogenesis of the syndrome is not well understood. The direct relationship between monoclonal plasma cells and the various manifestations of the syndrome has not been elucidated. To our knowledge, this is the first report to analyze the plasma cell clone size in patients with POEMS syndrome using NGS to understand the significance of clonal plasma cells in this disease.

We analyzed the *IGLV* gene rearrangement in patients with POEMS syndrome, and confirmed that clonal light chain gene usage is restricted to the *IGLV1-44* and *IGLV1-40* germline sequences, as others and we previously reported.[19–21] We have demonstrated a significant increase in unique *IGLV* gene rearrangements in patients with POEMS syndrome. In some cases, we did not detect significant differences. One possible reason is that bone marrow involvement in POEMS syndrome is usually found in 2/3 patients, and in 1/3 patients the disease is present only in focal bone lesions, therefore the frequency of the dominant clonotype was too low to be detected in some cases.[2] However, it should be noted that in 15 patients without significant increase of clonal *IGLV* gene rearrangement, existence of their clonal *IGLV* gene rearrangements were confirmed in cDNA samples by HD analysis. This discrepancy in results between NGS and HD analysis suggests that the quite small number of monoclonal plasma cells in POEMS syndrome may produce large quantity of mRNA of monoclonal proteins. This supports the hypothesis that even small monoclonal plasma cell clones have high impact on the pathogenesis of POEMS syndrome. Although the number of patients analyzed in this study is small, the fact that most cases had monoclonal *IGLV* genes by HD analysis supports the significance of the study.

VEGF plays an important role in the clinical manifestations of POEMS syndrome and correlates with disease activity.[7,15,34] Contrary

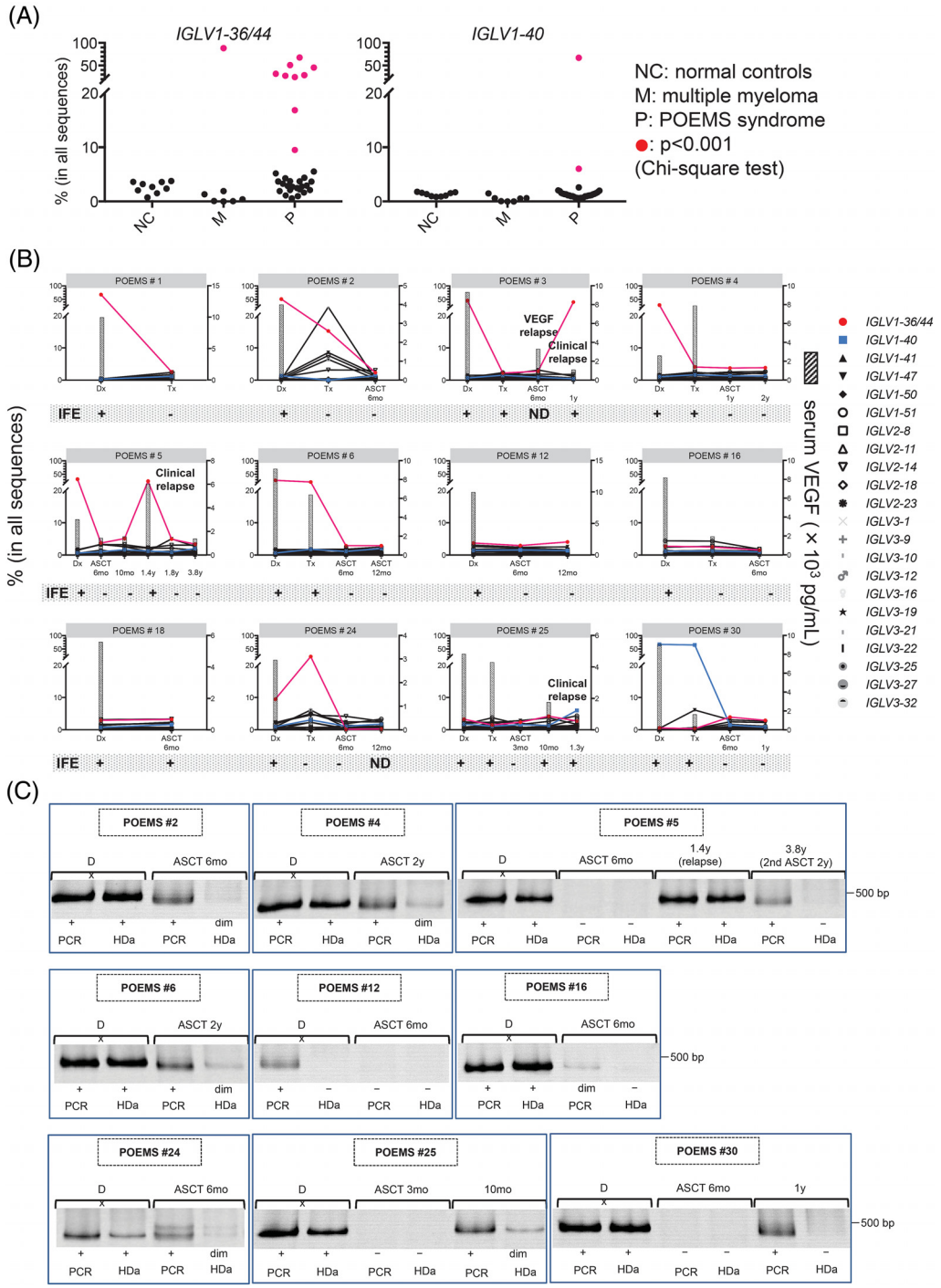

FIGURE 2 Clone size of *IGLV* gene rearrangements in each germline sequence group and changes in *IGLV* germline sequence frequency during the clinical course. A, Clone size of *IGLV* gene rearrangements in each germline sequence group. "Clone size" is the percentage number of the most frequent clonotype in each germline sequences, accounted for sequences in all germline sequence categories. Eleven out of 30 POEMS patients (36.7%; *IGLV1-36/44*: n = 9, *IGLV1-40*: n = 2) had a statistically significant increase in clonal rearrangements from each germline sequence group compared with that in the controls. NC, Normal control; M, Multiple myeloma; P, polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes (POEMS) syndrome. B, Changes in *IGLV* germline sequence frequency and VEGF levels during the clinical course. Each graph shows the clinical course of each patient with POEMS syndrome. The changes in the frequency of the most dominant rearrangement clonotypes are shown as line graphs for each germline sequence group. The column graph shows the serum VEGF levels. The number of the most frequent clonotypes had a similar tendency with disease status, which was assessed by serum VEGF levels and IFE in POEMS patients #1, #3, #5, #6, and #30. In some patients (#2, #4, and #24), the clonotype size and serum VEGF levels did not correlate, although both decreased with disease remission. Dx, At diagnosis; Tx, Post induction therapy; ASCT, Autologous stem cell transplantation; ISH, In situ hybridization; IFE, Immunofixation electrophoresis; ND, No data. C, Heteroduplex (HD) analysis of samples in clinical course. The homoduplex bands detected at diagnosis became weak at the time of remission and reappeared at the time of relapse in all patients excluding patient #12. From samples from patient #12, the homoduplex band could not be detected neither from sample at diagnosis and sample after ASCT. Total DNA amount for PCR was set even in each patient. HDa, heteroduplex analysis [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Comparison of the characteristics of patients with polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes (POEMS) syndrome with or without significantly increasing clonal *IGLV* gene rearrangements

		Significant, n = 11	Not significant, n = 19	*P*
Gender	Male	6	9	1
	Female	5	10	
Median age at diagnosis (years)	...	51.0 ± 11.5	61.0 ± 12.0	0.03
Median serum VEGF level at diagnosis (pg/mL)	...	4430 ± 1061.6	5570 ± 1483.3	0.83
Median % of plasma cell in bone marrow at diagnosis (%)	...	2.8 ± 2.4	3.6 ± 2.3	0.53
Light chain λ restriction of bone marrow plasma cells by ISH (n = 24)	Positive	5	5	0.6
	Negative	5	9	7

Abbreviation: ISH, in situ hybridization.

to our prediction, VEGF levels at diagnosis were not correlated with the clone sizes of *IGLV* gene rearrangements among our patients. VEGF is generally expressed in plasma cells,[35,36] megakaryocytes/platelets,[37,38] osteoblasts, macrophages, and tumor cells.[39] Our data indicate that VEGF may not be secreted mainly from monoclonal plasma cells in POEMS syndrome. Further investigation will be needed to clarify the source of VEGF.

In contrast, following each patient's clinical course, we could recognize that the frequency of *IGLV* gene rearrangements from *IGLV1-36/44* or *IGLV1-40* germline sequences correlated with disease course as assessed by serum VEGF levels and IFE in patients with significantly increased *IGLV* gene rearrangements. Serum VEGF levels are one of the most reliable markers for monitoring POEMS syndrome.[11,40] VEGF levels are increased at diagnosis and at the time of relapse, and are decreased when treatment is successful. A similar trend in the greatest frequency of the clonotype derived from *IGLV1-36/44* or *IGLV1-40* was detected in this study. Other frequent clonotypes derived from other germline sequences did not show such variations. Overall, our results show that the initial size of clonal plasma cell populations, which is very tiny in some cases, does not determine disease status. However, the clone size in each patient is linked to the clinical course at remission or relapse. These results confirm the association between the clonal plasma cell population and the clinical course of POEMS syndrome. Taken together with the discrepancy in results between NGS and HD analysis, it is suggested that small monoclonal plasma cell clones induce dynamic changes in the manifestations of POEMS syndrome.

In conclusion, our deep sequencing analysis of *IGLV* gene rearrangements has demonstrated the association between the size of clonal *IGLV* gene rearrangement clones and the clinical courses in each patient with POEMS syndrome. These findings strongly suggest that even a small number of monoclonal plasma cells can affect the pathogenesis and clinical features of POEMS syndrome.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All authors reviewed the draft manuscript and approved the final version for submission.

Designed the research: C.K-M., N.M., E.S., M.Takeuchi, A.K. and C.N.

Collected and analyzed patients' clinical data and performed statistical analysis: C.K-M. and E.S.

Performed next generation sequencing analysis: C.K-M., H.N-F., M.F., B.R. and A.K.

Interpreted data: H.N-F., M.F., K.Y., M.Tsuiji and A.K.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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