

A VH4-34⁺ myeloma protein with weak autoreactivity

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ABSTRACT

It has been suggested that VH4-34 gene segment expression is counter-selected in multiple myeloma (MM) due to a self-tolerance mechanism. We cloned and sequenced a VH4-34 gene segment from bone marrow mononuclear cells of a stage III MM patient. We show that VH4-34 was expressed by the serum IgA myeloma (M)-protein, as demonstrated by reactivity with the VH4-34 specific 9G4 mAb and mass spectrometry (MS). The M-protein had weak reactivity with nuclei. These results demonstrate that VH4-34 may be expressed in secreted IgA M-protein with weak autoreactivity. Thus, counter-selection of VH4-34 is pronounced but not absolute in MM. Mechanisms of how VH4-34 can occasionally be expressed in MM and clinical implications are discussed.

Key words: VH4-34, myeloma protein, autoreactivity.

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The B cell receptor repertoire is shaped during B cell differentiation. Thus, the variable heavy 4 (VH4) family member VH4-34 is frequently expressed in normal B cells (2-10%), but it is excluded from the plasma cell subset, presumably due to autoreactivity imparted by VH4-34 and negative selection during the germinal center reaction.¹ Supporting an association with autoreactivity, VH4-34 is over-represented in autoimmune diseases such as cold agglutinin (CA) disease² and systemic lupus erythematosus (SLE) suggesting a relationship with autoreactivity.³ Furthermore, VH4-34 is over-represented in B cell tumors associated with autoimmunity, such as chronic lymphocytic leukemia⁴ and diffuse large B cell lymphoma.⁵ The myeloma clone is thought to originate from post-germinal center B cells⁶ and there are few autoimmune phenomena in multiple myeloma (MM).⁷ For some years it was believed that MM did not express VH4-34 encoded myeloma (M)-proteins.⁷ However, among three large cohorts studied,⁷⁻⁹ VH4-34 was expressed in 3/219 MM patients (1.4%). Some of

these studies did not rule out that the VH4-34⁺ cDNA could have originated from contaminating B cells in the bone marrow aspirates. More importantly, none of the studies demonstrated that VH4-34 was expressed as part of a secreted M-protein or whether the M-protein had autoreactivity. This study shows that VH4-34 can, in fact, be expressed in a secreted M-protein with weak anti-nuclear activity.

Design and Methods

Bone marrow aspirate and a blood sample were obtained at diagnosis from a 65 year old stage III MM patient with >50% marrow involvement, serum IgAλ M-protein, and total IgA concentration of 55.5 g/L. The study was approved by The Regional Committees for Medical Research Ethics (Norway). The myeloma IgH and IgL (light) V regions were amplified from bone marrow mononuclear cells, cloned and sequenced essentially as previously described.¹⁰ IgA1 M-protein was affinity purified from patient serum on an

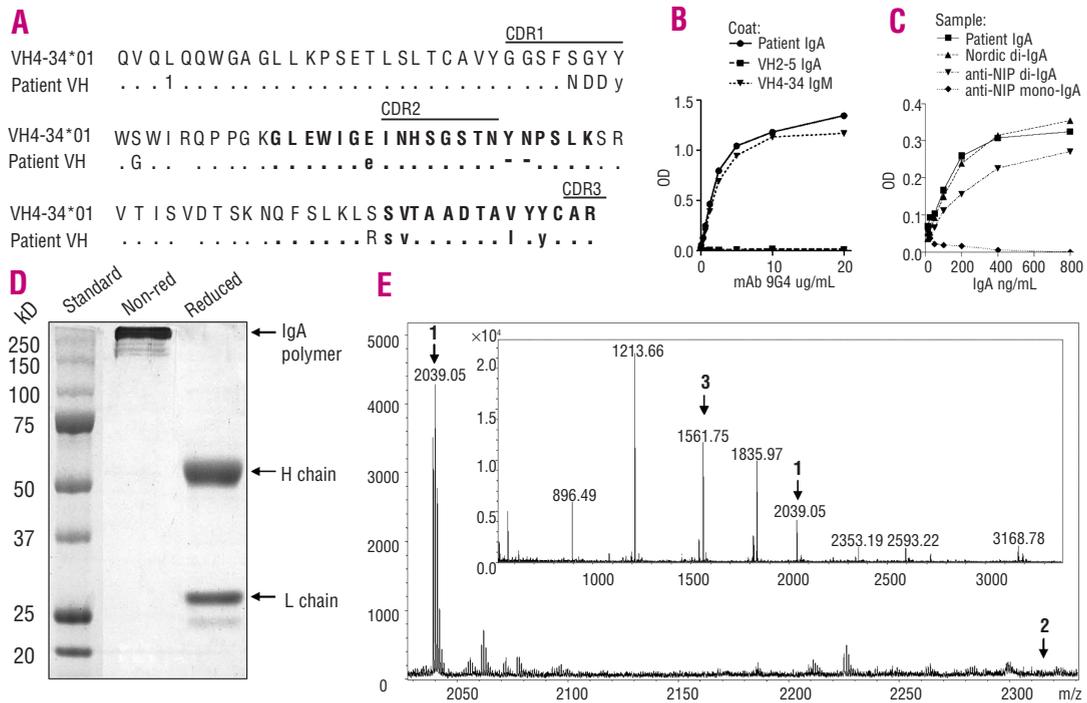


Figure 1. VH4-34 gene segment expressed as part of a polymeric IgA myeloma protein. **(A)** Comparison of the aa sequence, translated from nucleotide sequence, of the patient VH with the closest matching germline VH4-34 (allele 01). Dots (.) represent an identity and dashes (-) represent gaps for optimal alignment. Silent and replacement mutations are indicated with letters in lower- and uppercase, respectively. Two tryptic segments of particular interest are boxed. Nucleotide sequence of the whole VDJ rearrangement is shown in Supplementary Figure 1. **(B)** The M-protein was tested for expression of the VH4-34 gene segment by its ability to be bound by the VH4-34 specific mAb 9G4 in ELISA. A mAb VH4-34 IgM known to be bound by 9G4 and an MM VH2-5 IgA served as positive and negative controls, respectively. **(C)** The myeloma IgA was tested for the presence of J chain by its ability to bind human SC in ELISA. Dimeric (Nordic di-IgA and anti-NIP di-IgA) and monomeric (anti-NIP mono-IgA) IgA served as positive and negative controls, respectively. **(D)** Coomassie-stained 10% SDS-PAGE of 5 μ g non-reduced and β -mercaptoethanol reduced purified myeloma IgA. IgA polymer, H chain and L chain are indicated. **(E)** MS analysis of a tryptic digest of the patient IgA H chain from SDS-PAGE. The inset shows the whole spectrum, while an area of particular interest is shown enlarged. The arrows indicate three molecular weights of particular interest connected to the boxed segments in A as outlined in the text.

immobilized Jacalin agarose column (Pierce). Binding of the VH4-34 specific rat mAb 9G4¹¹ to M-protein was tested in enzyme-linked immunosorbent assay (ELISA) with alkaline phosphatase-conjugated sheep anti-mouse/rat IgG (whole molecule) (Sigma). Binding of myeloma IgA to human secretory component (SC) was detected by ELISA essentially as previously described¹² but rabbit anti-human IgA (DAKO) was used as the coating antibody.

Both non-reduced and reduced purified M-protein was subjected to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie blue. In-gel trypsin digestion of the excised H chain, and purification, elution and analysis by mass spectrometry (MS) of the tryptic peptide mixture was performed as previously described.¹³ Binding of M-protein to HEp-2 cells (BioSystems), monkey kidney (The Binding Site) and esophagus cryostat sections (Orgentec) was detected with goat anti-human IgA FITC conjugate prediluted with eriochrome black counterstain (Panbio).

Results and Discussion

Three independent polymerase chain reactions and sequencing showed the rearranged MM IgH V region to be composed of VH4-34/D4-4/JH6 gene segments. The in-frame VH4-34 sequence revealed 9 silent and 6 replacement mutations, as well as the deletion of six nucleotides resulting in the loss of two amino acids (aa) (Figure 1A, *Supplementary Figure 1*, GenBank accession number DQ916278). The IgL V region was generated by a V λ 3-1 and a J λ 2/3 rearrangement (*Supplementary Figure 2*, GenBank accession number DQ916279). The mAb 9G4 has a unique specificity for a VH4-34 encoded determinant, binding aa residues 6-12 and 23-25 of FR1.¹⁴ 9G4 bound the purified M-protein (Figure 1B), thereby demonstrating expression of VH4-34 at the protein level. The myeloma IgA bound SC, indicating the presence of a J chain (Figure 1C).¹² Consistent with this, the IgA was polymeric under non-reducing conditions in SDS-PAGE (Figure 1D).

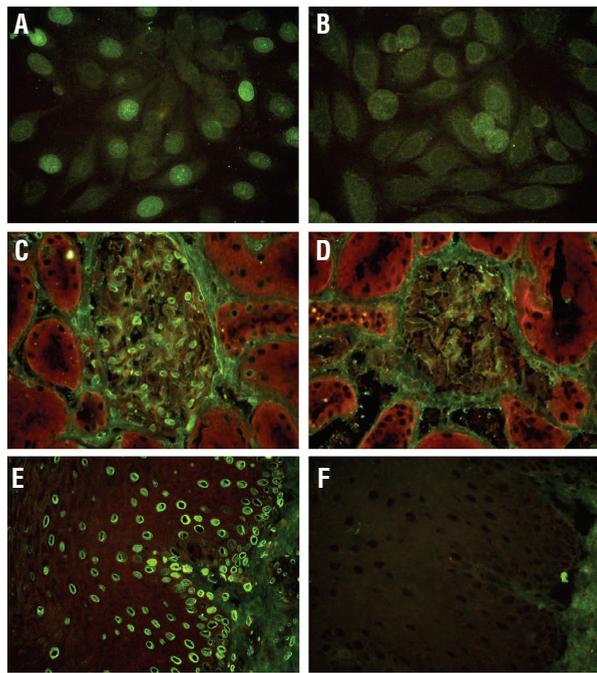


Figure 2. Tissue reactivity of the VH4-34 M-protein. Purified VH4-34 patient IgA M-protein was tested for tissue reactivity by immunofluorescent staining of HEP-2 cells (A), monkey kidney (C) and monkey esophagus tissue (E). A control IgA M-protein expressing VH2-5 served as isotype control for each tissue (B, D, F). Stainings with 100 µg/mL IgA are shown, but the pattern was also weakly observed at 10 µg/mL. Secondary reagent was goat anti-human IgA FITC conjugate prediluted with eriochrome black counterstain. A Leica DM RB microscope with a Leica PL FLUOTAR 40x/0.70 lens was used at room temperature in air. Images were acquired through a Leica DFC 320 Digital Camera using Leica DFC Twain camera software and processed using Adobe Photoshop Elements 3.0.

The H chain of the reduced M-protein was excised from the gel, digested with trypsin, and subjected to MS (Figure 1E). The first tryptic peptide detected (boxed with solid line in Figure 1A) covers a sequence that spans the two deleted aa and should have a mass of 2039.02 Da. Consistent with this, the MS spectrum showed a peak of 2039.05 Da (arrow 1 in Figure 1E). The germline VH4-34 should have yielded a tryptic peptide of 2316.13 Da, due to the inclusion of two extra aa (YN in Figure 1A). No such peak was detected in the MS spectrum (arrow 2 in Figure 1E). The second tryptic peptide detected (boxed with dashed line in Figure 1A) is dependent on an S→R aa exchange that introduces a new tryptic cleavage site. This fragment also contains a V→I aa replacement, and the mass should be 1561.73 Da. Consistent with this, the MS spectrum showed a peak of 1561.75 Da (arrow 3 in Figure 1E). In the absence of both or only one of these mutations the mass of the tryptic fragment should have been either 1761.85, 1747.83 or 1547.72 Da, but such peaks were not seen in the spectrum. The MS spectrum conclusively demonstrates that the translated VH4-34 aa sequence given in Figure 1A is indeed

expressed as part of the secreted M-protein.

VH4-34 has been closely associated with IgM CAs that bind erythrocyte I/i surface N-acetylglucosamine glycoprotein structures.² However, the present VH4-34 polymeric IgA M-protein did not agglutinate adult erythrocytes at 4°C, and only very weakly agglutinated papain-treated cord erythrocytes (*data not shown*).¹⁵ The M-protein did not bind to erythrocytes in flow cytometry (*data not shown*). Binding to I/i is determined by a hydrophobic patch in FR1, involving residue W7 and the AVY motif (residues 23-25), identical to the epitope bound by the 9G4 antibody.¹⁴ In addition, aa in the carboxyl-terminal region of CDR3 form a surface region in proximity of this hydrophobic patch, and it is proposed that the I/i carbohydrate antigen interacts simultaneously with the hydrophobic patch and CDR3.¹⁴ Since the M-protein had an intact FR1 motif for I/i binding (Figure 1A), it is possible that the CDR3 of the M-protein does not allow significant binding to the I/i antigen.

Anti-dsDNA antibodies are a hallmark of SLE, and often display arginine-rich CDR3 regions involved in the DNA binding.¹⁶ The M-protein had three arginines (R) in the CDR3 region (*Supplementary Figure 1*). It showed weak anti-nuclear activity using HEP-2 cells and monkey kidney and esophagus sections, also with perinuclear accentuation (Figure 2). The nuclear pattern in kidney was different to the patchy tubule staining observed with VH4-34 anti-I/i antibodies.¹⁵ Collectively, the M-protein resembled those VH4-34 encoded anti-DNA antibodies that lack agglutinating activity despite expressing the hydrophobic patch essential for CA reactivity.^{14,16}

The patient (now deceased) had no recorded symptoms or findings of autoimmune disease despite a high level of the autoreactive VH4-34⁺ IgA M-protein (55.5 g/L). The patient did have kidney failure, but as this is a common complication in MM, it is difficult to establish whether the anti-nuclear activity of the M-protein was a contributing factor. Monoclonal IgA has few biological effector functions, perhaps explaining the lack of autoimmune phenomena in the patient. Myeloma clones are thought to originate from post-germinal center B cells, as they are isotype switched and display extensive somatic hypermutation.⁶ VH4-34 expressing B cells are physiologically excluded during the early stages of the germinal center reaction, and appear to be completely absent from normal plasma cells in bone marrow and tonsil.^{1,17} Consistent with these two observations, VH4-34 expression is under-represented in MM,⁷⁻⁹ and until now VH4-34⁺ M-proteins have not been described. How then can the current finding of a heavily mutated VH4-34⁺ M-protein be explained? One explanation is that tolerance induction is not complete and that VH4-34⁺ B cells may very rarely proceed through the germinal center and later become malignantly transformed. Alternatively,

somatic hypermutation and generation of myeloma clones may occur extra-follicularly, for example, in the mantle zone of tonsils where VH4-34⁺ cells are abundant.¹ It has recently been shown that autoreactive mouse splenic B cells can proliferate and undergo somatic hypermutation at the T zone-red pulp border.¹⁸ Furthermore, somatic hypermutation can occur in non-lymphoid tissues such as in the inflamed synovial tissues of rheumatoid arthritis patients.¹⁹ In normal lymphoid tissue, VH4-34 expressing B cells are concentrated in the follicular mantle zone, and it seems likely that under appropriate conditions they are capable of generating vigorous but self-limited plasma cell responses.¹⁷ Selection against autoreactive VH4-34 expressing B cells may be absent or less efficient in these areas compared to the germinal centers. The M-protein in the current study may thus have originated not from a post-germinal center B cell, but

rather from a VH4-34⁺ extra-follicular B cell, and may therefore represent a distinct subdivision of multiple myeloma. Indeed, studies of chromosomal translocations and gene expression have indicated that multiple myeloma can be classified into discrete entities with different prognoses.²⁰ Whether these different entities may be related to different B cell origins of the myeloma clones is as yet unknown.

Authors' Contributions

MF, KMT, SSS and BB designed research; MF, KMT and SJT performed research; TGD collected patient samples and data; MF, KMT, SJT, SSS and BB analyzed data; MF, KMT and BB wrote the paper; all authors discussed and contributed to the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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