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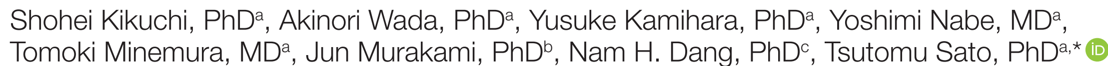
Involvement of activation induced cytidine deaminase in malignant B-cells expressing two distinct M-components as an etiology of biclonal gammopathy



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Abstract

Biclonal gammopathy (BG) is a rare phenomenon in which 2 M proteins are detected in the same patient, with 2 major hypotheses regarding its etiology. One potential explanation is that completely different malignant B-cell clones produce different M proteins, while the other is that there is a malignant clone that produces both M proteins simultaneously. In this study, we examined 2 cases of B-cell malignancy with BG and found that some cells were double positive for both M proteins by immunofluorescence and flow cytometry. However, most of the remaining cells were single positive cells that produced only one of the M proteins. We hypothesized that double positive cells were in the process of transitioning from 1 single positive cell to another single positive cell, and that class switch recombination (CSR) would be involved as a mechanism. We then examined the expression of activation induced cytidine deaminase (AICDA), which is responsible for CSR, and found that lymphoma/myeloma cells in 2 BG patients were positive for AICDA by immunostaining. Our study is the first report suggesting that AICDA may be involved in the pathogenesis of BG.

Abbreviations: AICDA = activation induced cytidine deaminase, BG = biclonal gammopathy, CSR = class switch recombination, MG = monoclonal gammopathy, SHM = somatic hypermutation, SSB = single-strand break.

Keywords: activation induced cytidine deaminase, biclonal gammopathy, class switch recombination

1. Introduction

Monoclonal gammopathy (MG) is characterized by the presence of a single M protein produced by malignant B-cells that expand clonally. M protein is detected in MG of undetermined significance (MGUS) and multiple myeloma (MM), but is also frequently found in various types of B-cell non-Hodgkin's lymphomas, with 1 study reporting a frequency of 17.2% (44/166).[1] Although most cases involve the production of a single M protein, 2 M proteins are detected in a minority of patients, a condition called biclonal gammopathy (BG). The mechanism involved in the production of these 2 M proteins has long been a subject of interest, specifically whether the 2 M proteins are derived from completely separate malignant clones or from the same one.

BG with different heavy chain (HC) isotypes and different light chain (LC) isotypes is called true BG, and while it is commonly assumed that the 2 M proteins originate from different malignant clones, there has been no study to validate this hypothesis. On the other hand, there have been published reports that analyzed BG with different HC isotypes and the same LC isotypes.

One is a case report by Bakkus et al,[2] in which sequence analysis of the variable regions of the immunoglobulin heavy chain genes was performed on a MM patient with IgA-κ/IgE-κ. Their results showed that the sequences of the variable regions of both IgA and IgE were exactly the same, including the somatic mutations, hence strongly suggesting that the 2 M proteins may be derived from a single malignant clone. However, they reported that no malignant cells expressed concurrently both IgA and IgE, based on data demonstrating that 9 of the cloned MM cells were positive for IgE and 7 for IgA, but none were double positive.

SK and AW contributed equally to this work.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Using the same sequence analysis technique as Bakkus et al, Tschumper et al[3] demonstrated that the variable regions of the 2 M proteins were identical in 9 of 14 BGs with different HCs and the same LC. Furthermore, immunofluorescence performed on one of these 9 cases showed no cell with simultaneous expression of the 2 M proteins.

These 2 reports therefore came to the same conclusion that although the 2 M proteins were derived from a single clone, no cell expressed them simultaneously. However, using the same immunofluorescence technique as Tschumper et al, several studies have demonstrated malignant cells that produced both M proteins simultaneously.[4–6] Specifically, Rudders et al reported IgG-λ/IgA-λ phenotype,[4] Costea et al reported IgG-κ/IgA-κ phenotype,[5] and Gallart et al reported IgG-λ/IgA-λ phenotype.[6]

It is to be expected that some clues can be obtained from the study of BG in animals. We found only 1 report of mice with BG of IgG2a-κ/IgG2b-κ. Sequence analysis of the variable regions of the immunoglobulin heavy chain genes in this report showed an interesting result that although both IgG2a and IgG2b are from the same small 36-60 family, there are at least 3 distinct clones of IgG2a. However, this result does not provide a clue to our question.[7]

Given the lack of clarity regarding this issue, a primary goal of our present work is to investigate whether there are malignant cells that produce both M proteins concurrently. In addition, we were interested in understanding the potential role of molecules involved in the process of class switch recombination (CSR).

2. Materials and Methods

2.1. Patients

Between April 2010 and July 2021, M protein was detected in 172 patients by immunofixation examinations at the Division of Hematology, Toyama University Hospital. Immunofixation assays were performed using an agarose gel-based commercially available kit from Helena Biosciences (Beaumont, TX). The average age of the 172 patients was 70 years (24–94), with 100 males and 72 females. The patients' diagnoses were as follows: chronic lymphocytic leukemia (CLL) in 3, diffuse large B-cell lymphoma (DLBCL) in 8, follicular lymphoma (FL) in 2, lymphoplasmacytic lymphoma/Waldenström's macroglobulinemia (LPL/WM) in 11, mucosa-associated lymphoid tissue lymphoma (MALT) in 9, mantle cell lymphoma (MCL) in 1, methotrexate-associated lymphoproliferative disorders (MTX-LPD) in 1, marginal zone lymphoma (MZL) in 4, MGUS in 55, MM in 75, POEMS syndrome in 2, and amyloidosis in 1. The sum of MGUS and MM accounted for 75.6% (130/172) of the total.

2.2. Case presentation

Patient 12 (Table 1) was referred to our hospital for an examination of hyperproteinemia. Total protein was 9300 mg/dL, IgG was 3157 mg/dL, IgA was 52 mg/dL, IgM was 1313 mg/dL. Immunofixation analysis detected both IgG-κ and IgM-κ (Fig. 1). Bone marrow examination showed an increased number of lymphocytes (51%), which were positive for kappa, CD19, CD20, and CD25 expression by flow cytometry. The patient was diagnosed with splenic MZL with bone marrow involvement. Systemic therapy with bendamustine plus rituximab was initiated, and the soluble interleukin 2 receptor (sIL-2R) level was reduced from 771 U/mL to 265 U/mL. No lymphoma cells were detected in the bone marrow following the sixth course of treatment. Immunofixation analysis revealed the disappearance of the IgM-κ M-protein, but IgG-κ was still present.

Patient 13 (Table 1) was referred to our hospital for an examination of plasmacytoma of the sacral bone. Total protein was 6600 mg/dL, IgG was 945 mg/dL, IgA was 94 mg/dL, IgM was 36 mg/dL. In addition to IgG-λ, IgD-λ was also detected by immunofixation analysis (Fig. 1). Bone marrow examination showed an increased number of plasma cells (17%), which were positive for CD38 and CD56, and negative for CD19 by flow cytometry. The patient was diagnosed with multiple myeloma. Systemic therapy with daratumumab plus bortezomib, melphalan, and prednisone was initiated, and the λ chain level decreased from 1175 mg/L to 6 mg/L. At the end of the fifth course, IgD-λ was undetectable but a thin band of IgG-λ was identified by immunofixation analysis. IgG-λ and IgD-λ were detected prior to initiation of therapy with daratumumab.

2.3. Flow cytometry

Total bone marrow mononuclear cells (BM MNCs) were analyzed by flow cytometry using BD FACSLyric with BD FACSuite ver.1.5 (BD Biosciences, Franklin Lakes, NJ). BM MNCs of patient 12 were stained with polyclonal rabbit anti-human IgG/FITC, F0185 (Dako, Glostrup, Denmark), polyclonal rabbit anti-human IgM/RPE, R5111 (Dako), polyclonal rabbit anti-human kappa light chains/APC, C0222 (Dako), and 7-AAD (7-Aminoactinomycin D), A1310 (Life Technologies, Carlsbad, CA). BM MNCs of patient 13 were pretreated with IntraStain, K2311 (Dako) and stained with polyclonal rabbit anti-human IgD/FITC, F0189 (Dako), monoclonal mouse anti-human CD38/PE, clone HB7 (BD Pharmingen, San Diego, CA), polyclonal rabbit anti-human lambda light chains/PerCP-Cy5.5, PR712 (Dako), and monoclonal mouse anti-human IgG/APC, clone G18-145 (BD Pharmingen).

2.4. Immunofluorescence

BM MNCs were fixed with 4% paraformaldehyde for 30 minutes and blocked with Fc-block, AB_2728082 (BD Biosciences) for another 30 minutes. Cells from patient 12 were then stained for 60 minutes with monoclonal mouse anti-human kappa light chains/PerCP-Cy5.5, clone G20-193 (BD Biosciences), monoclonal mouse anti-human IgG/PE, clone G18-145 (BD Biosciences), and monoclonal mouse anti-human IgM/FITC, clone G20-127 (BD Biosciences). Cells from patient 13 were stained with monoclonal mouse anti-human CD138/Alexa Fluor-647, clone MI15 (BD Biosciences), monoclonal mouse anti-human IgG/PE, clone G18-145 (BD Biosciences), and

Table 1

Characteristics of biclonal gammopathy patient cohort.

Patient	Age, Yr	Sex	Diagnosis	Immunofixation	
1	**92**	**M**	**MGUS**	**IgA-λ**	**IgA-λ**
2	80	F	MGUS	IgA-κ	IgA-κ
3	70	M	MM	IgG-κ	IgG-κ
4	68	F	MGUS	IgA-κ	IgG-λ
5	65	F	MALT	IgA-κ	IgM-λ
6	71	M	MM	IgG-κ	IgG-λ
7	62	M	MM	IgG-κ	BJP-κ
8	77	M	DLBCL	BJP-κ	BJP-λ
9	77	M	MM	BJP-κ	IgG-κ
10	73	M	MM	IgG-λ	BJP-λ
11	65	M	MGUS	IgM-κ	IgG*
12	53	F	MZL	IgG-κ	IgM-κ
13	73	F	MM	IgD-λ	IgG-λ

BJP = Bence-Jones protein, DLBCL = diffuse large B-cell lymphoma, MALT = mucosa-associated lymphoid tissue lymphoma, MGUS = monoclonal gammopathy of undetermined significance, MM = multiple myeloma, SMZL = marginal zone lymphoma.
*Unable to determine whether κ or λ.

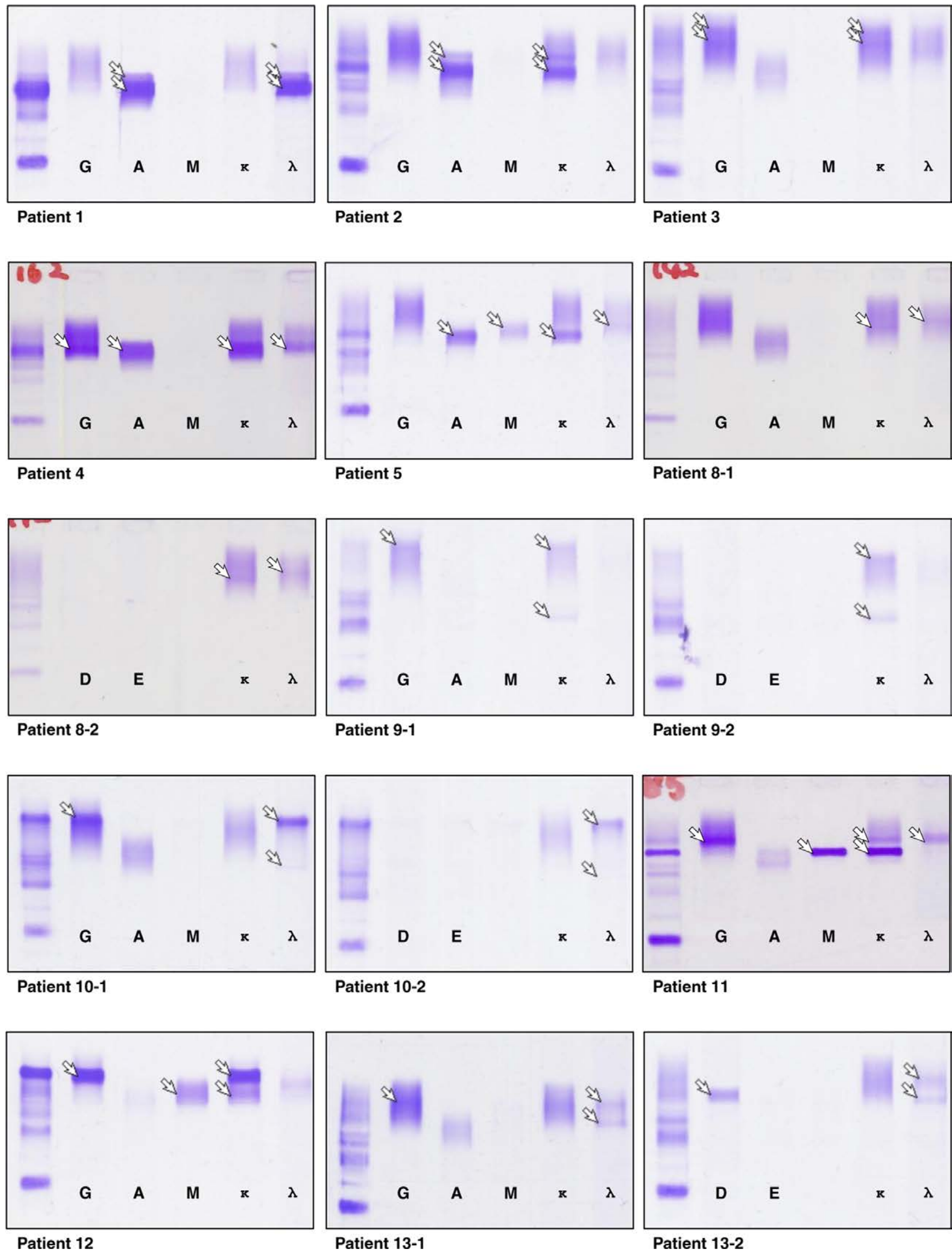
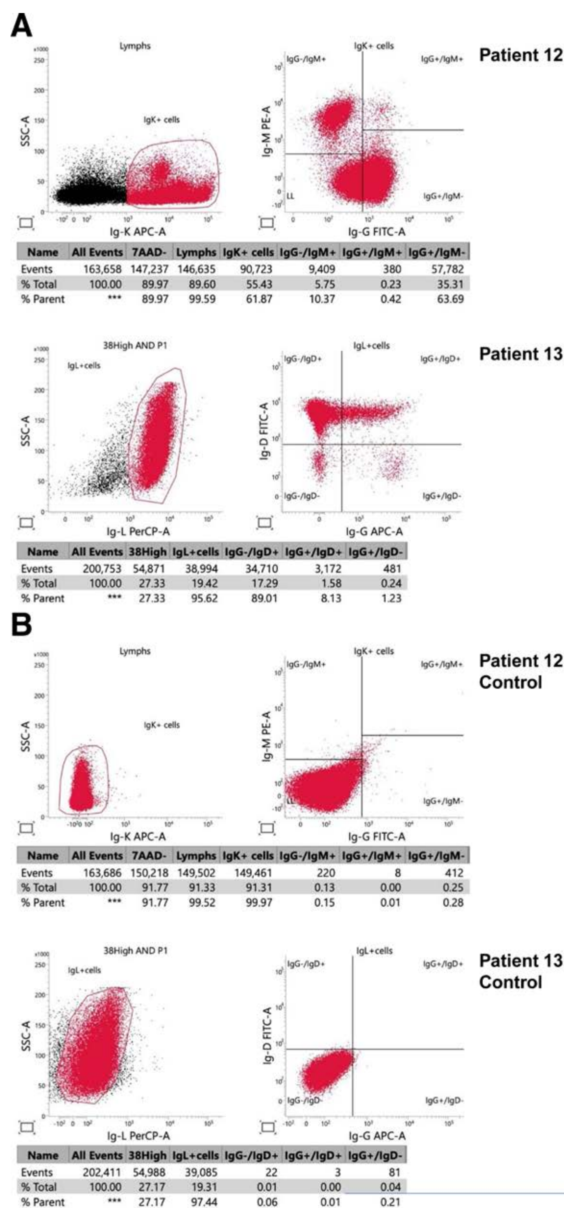


Figure 1. Immunofixation results of biclonal gammopathy patient cohort. White arrows indicate M proteins.

monoclonal mouse anti-human IgD/FITC, clone IA6-2 (BD Biosciences). Following incubation, cells were visualized using a fluorescent microscope, equipped with necessary fluorescent filters, and images were acquired with a digital camera.

2.5. Immunohistochemical staining

Expression of AICDA in sections of bone marrow was assessed by immunohistochemistry using the standard protocol. In brief, following heat-induced antigen retrieval at 95°C to 98°C in water



Name	All Events	7AAD-	Lymphs	IgK+ cells	IgG-/IgM+	IgG+/IgM+	IgG+/IgM-
Events	163,658	147,237	146,635	90,723	9,409	380	57,782
% Total	100.00	89.97	89.60	55.43	5.75	0.23	35.31
% Parent	***	89.97	99.59	61.87	10.37	0.42	63.69

Name	All Events	38High	IgL+cells	IgG-/IgD+	IgG+/IgD+	IgG+/IgD-
Events	200,753	54,871	38,994	34,710	3,172	481
% Total	100.00	27.33	19.42	17.29	1.58	0.24
% Parent	***	27.33	95.62	89.01	8.13	1.23

Name	All Events	7AAD-	Lymphs	IgK+ cells	IgG-/IgM+	IgG+/IgM+	IgG+/IgM-
Events	163,686	150,218	149,502	149,461	220	8	412
% Total	100.00	91.77	91.33	91.31	0.13	0.00	0.25
% Parent	***	91.77	99.52	99.97	0.15	0.01	0.28

Name	All Events	38High	IgL+cells	IgG-/IgD+	IgG+/IgD+	IgG+/IgD-
Events	202,411	54,988	39,085	22	3	81
% Total	100.00	27.17	19.31	0.01	0.00	0.04
% Parent	***	27.17	97.44	0.06	0.01	0.21

Figure 2. (A) Flow cytometry results of bone marrow mononuclear cells. In patient 12 (upper), both 7AAD-negative and Igκ (APC)-positive cells were gated and analyzed with IgM-PE and IgG-FITC antibodies. In patient 13 (lower), both CD38 (PE) and Igλ (PerCP)-positive cells were gated and analyzed with IgD-FITC and IgG-APC antibodies. (B) Flow cytometry results of bone marrow mononuclear cells (control analysis of [A]). In patient 12 (upper), both 7AAD-negative and Igκ (APC)-negative cells were gated and analyzed with IgM-PE and IgG-FITC antibodies. In patient 13 (lower), both CD38 (PE)-positive and Igλ (PerCP)-negative cells were gated and analyzed with IgD-FITC and IgG-APC antibodies.

bath with Tris-EDTA buffer, pH 9.0 for 20 minutes, specimens of decalcified bone marrow core biopsy or aspirate clot were incubated with monoclonal rabbit anti-human AICDA, EPR23436-45 (abcam, Cambridge, MA) at dilution of 1:1000 for 1 hour at room temperature. The immune complex of AICDA/anti-AICDA antibody on the tissue section was detected using the second antibody conjugated with biotin, which was visualized with the 3,3'-diaminobenzidine peroxidase substrate kit, SK-4105 (Vector Labs, Burlington, ON).

2.6. Ethics approval and consent to participate

This study was conducted according to the Declaration of Helsinki and was approved by the ethics committees of Toyama University Hospital (reference number R2021124). Written informed consent was obtained from all patients prior to study participation.

3. Results

3.1. BG patient cohort

Of the 172 patients with M-protein, 13 (7.6%) were suspected to have BG (Fig. 1) (Table 1). The average age of these13 patients was 71 years (53–92), with 8 males and 5 females. The patients' diagnoses were as follows: DLBCL in 1, MALT in 1, MZL in 1, MGUS in 4, and MM in 6. The sum of MGUS and MM accounted for 77.0% (10/13) of the total. Three patients had the same isotypes for both HC and LC (patients 1, 2 and 3). Patients 1 and 2 had IgA isotype, and patient 3 had IgG isotype. Patient 3 had a 13-year history of MM without treatment. During this period, immunofixation analyses were performed a total of 47 times, and the second M protein was detected only once. There were 2 patients with different isotypes for both HC and LC, so-called true BG (patients 4 and 5). One patient had the same HC and different LC isotypes (patient 6). Four patients had Bence-Jones protein (BJP) as one or both M proteins (patients 7, 8, 9, and 10). Patient 11 had IgM-κ as the first M protein, but it was impossible to determine whether the LC of the second M protein (an IgG isotype), was κ or λ. Patients 12 and 13 had BG of different HC and the same LC isotypes, and were the subjects of our present study.

3.2. Detection of a double positive cell population

M protein expression in tumor cells was analyzed by both flow cytometry (Fig. 2A) and immunofluorescence (Fig. 3). Flow cytometric analysis of BM MNCs from patient 12 demonstrated a predominance of IgG+/IgM– cells (63.7%) over IgG–/IgM+ cells (10.4%) in Igκ-positive cells (Fig. 2A). In addition, a double positive IgG+/IgM+ cell population was clearly identified (0.4%) (Fig. 2A); however, there were no double positive cells in the control Igκ-negative cells (Fig. 2B). In patient 13, while the dominant M protein was IgG (Fig. 1), IgG–/IgD+ cells (89.0%) were overwhelmingly predominant over IgG+/IgD– cells (1.2%) in Igλ-positive cells (Fig. 2A). In addition, double positive IgG+/IgD+ cells were more clearly detected in this patient (8.1%) (Fig. 2A) than in patient 12; however, there were no double positive cells in the control Igλ-negative cells (Fig. 2B). In addition to flow cytometry, immunofluorescence analysis was also performed (Fig. 3). For the BM MNCs from patient 12, IgG-positive cells were stained red and IgM-positive cells were stained green. Although the majority of cells were positive for IgG alone or IgM alone, a small number of cells clearly exhibited yellow fluorescence, indicating double positivity. In addition, in patient 13, IgG-positive cells were stained red and IgD-positive cells were stained green. Similarly, a small number of double positive yellow cells were detected in this sample.

3.3. Involvement of CSR mechanism in BG etiology

Bone marrow core biopsy or aspirate clot specimens from patient 12 and patient 13 were immunostained for activation induced cytidine deaminase (AICDA) (Fig. 4A). Some of the CD20-positive lymphoma cells infiltrating the bone marrow of patient 12 expressed AICDA, while some of the CD138 positive myeloma cells in the bone marrow of patient 13 were positive for AICDA. As negative controls, we examined 1 case of MZL (negative control 1) and 2 cases of MM (negative control 2 and 3) without BG, and detected no AICDA-positive cells in these samples. As positive controls, we used tonsil and lymph node tissues from individuals without hematological malignancies as shown in Figure 4B.

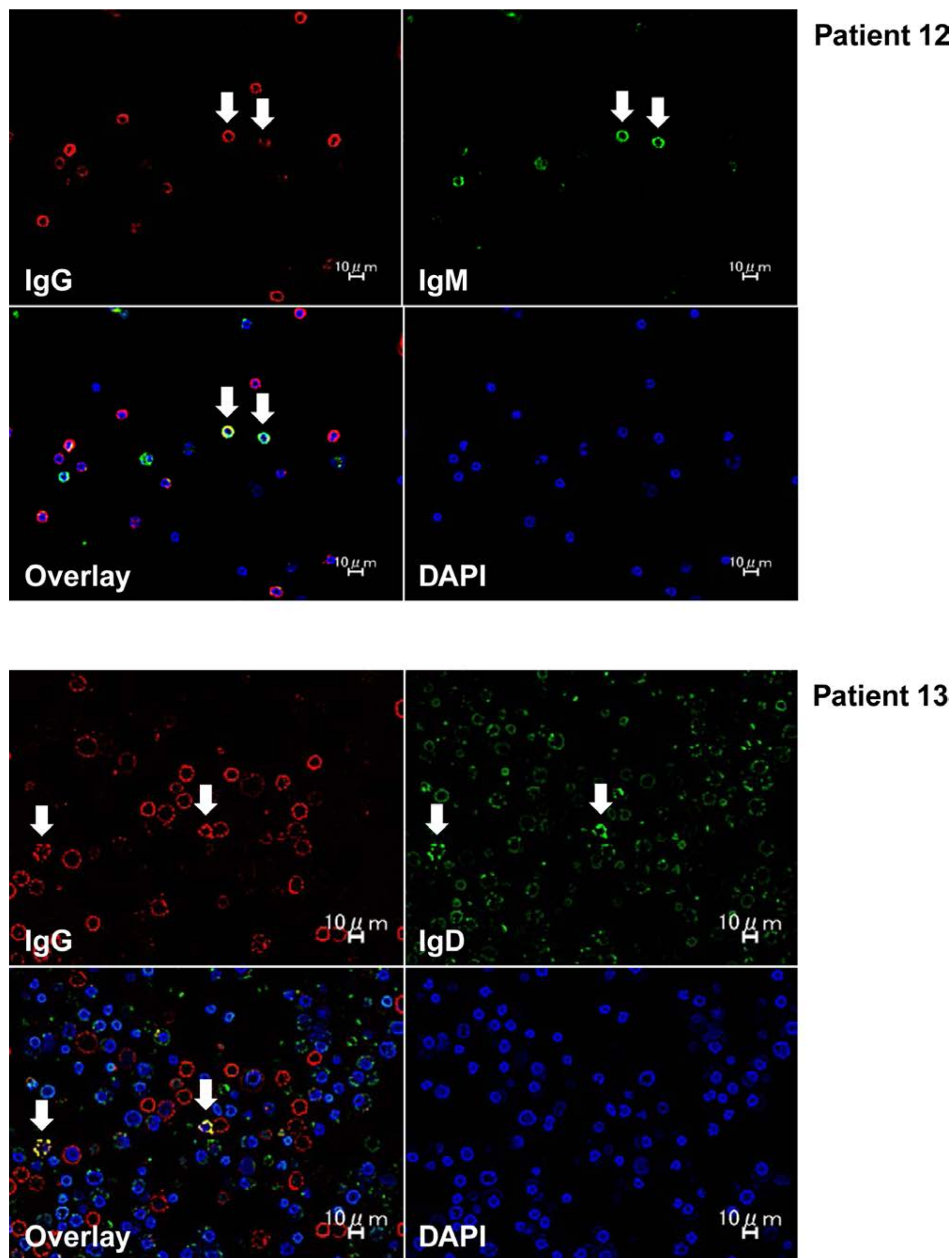


Figure 3. Immunofluorescence results of bone marrow mononuclear cells. In patient 12 (upper), IgG-positive cells were stained red and IgM-positive cells were stained green. In patient 13 (lower), IgG-positive cells were stained red and IgD-positive cells were stained green. The yellow fluorescence indicates double positivity.

4. Discussion

There have been several reports on the frequency of BG in patients with M protein. Sharma et al reported a rate of 1.6% (15/916).[8] Meanwhile, Kyle et al reported a rate of 1.5% (57/3447),[9] but since they did not include the BG patients with Bence Jones proteinemia, the actual rate may be higher. The frequency reported by Guastafierro et al was 4% (49/1214),[10] but they excluded cases of double IgA-κ and double IgA-λ, citing the possibility of false positives due to IgA glycosylation and its tendency to polymerize. They also excluded cases without clear confirmation of a monoclonal component. If we follow their approach and exclude the 2 cases of double IgA in our series (patients 1 and 2) and the 2 cases without clear confirmation (patients 3 and 11), the frequency observed in our study was 5.2% (9/172), similar to the rate seen by Guastafierro et al.

There is a long-standing controversy over whether or not there are malignant B-cells that produce 2 M proteins simultaneously.[2–6] On this issue, we were able to clearly demonstrate the existence of double positive cells that simultaneously produced 2 M proteins with different HC, in addition to 2 distinct cell groups each producing a separate M protein. We hypothesized that these double positive cells may be a manifestation of the process of CSR. Specifically, they are in the process of switching from the IgM to IgG isotype in patient 12 and from the IgD to IgG isotype in patient 13. In view of these observations, we focused our attention on AICDA, given its central role in CSR.

AICDA is expressed in antigen-stimulated mature B cells and is essential to both somatic hypermutation (SHM) and CSR. Mechanistically, AICDA has dual functions in the immunoglobulin locus, that is, DNA cleavage and recombination. DNA cleavage in the switch (S) and variable (V) regions initiates CSR and

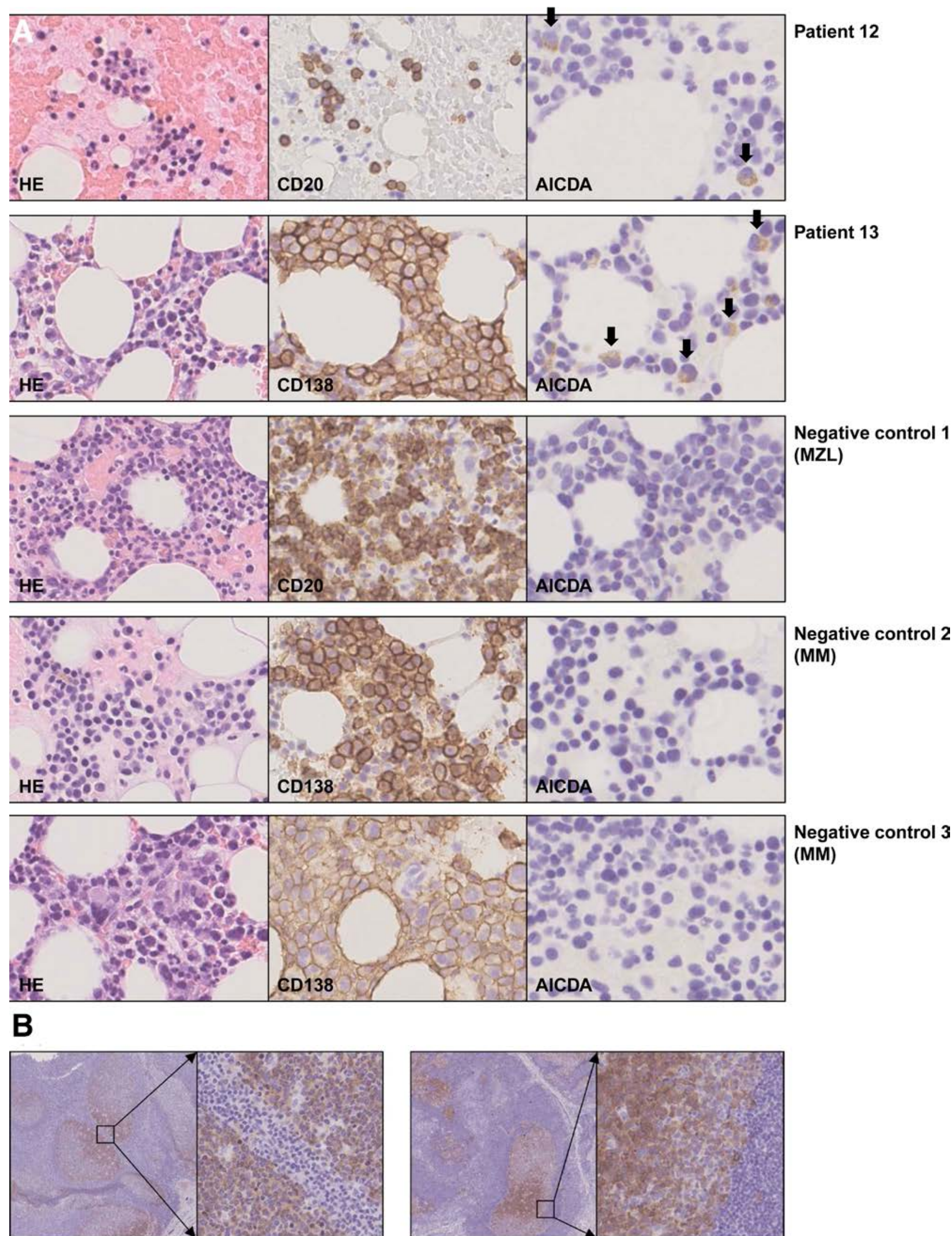


Figure 4. (A) Immunostaining results of bone marrow core biopsy or aspirate clot. MZL patients (patients 12 and negative control 1) were stained with anti CD20 antibody and anti AICDA antibody. MM patients (patients 13 and negative control 1 and 2) were stained with anti CD138 antibody and anti AICDA antibody. (B) Immunostaining results of positive controls with anti AICDA antibody (left) tonsil and (right) lymph node tissues from individuals without hematological malignancies.

SHM, respectively. In SHM, error-prone DNA synthesis introduces point mutations during single-strand break (SSB) repair. During CSR, SSBs located at donor and acceptor S regions are converted to double-strand breaks, followed by recombination between the 2 double-strand breaks ends to complete CSR.[11]

Our immunostaining analysis clearly demonstrated expression of AICDA in samples from patients 12 and 13, compared with negative controls, findings that support our hypothesis that double positive cells are undergoing CSR. We also attempted to confirm our findings regarding AICDA expression in double positive cells by flow cytometric isolation. However, we were unsuccessful in this effort due to insufficiency of samples and reduced cell viability during the experimental process. A potential solution to this issue may be the tumor cell cloning technique used by Bakkus et al.[2] While these authors did not detect double positive cells among the 16 clones examined, since our quantitative study by flow cytometry demonstrated only a low percentage of double positive cells, the number of clones to be examined likely needed to be much higher to detect the few double positive cells.

The issue of whether 2 cell populations producing different M proteins are equally or differentially sensitive to chemotherapy has important clinical relevance. Mullikin et al[12] reviewed 13 BGs that had responded to treatment and then relapsed and found that the M protein that was dominant prior to chemotherapy was still dominant following relapse, suggesting that there was no difference in chemotherapy sensitivity between the 2 cell populations. On the other hand, Gallart et al[6] reported a case of BG in which the dominant M protein switched prior to therapy and following treatment, implying that the 2 cell populations have different chemotherapy sensitivity. Both our patient 12 and 13 responded well to treatment, but the IgG that was

originally the dominant M protein remained. If relapse occurs, we plan to examine whether the IgG isotype remains dominant.

We do acknowledge that there are limitations with our findings. One is that we did not demonstrate clonal relatedness by sequence analysis technique used by Tschumper et al.[3] Another is that the possible dual positivity of the background normal cells[13] could not be formally ruled out by our experimental procedures.

In conclusion, our present study demonstrates the existence of malignant B-cells that simultaneously produce 2 M proteins in BG patients, associated with AICDA expression, a key molecule in regulating CSR. Importantly, our paper is the first to evaluate the relationship between BG and AICDA.

Author contributions

All authors read and approved the final article. We gratefully thank Ms. Toyomi Kozawa for her help in performing this study.

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