Lower number of 5-hydroxymethylcytosine-expressing cells in plasma cell myeloma than in reactive plasma cell hyperplasia: A useful immunohistochemical approach for identification of neoplastic plasma cells

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Running title: 5-hmC expression in myeloma and reactive plasma cells

Abstract

Plasma cell myeloma (PCM) is a hematological malignancy defined by aberrant proliferation of plasma cells in the bone marrow. For the diagnosis of PCM, immunostaining of light chains or surface marker analyses by flow cytometry is needed but sometimes difficult. Aberrant methylation at the carbon-5 position of cytosine results in 5-methylcytosine (5-mC), which is a well-known epigenetic hallmark of cancer in mammals. 5-mC is oxidized to 5-hydroxymethylcytosine (5-hmC), and low 5-hmC levels occur in several cancers and hematopoietic malignancies. We compared the expression of 5-hmC by immunohistochemistry (IHC) in neoplastic plasma cells in 31 PCM cases and in non-neoplastic plasma cells in 14 benign reactive lesions. The mean percentage of 5-hmC-positive cells was 7.8% and 81.5% in PCM and plasma cell hyperplasia, respectively. Thus, the frequency of 5-hmC-positive cells in PCM specimens was significantly lower than that in reactive plasma cell hyperplasia ($p < 0.001$ by Student's t-test). IHC of 5-hmC is a useful method for differentiating neoplastic plasma cells from non-neoplastic plasma cells in reactive lesions even in tiny tissue samples unsuitable for flow cytometric analyses or for immunoglobulin light chain IHC.

Introduction

Plasma cell myeloma (PCM) is a hematological malignancy defined by aberrant proliferation of plasma cells in the bone marrow. To diagnose PCM, monoclonal gammopathy from neoplastic plasma cells needs to be identified [1]. It is difficult to histopathologically identify immunoglobulin monoclonality, but an imbalance of antibody light chain, such as more than 5:1 lambda chain over kappa chain or 10:1 kappa chain over lambda chain, is generally accepted as an index of monoclonal proliferation in plasma cells. However, judging light chain immunohistochemical staining is sometimes difficult because of high background staining from serum immunoglobulins in tissue specimens. Additionally, the immunohistochemical approach to analyze the imbalance of light chains cannot be applied to the diagnosis of small tissue specimens containing a small aggregate of plasmacytoid cells such as biopsy samples during chemotherapy. Recent advances in flow cytometry technologies can distinguish non-neoplastic plasma cells from myeloma cells because of different immune phenotypes such as CD38+/CD56+/CD19− in most myeloma cells and CD38+/CD56−/CD19+ in non-neoplastic plasma cells. In addition to these immunophenotypic analyses, the imbalance between cytoplasmic kappa and lambda immunoglobulin light chains enables identification of neoplastic plasma cell populations. However, judging an imbalance between kappa and lambda light chains is limited when the number of neoplastic cells is low [2].

Genomic analysis has revealed genome-wide hypomethylation and specific hypermethylation of tumor suppressor genes in PCM cases [3, 4]. In particular, genome-wide

hypomethylation occurs during the progression of monoclonal gammopathy of undetermined significance (MGUS) to PCM [3]. However, because genome analysis requires advanced molecular biological techniques, it is difficult to perform in patients in routine clinical settings, and small specimens impose technical limitations. Aberrant methylation at the carbon-5 position of cytosine results in 5-methylcytosine (5-mC), which is a well-known epigenetic hallmark of cancer in mammals. 5-mC is oxidized to 5-hydroxymethylcytosine (5-hmC) through a family of Ten Eleven Translocation (TET) proteins, Fe2+, and α-ketoglutaric acid demethylation [5, 6]. 5-hmC may have a role in epigenetic regulation because hydroxymethyl radicals are involved in switching gene expression on and off [6]. Decreased 5-hmC levels contribute to the development and progression of several human solid tumors (malignant melanoma [7], prostate cancer [8], colorectal cancer [8], lung cancer [8], and hepatocellular carcinoma [9]) and hematological malignancies (acute myeloid leukemia [10], myelodysplastic syndrome [10], and several T-cell lymphomas [11]) and can be observed in some cases by immunohistochemistry (IHC) [7-10]. Furthermore, decreased 5-hmC expression leads to poor prognosis of patients with malignant melanoma [7], hepatocellular carcinoma [9], and myelodysplastic syndrome [10]. However, there are no reports of 5-hmC immunohistochemical expression in PCM cells.

We examined the expression of 5-hmC in PCM cells and plasma cells from benign reactive lesions by IHC.

Materials and Methods

Cases and tissue samples

Consecutive histopathological slides of bone marrow aspirates in 31 cases of PCM were retrieved from the pathology files at the Department of Laboratory Medicine, Matsumoto Medical Center Matsumoto Hospital, Matsumoto, Japan between 2010 and 2015. Lymph node tissues in 14 cases of reactive plasma cell hyperplasia were also used. Patients with reactive plasma cell hyperplasia were restricted to those who immunohistochemically exhibited CD138-positive plasma cell hyperplasia, did not have an imbalance in immunostaining of immunoglobulin light chains, and had not developed any hematological tumors, such as malignant lymphoma, during an at least 2-year follow-up period after biopsy. The characteristics of the patients enrolled are presented in Tables 1 and 2. In PCM patients, there were 16 male and 15 female patients, and the median age was 72.1 years (range 46-90 years). In patients with reactive plasma cell hyperplasia, there were 7 male and 7 female patients, and the median age was 60.6 years (range 17-85 years). All tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial tissue sections were prepared for hematoxylin and eosin (HE) staining and IHC. This study was approved by the ethics committee of the National Hospital Organization Matsumoto Medical Center, Matsumoto, Japan.

Immunohistochemistry

IHC was performed using the following mouse monoclonal antibodies: anti-CD138 (clone M115, Dako, Glostrup, Denmark), anti-human immunoglobulin light chains kappa

(clone A0418, Dako) and lambda (clone A0417, Dako), and the rabbit polyclonal antibody: anti-5-hmC (No.39770, Active Motif, Carlsbad, CA, USA). Antigen retrieval for CD138 and kappa and lambda light chain was performed by heating sections in 10 mM EDTA buffer (pH 8.0) in a microwave oven at 600 W for 30 minutes. For 5-hmC, antigen retrieval was performed by heating sections in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 15 minutes, and slides were subsequently placed in 2 N HCl for 30 minutes, rinsed in distilled water, and placed in 100 mM Tris-HCl (pH 8.5) for 10 minutes. Incubation with primary antibodies was performed for 1 hour at room temperature, and subsequent signal development was performed using the immunoenzyme polymer method with 3,3'-diaminobenzidine as a chromogen.

Cells with brown nuclear staining were assessed as 5-hmC-positive regardless of staining intensity. The numbers of 5-hmC-positive cells and CD138-positive cells were counted in the same field in samples for both MM and reactive plasma cell hyperplasia. At least 500 CD138-positive cells were counted for each tissue sample.

Statistical analysis

Statistical analysis was performed using a Student's *t*-test.

Results

HE staining of the bone marrow of PCM patients revealed a nodule-forming proliferation of neoplastic plasma cells with eccentric swelling nuclei and mildly basophilic cytoplasm, and various degrees of decreased normal hematopoietic foci (Fig. 1A). For

CD138 IHC, PCM cells had strong staining of cytoplasmic membranes (Fig. 1B). For immunoglobulin light chains, positive staining for either kappa or lambda chain was observed in 16 and 15 cases, respectively (Table 1). On the other hand, HE staining of lymph node specimens of reactive plasma cell hyperplasia revealed small-to-moderate nodular clusters of cells with relatively small eccentric nuclei and mildly basophilic cytoplasm, distributed in medullary sinuses and paracortex (Fig.1D). For CD138 IHC, the non-neoplastic cells had strong staining of cytoplasmic membranes (Fig. 1E).

In the IHC for 5-hmC of the bone marrow of PCM patients (Fig. 2A,B), the strong nuclear staining of the normal mature hematopoietic cells was used as an internal control [10], and using it as an indicator, we stopped signal generation. In the IHC for 5-hmC of the lymph nodes (Fig. 2C,D), the negative staining of most of lymphoid cells in the germinal center and the strong positive staining of lymphoid cells in the mantle zones were used as an internal control [12], and using it as an indicator, we stopped signal generation. The frequency of 5-hmC-positive plasma cells in PCM specimens was lower than that in specimens of reactive plasma cell hyperplasia (Fig. 1C and 1F). The mean percentage of 5-hmC-positive cells was 7.6% (range 2.0-15.9%) and 81.5% (range 71.9-88.0%) for PCM and plasma cell hyperplasia, respectively (Tables 1 and 2). A Student's t-test revealed a significant difference in the percentage of 5-hmC-positive plasmacytoid cells between PCM and reactive plasma cell hyperplasia ($p < 0.001$).

Discussion

In the current study, with setting up internal controls in the immunohistochemistry of 5-hmC as mentioned above, we interpreted brown nuclear staining regardless of staining intensity as 5-hmC positive cells. Even without evaluating the difference in staining intensity, the frequency of 5-hmC positive cells in PCM specimens was significantly lower than that in specimens of reactive plasma cell hyperplasia.

The following disease progression model in plasma cell neoplasms has been proposed in recent studies: post-germinal center cells, which differentiate into plasma cells, are received several genetic hits and develop into MGUS, the first stage of neoplastic transformation of plasma cells. Transformed plasma cells become proliferative through further genetic alterations, and MGUS progresses into smoldering multiple myeloma, PCM, and plasma cell leukemia (PCL) [13, 14]. In addition, various factors, such as adaptation to the bone marrow microenvironment and resistance to treatment, are involved [15]. Thus, PCM is a heterogeneous disease because individual patients have different genetic and cytological makeups, and the progression of clinical malignancy is unique [16, 17]. Epigenetic changes in PCM, including DNA methylation and alterations in microRNA, have been examined [4, 18]. DNA microarray analyses in MM found global hypomethylation of DNA and gene-specific hypermethylation, similar to those in other malignancies [3, 4]. Hypomethylation in genes, especially for genes that influence the cell cycle, frequently occurs during the progression from MGUS to PCM, whereas gene-specific hypermethylation occurs in genes related to cell adhesion along with global hypomethylation during the progression to PCL [3, 4]. Therefore,

epigenetic dysregulation is thought to be involved in the development and progression of plasma cell neoplasms. Decreased expression of 5-hmC in PCM may reflect insufficient demethylation of genes during the progression to PCM. The degree of decreased 5-hmC expression (percent change in 5-hmC-positive cells) varied among individual PCM cases, which may be related to the genetics and clinical malignancy of individual PCM cases.

Genome-wide hypomethylation in PCM is associated with poor prognosis [19]. On the other hand, an inhibitor of DNA methylation was approved for the treatment of PCM in 2015. The DNA methylation inhibitor has an anti-tumor effect by inhibiting histone deacetylase, which is involved in DNA methylation pathways and aberrant methylation of cytosine to 5-mC. The degree of aberrant DNA methylation may be an important indicator for determining prognosis and selecting treatment for PCM. Therefore, immunohistochemical expression of 5-hmC can help predict the degree of aberrant DNA methylation in PCM cells.

We evaluated the expression of 5-hmC in neoplastic and reactive plasma cells by IHC. Decreased expression of 5-hmC was observed in CD138-positive neoplastic plasma cell foci in PCM patients. Thus, IHC for 5-hmC is a useful method for differentiating neoplastic plasma cells from non-neoplastic plasma cells in reactive lesions even in tiny tissue samples unsuitable for flow cytometry analyses and IHC of immunoglobulin light chains.

Acknowledgments

Compliance with ethical standards

This study was approved by the medical ethics committee at the National Hospital Organization Matsumoto Medical Center (Project #27-11 was approved on August 31, 2015).

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 Summary of the clinical characteristics, the number of immuno-positive cells, and the percentages of 5-hmC/CD138 immuno-positive cells in 31 cases of multiple myeloma.

Case no.	Age(years)	Sex	light chain restriction	Number of immuno-positive cells		5-hmC+cells/CD138+cells (percentage)
				CD138+cells	5-hmC+cells	
M-1	66	M	κ	584	54	9.3
M-2	71	M	λ	501	50	10.0
M-3	62	M	κ	642	72	11.2
M-4	78	M	λ	508	13	2.6
M-5	86	F	λ	510	63	12.4
M-6	68	F	λ	533	49	9.2
M-7	73	M	κ	724	38	5.3
M-8	84	M	κ	507	81	15.9
M-9	55	F	κ	523	45	8.6
M-10	78	F	λ	550	58	10.5
M-11	65	M	κ	529	11	2.0
M-12	54	M	λ	531	34	6.4
M-13	73	F	λ	556	47	8.5
M-14	82	F	λ	733	41	5.6
M-15	82	F	κ	540	27	5.0
M-16	70	F	κ	712	31	4.4
M-17	69	F	κ	734	58	7.9
M-18	75	F	κ	880	66	7.5
M-19	69	F	κ	862	81	9.4
M-20	69	M	κ	580	66	11.4
M-21	90	F	κ	540	17	3.2
M-22	73	M	κ	816	40	4.9
M-23	72	F	λ	609	52	8.5
M-24	89	M	λ	591	68	11.5
M-25	75	M	λ	509	33	6.5
M-26	59	F	κ	668	14	2.0
M-27	75	M	λ	711	68	9.6
M-28	46	F	λ	713	13	1.8
M-29	79	M	λ	646	32	5.0
M-30	72	M	λ	605	86	14.2
M-31	75	M	κ	509	54	10.6

Table 2 Summary of the clinical characteristics, the number of immuno-positive cells, and the percentages of 5-hmC/CD138 immuno-positive cells in 14 cases of reactive lymphoid hyperplasia.

| Case no.	Age(years)	Sex	Site of the lymph nodes	Number of immuno-positive cells		5-hmC+/CD138+cells （percentage）
				CD138+cells	5-hmC+cells	
R-1	34	M	cervical	727	639	87.9
R-2	17	M	cervical	552	456	82.6
R-3	81	M	supraclavicular	543	466	85.8
R-4	53	F	inguinal	550	405	73.6
R-5	77	M	cervical	552	486	88
R-6	49	F	inguinal	764	549	71.9
R-7	62	M	para-aorta	694	593	85.4
R-8	71	F	cervical	519	414	79.8
R-9	67	F	inguinal	505	413	81.8
R-10	74	F	axillary	576	480	83.3
R-11	55	F	axillary	549	409	74.5
R-12	40	M	submaxillary	564	436	77.3
R-13	85	M	abdominal	815	667	81.8
R-14	83	F	abdominal	673	587	87.2

Figure 1
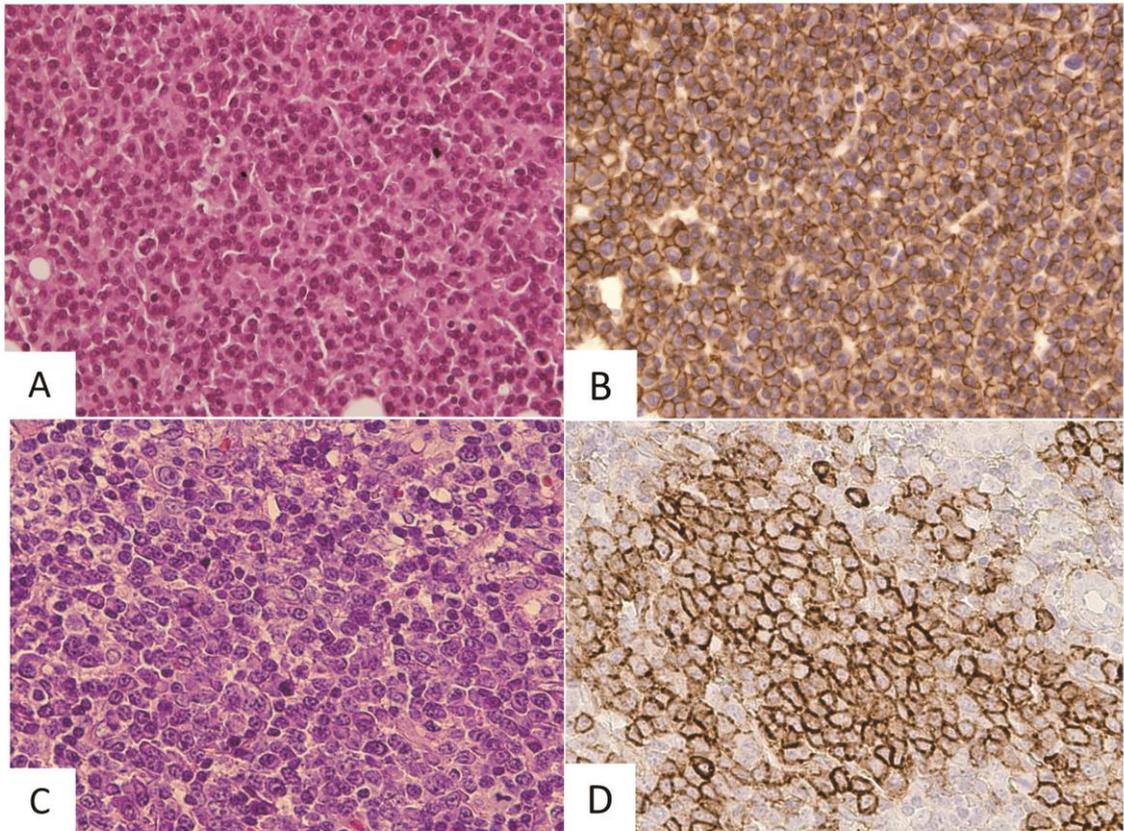
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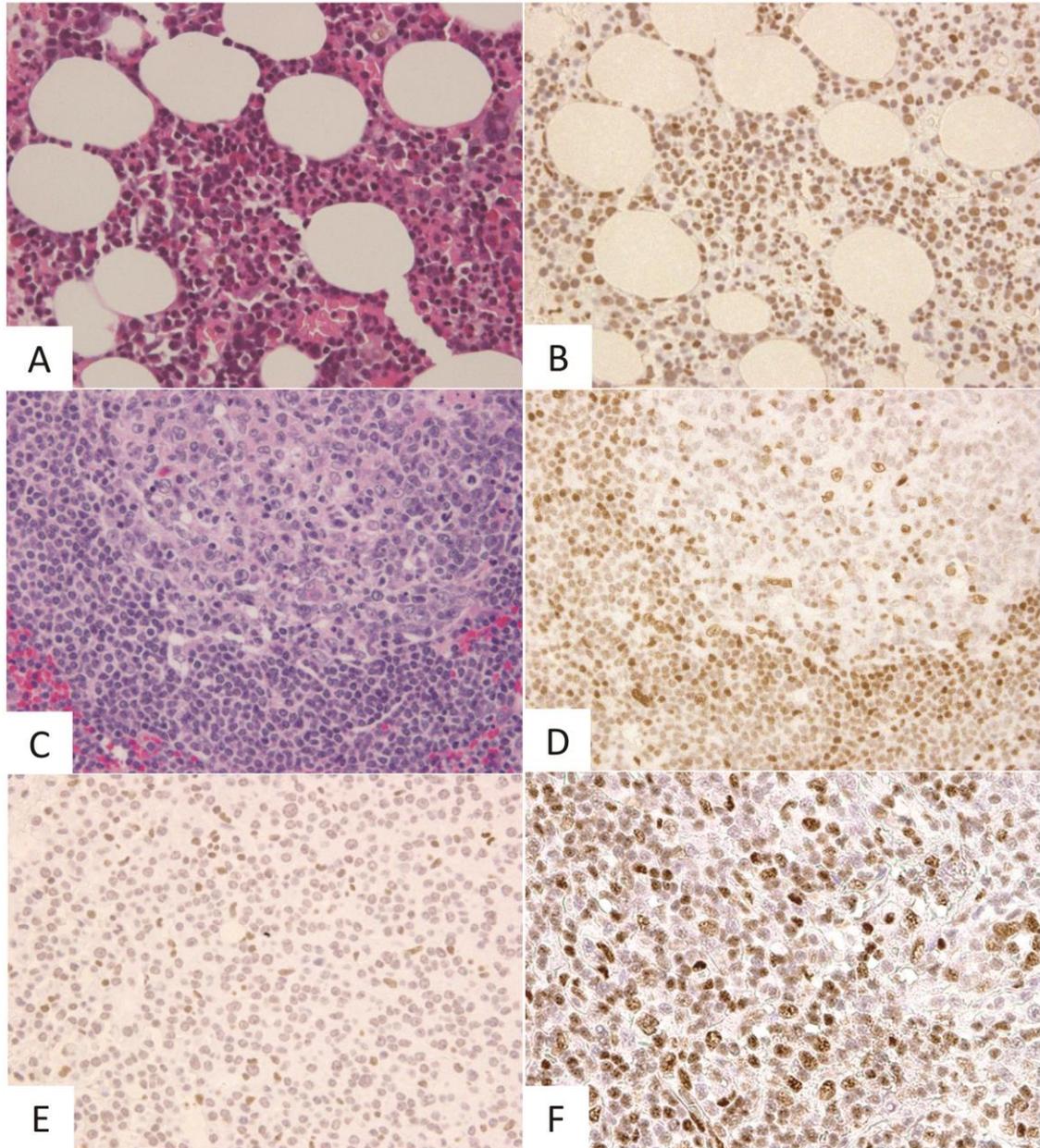
Legends to figures

Fig. 1 Representative images of histopathology and immunohistochemistry of CD138 and 5-hmC of plasma cell myeloma (A-C) and reactive plasma cell hyperplasia (D-F). A and D: HE stain; B and E: immunostaining for CD138; C and F: immunostaining for 5-hmC. Original magnification: 400×.

Fig. 2 Representative images of histology and immunohistochemistry of 5-hmC of bone marrow (A,B) and lymph node (C,D). A and C: HE stain; B and D: immunostaining for5-hmC;. Original magnification: 400×(A,B) and 200×(C,D).