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Triple immunostaining demonstrates the possible existence of segregated-nucleus-containing atypical monocytes in human primary myelofibrosis bone marrow: a case report

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Abstract

Background Segregated-nucleus-containing atypical monocytes have recently been identified in mice. Segregated-nucleus-containing atypical monocytes are thought to originate from the bone marrow and induce fibrosis in the drug-injured lung. The Lyc6c^{-} murine monocyte subset is the counterpart to human CD14^{-}CD16^{++} non-classical monocytes; however, the human counterpart to murine segregated-nucleus-containing atypical monocytes has not yet been identified. Primary myelofibrosis is a well-known disease of progressive marrow fibrosis, and atypical megakaryocytes are thought to be closely related to fibrosis in primary myelofibrosis bone marrow. However, recently, monocytes have been reported to play an important role in marrow fibrosis in primary myelofibrosis. We speculated that, if there is a human counterpart to murine segregated-nucleus-containing atypical monocytes, it would present the same markers as murine segregated-nucleus-containing atypical monocytes, such as CD14^{-}CD16^{+} macrophage-1 antigen (CD11b/CD18 complex)$^{+}$, MSR1^{+}, and CEACAM1^{+}, and it might exist in the bone marrow of patients with primary myelofibrosis.

Case presentation A 74-year-old Japanese male visited our hospital for clinical follow-up after total prostatectomy for prostatic cancer. Anemia, thrombocytosis, and elevated lactate dehydrogenase were suddenly observed in a periodic examination. CALR mutation type 2 (p.K385fs*47) was observed. The histological features of the patient's bone marrow were consistent with fibrotic primary myelofibrosis. We immunohistochemically studied the bone marrow in an attempt to identify a human counterpart to murine segregated-nucleus-containing atypical monocytes. We detected a few CD16^{+}MSR1^{+}CEACAM1^{+} cells, but not CD14^{+}MSR1^{+}CEACAM1^{+} cells, by triple immunostaining. The patient is in a good condition and does not require treatment for primary myelofibrosis.

Conclusion There is a possibility that human segregated-nucleus-containing atypical monocytes exist in the bone marrow of primary myelofibrosis patients and might be related to marrow fibrosis.

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Background

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm (MPN) of progressive bone marrow fibrosis that often results from *JAK2* V617F, *CALR*, or *MPL* mutations. Among the three mutations, *CALR* mutations cause PMF, essential thrombocythemia (ET) and myelodysplastic (MDS)/MPN neoplasm with *SF3B1* mutation and thrombocytosis (MDS/MPN-SF3B1-T) according to the *WHO Classification of Haematolymphoid Tumours*," Fifth Edition [1]. Among the diseases caused by *CALR* mutations, it is likely that fibrosis occurs in the bone marrow [1]. However, the most important characteristic of PMF is atypical megakaryocytes (MgKs), which have hyperchromatic nuclei and atypical localization [2]. However, no atypical MgKs have been reported in the bone marrow of patients with ET and MDS/MPN-SF3B1-T. Of note, the most important characteristic of MDS/MPN-SF3B1-T is the presence of ring sideroblasts in the bone marrow [4]. The incidence of fibrotic PMF is lower than that of ET [2, 3], whereas the incidence of MDS/MPN-SF3B1-T is unknown [4].

It was thought that atypical MgKs in the PMF contribute to bone marrow fibrosis [5]; however, it has recently been shown that CD45/procollagen-double-positive fibrocytes, which are derived from $CD14^+$ monocytes, could be related to bone marrow fibrosis in patients with PMF [6]. Moreover, it has been reported that both neoplastic PMF monocytes and healthy marrow monocytes form MgK colonies *in vitro*, meaning that marrow monocytes could potentially differentiate into MgKs [7]. These studies suggest that $CD14^+$ marrow monocytes could contribute to marrow fibrosis in PMF.

Satoh *et al.* identified a unique subset of murine monocytes in the bleomycin-injured fibrotic murine lung. These cells demonstrated morphological and proteomic features common to both monocytes and granulocytes. Therefore, they were named segregated-nucleus-containing atypical monocytes (SatMs). Satoh *et al.* also discovered that SatMs originate from the bone marrow and activate lung fibroblasts to induce fibrosis in bleomycin-induced lung injury, and that SatMs present the following staining pattern: $Ly6C^-Mac1^+F4/80^-MSR1^+CEACAM1^+$ [8]. Before SatMs were identified, it was unknown whether murine $Ly6C^{low/-}$ monocytes could differentiate into prefibrotic macrophages or promote tissue remodeling [9]. Therefore, SatMs are the first $Lyc6c^{low/-}$ monocytes to be identified that could induce tissue fibrosis.

Accordingly, we hypothesized that there might be human SatMs in the bone marrow of humans with PMF. Human monocytes are classified into three groups: $CD14^{++}CD16^-$, $CD14^{low/-}CD16^{++}$, and $CD14^{++}CD16^+$ [10]. Mouse monocytes are classified as either $Ly6C^{high/+}$ or $Ly6C^{low/-}$ [9]. The human counterpart to murine $Ly6C^{low/-}$ monocytes is reported to be $CD14^{low/-}CD16^{++}$ monocytes [10, 11]. As SatMs show $Ly6C^-Mac1^+F4/80^-CEACAM1^+MSR1^+$, and F4/80 is a mouse-specific monocyte marker, human SatMs, if they exist, should show the following characteristics: $CD14^{low/-}CD16^+Mac1^+CEACAM1^+MSR1^+$. Here, we immunohistochemically investigated whether human SatMs exist in the bone marrow of a patient with PMF.

Case presentation

Clinical summary

A 74-year-old Japanese male visited our hospital for clinical follow-up after total prostatectomy for prostatic cancer. His nutritional condition was good (height, 155 cm; weight, 58 kg). His peripheral blood counts were within normal ranges. Hemoglobin (Hb) was 14.8 g/dL (normal range at our hospital is 13.7–16.8 g/dL), and lactate dehydrogenase (LDH) was 250 U/L (normal range: 142–250 U/L). A total of 5 years postoperatively, at the age of 79 years, periodic examination of peripheral blood revealed anemia [Hb, 12.6 g/dL (anemia was decided according to the WHO definition, https://iris.who.int/handle/10665/85839)], mild thrombocytosis [435×10^9/L (normal range at our hospital is $100–400\times10^9$/L)], and elevated LDH (561 U/L). His white blood cell (WBC) count was within the normal range. His peripheral blood count and LDH concentration were stable during periodic monitoring of his condition. Another 8 years after his first visit, at the age of 82 years, the patient experienced a nasty cough. Anemia (Hb, 12.1 g/dL), mild thrombocytosis (448×10^9/L), and the normal WBC count had not changed, and the LDH concentration was high (598 U/L). We thoroughly examined his condition. Computed tomography showed mild interstitial pneumonia in both lungs, and the spleen volume was approximately 640 mL. Some peripheral erythrocytes showed dacryocytes and schistocytes. His nucleated blood cells presented a *CALR* mutation type 2 (p.K385fs*47). Neither the *JAK2* V617F mutation nor the *MPL* W515L/K mutation were detected. Cytogenetic analysis of the

patient's bone marrow cells showed 46, XY, del (20) (q11.2q13.3) in 20 of 20 cells. According to laboratory, genetic, cytological, and radiological examinations, we suspected he suffered from PMF, ET, or MDS/MPN-SF3B1-T, which necessarily presents ring sideroblasts if molecular analysis of SF3B1 is unavailable and simultaneously shows thrombocytosis with *JAK2* V617F, *MPL* or *CALR* mutation [1]. To determine the diagnosis, we attempted a histopathological examination of the bone marrow. We could not aspirate any marrow particles to analyze the histological features of the bone marrow (dry tap) although cytogenic analysis succeeded. We suspected that fibrosis had occurred in the bone marrow [1, 12], and we harvested a trephine biopsy from the bone marrow. The biopsy specimen showed histological features of fibrotic PMF. A total of 2 years later, his platelet count had declined to normal (275×10^9/L), while his peripheral WBC count, anemia, and blood chemistry, as well as his condition, did not change during the 2 years. At present, 14 years after his first visit, the patient is doing well, and his platelet count is within the normal range. Anemia (Hb, 10 g/dL) and high LDH concentration (616 U/L) remain unchanged. Computed tomography revealed a spleen volume of approximately 900 mL. We have followed his condition and, at present, have decided that treatment for PMF is not necessary yet. The patient's perspective is that he understands his disease and condition, and that no treatment for his PMF is now required. Neither metastasis nor recurrence of prostatic cancer has been found, and no blasts have been observed in his peripheral blood during his clinical course at our hospital. The clinical timeline is shown in Fig. 1.

Histological and immunohistochemical findings

The bone marrow biopsy specimen was fixed with 10% buffered formalin, demineralized under neutral conditions, and embedded in paraffin. We analyzed hematoxylin and eosin (H&E)-stained, silver impregnation-stained, iron-stained, and naphthol ASD–Giemsa-stained sections. The bone marrow was markedly hypercellular with proliferation of atypical MgKs, which had large, hyperchromatic, and bizarre nuclei (Fig. 2A). The atypical MgKs formed clusters in the intertrabecular space and paratrabecular space (Fig. 2A, B). MgK localization in the paratrabecular space was atypical [1] because this is where the differentiation of myeloid cells occurs in normal/healthy bone marrow. The reticulin fibers diffusely proliferated, and parts of the reticulin fibers formed thick collagen fibers (Fig. 2C). No ring sideroblasts were observed. We classified the marrow fibrosis as myelofibrosis (MF)-3 according to the marrow fibrosis grading. These findings are consistent with those of fibrotic PMF [2].

Although macrophage-1 antigen (Mac1) is a complex of CD11b and CD18, CD18 can also combine with CD11a, CD11c, and CD11d [13]. Therefore, we stained for CD11b instead of Mac1. We immunohistochemically identified the expression of CD14, CD16, CD11b, MSR1, and CEACAM1 using Ventana Benchmark GX (Roche Diagnostics, Tucson, AZ, USA). Antigens were retrieved with CC1 alkaline buffer (Roche Diagnostics) for autostaining with the Ventana BenchMark series. To explore the possibility that CD16+CEACAM1+MSR1+cells were present in the PMF bone marrow, we detected CD14, CD16, CD11b, MSR1, or CEACAM1, immunohistochemically

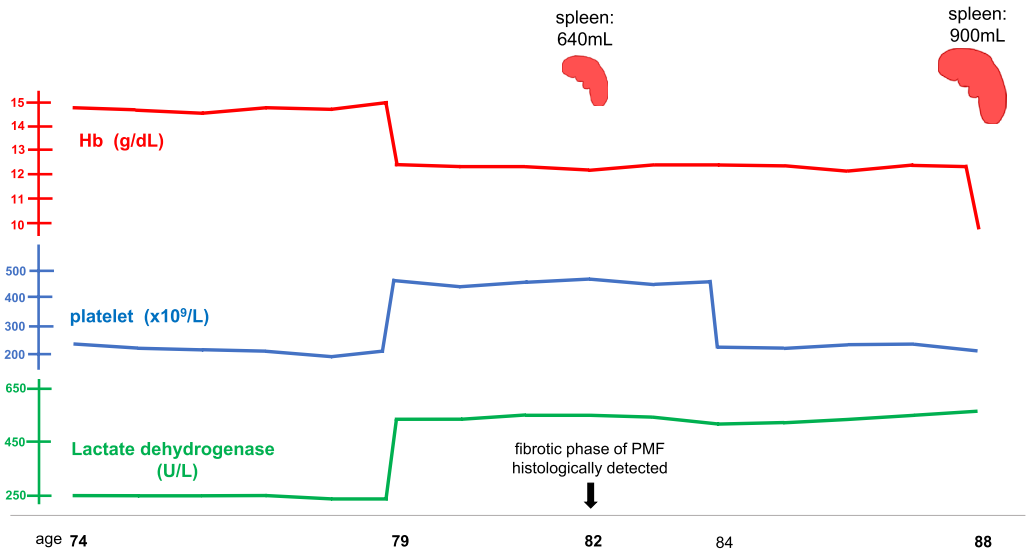

Fig. 1 Timeline of this case. Anemia, thrombocytosis, and increase in serum lactate dehydrogenase suddenly occurred

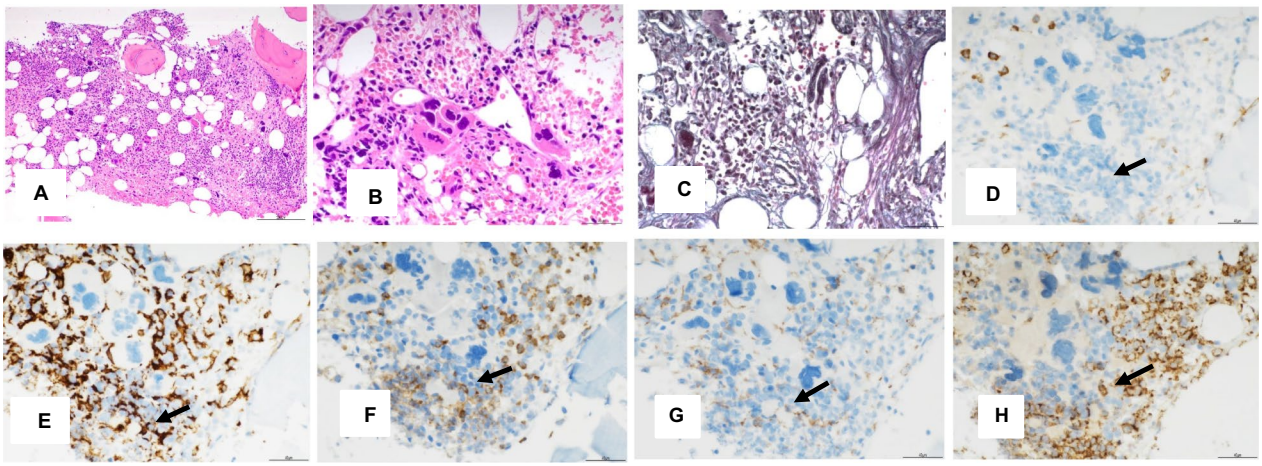

Fig. 2 Histological and immunohistochemical sections. **A** The bone marrow was hypercellular with marked proliferation of atypical megakaryocytes (MgKs). Scale bar = 200 μm. **B** The MgKs formed clusters and showed extremely hyperchromatic and bizarre nuclei. Scale bar = 40 μm. **C** Silver impregnation-stained section showing that the reticulin fibers formed a dense meshwork, with the presence of collagen fibers. Scale bar = 40 μm. **D** The $CD14^+$ cells were scattered. **E** Many $CD16^+$ cells formed a dense meshwork. **F** Many $CD11b^+$ cells were granulocytic cells, while some dendrite-shaped $CD11b^+$ cells appeared to be macrophages or dendritic cells. **G** $MSR1^+$ cells showed a dendritic form and seemed to form a loose meshwork. **H** Many $CEACAM1^+$ cells surrounded the MgKs and erythroblastic islands. The arrows in D–H indicate the possibility that $CD14^-CD16^+CD11b^+MSR1^+CEACAM1^+$ cells may exist in the patient's bone marrow. Scale bar = 40 μm

using serial sections. A few cells appeared to be simultaneously positive for CD16, CEACAM1, CD11b, and MSR1, which were present in the paratrabecular space and located near a cluster of atypical MgKs (Fig. 2D–H).

To determine whether SatMs were present in the bone marrow, we performed triple immunohistochemical staining of CD16/CEACAM1/MSR1 and CD14/CEACAM1/MSR1. We attempted to perform quadruple immunohistochemistry of CD14, CD16, CEACAM1, and MSR1. However, the immunofluorescent wavelengths overlapped between two dyes of similar wavelengths. Therefore, we instead selected triple staining. Neutrophils express CD16 and CEACAM1, but not MSR1 [14]. Human monocytes/histiocytes have not been reported to express CEACAM1, although a subset of monocytes/histiocytes express CD16 and MSR1 [10]. MSR1 is a well-known marker of macrophages/histiocytes. SatMs are thought to be $CD14^-CD16^+$ monocytes/histiocytes, which simultaneously express CEACAM1 [8]. Therefore, after antigen retrieval in 1 mM EDTA (pH 9.0) at 95 °C for 40 minutes, we stained the sections with either anti-CD14 antibody or anti-CD16 antibody, which was visualized with a fluorescence-based tyramide signal amplification technique with tyramide–biotin (Agilent Technologies Inc., Santa Clara, California, USA) and streptavidin AMCA (Vector Laboratories Inc., Newark, California, USA). The same sections were continuously stained with anti-CEACAM1 antisera and visualized with a fluorescein-labeling kit (Dojindo Laboratories, Kumamoto, Japan), as well as anti-MSR1 antibody and visualized with a R-phycoerythrin-labeling kit (Dojindo Laboratories). The triple-stained images were obtained by fluorescence microscopy (BZ-9000, KEYENCE, Osaka, Japan, or Zeiss Celldiscoverer 7, Carl Zeiss Microscopy GmbH, Jena, Germany) and analyzed using ImageJ software (https://imagej.nih.gov/ij). Of the almost 5000 nucleated cells in the CD16/CEACAM1/MSR1 triple-stained slide, we rarely detected cells that simultaneously expressed CD16, CEACAM1, and MSR1 (Fig. 3A–D). We detected a few CEACAM1 and MSR1 double-positive cells, which are candidate SatMs, scattered near to $CD16^+CEACAM1^+MSR1^+$ triple-positive cells in the patient's bone marrow. We also detected $CEACAM1^+MSR1^+$ double-positive cells in the CD14/CEACAM1/MSR1 triple-stained slide of the patient. Both the $CEACAM1^+MSR1^+$ double-positive cells and $CD16^+CEACAM1^+MSR1^+$ triple-positive cells were scattered throughout the patient's bone marrow (Fig. 3A–E). However, $CD14^+CEACAM1^+MSR1^+$ cells were not observed in the CD14/CEACAM1/MSR1 triple-stained slide (Fig. 3E). We also triple-stained healthy bone marrow harvested from a healthy volunteer aged 60 years and found no $CD16^+CEACAM1^+MSR1^+$ cells or $CEACAM1^+MSR1^+$ cells (Fig. 3F).

Table 1 presents the primary antibodies and antisera.

Discussion

Our patient developed the sudden onset of anemia and thrombocytosis without any symptoms. Therefore, we immediately examined whether *JAK2*, *MPL* and *CALR*

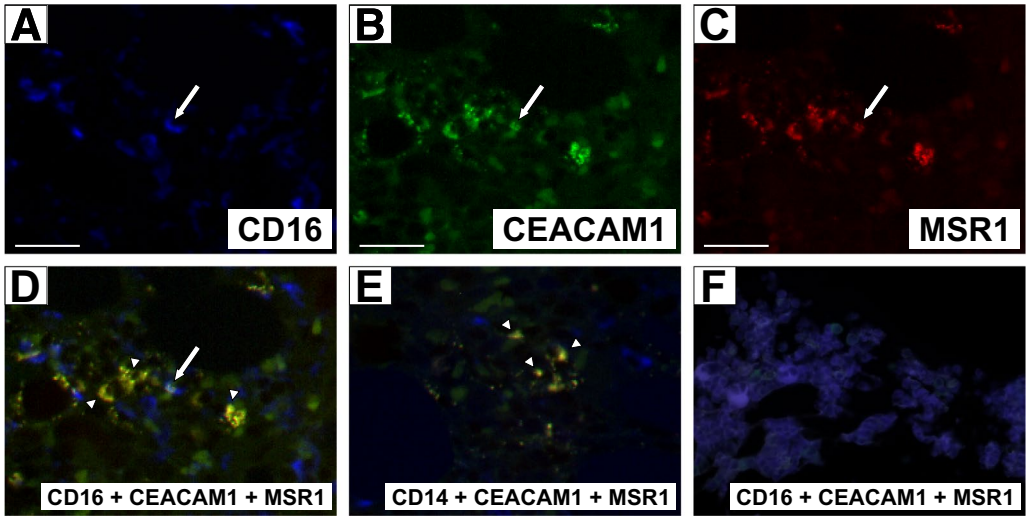

Fig. 3 Multiple fluorescence staining for CD16, CD14, CEACAM1, and MSR1. Immunocytochemical detection of the expression of CD16 or CD14 (blue; AMCA), CEACAM1 (green; fluorescein), and MSR1 (red; R-phycoerythrin). **A**–**D** Expression analysis of CD16, CEACAM1, and MSR1 in the patient's bone marrow. The overlay image shows $CD16^{+}CEACAM1^{+}MSR1^{+}$ cells (white color), indicated by the arrow. The cytoplasm of $CEACAM1^{+}MSR1^{+}$ cells (yellow color) is indicated by the arrowheads. **E** The overlay image of CD14, CEACAM1, and MSR1 expression in the patient's bone marrow. No $CD14^{+}CEACAM1^{+}MSR1^{+}$ cells (white color) were seen, but $CEACAM1^{+}MSR1^{+}$ cells (yellow color) were detected (arrowheads). **F** The overlay image of CD16, CEACAM1, and MSR1 expression in the bone marrow of a healthy volunteer. No $CD16^{+}CEACAM1^{+}MSR1^{+}$ cells (white color) or $CAECAM1^{+}MSR1^{+}$ cells (yellow color) were detected. Scale bar = 50 μm

Table 1 Monoclonal antibodies or antiserum used in immunohistochemical staining

Antigen	Clone or catalog number	Species	Manufacturer	Dilution	Antigen retrieval conditions
CD11b	Clone EPR1344	Rabbit	Abcam plc (Cambridge, UK)	1:1000 (autostaining) 1:100 (immunofluorescent staining)	CC1, 60 minutes (autostaining) 1 mM EDTA (pH 9.0), 95 °C, 40 minutes (immunofluorescent staining)
CD14	Clone EPR3653	Rabbit	Roche Diagnostics (Rotkreuz, Switzerland)	Prediluted	CC1, 60 minutes (autostaining) 1 mM EDTA (pH 9.0), 95 °C, 40 minutes (immunofluorescent staining)
CD16	Clone SP175	Rabbit	Abcam plc	1:200 (autostaining) 1:200 (immunofluorescent staining)	CC1, 60 minutes (autostaining) 1 mM EDTA (pH 9.0), 95 °C, 40 minutes (immunofluorescent staining)
CEACAM1/CD66a	Cat. No. 113392	Rabbit	GeneTex Inc. (Irvine, CA, USA)	1:300 (autostaining) 1:40 (immunofluorescent staining)	CC1, 60 minutes (autostaining) 1 mM EDTA (pH 9.0), 95 °C, 40 minutes (immunofluorescent staining)
MSR1/CD204	Clone UMAB247	Mouse	OriGene Technologies Inc. (Rockville, MD, USA)	1:1200 (autostaining) 1:400 (immunofluorescent staining)	CC1, 60 minutes (autostaining) 1 mM EDTA (pH 9.0), 95 °C, 40 minutes (immunofluorescent staining)

CC1 is the alkaline antigen-retrieval buffer for autostaining (Roche Diagnostics). Cat. No., catalog number; EDTA, ethylenediaminetetraacetic acid

mutations were present to differentiate primary thrombocytosis from secondary thrombocytosis [15]. We detected a *CALR* mutation. *CALR* mutations and thrombocytosis were reported to occur in patients who suffered from PMF, ET, or MDS/MPN-SF3B1-T according to the *WHO Classification of Haematolymphoid Tumours*," Fifth Edition [1]. To differentiate PMF from other hematopoietic diseases with an increase in one or more blood cell lineages, a bone marrow biopsy is required. This is because the only difference between these diseases is the atypical morphology of MgKs, which have hyperchromatic nuclei, and their atypical localization in the marrow as seen in PMF [2]. Moreover, the bone marrow of patients with MDS/PMN-SF3B1-T contains ring sideroblasts [4], whereas the bone marrow of those with PMF and ET does not. Our case had a *CALR* mutation determined by genetic analysis. After histological examination, we detected atypical MgKs, their atypical location, and massive fibrosis but no ring sideroblasts in the bone marrow. Therefore, we diagnosed our case as fibrotic PMF. Our case showed del20q. Chromosomal anomaly of del20q is reported to be the most common recurrent abnormality in the PMF patients [2]. We think that the recent decrease in the platelet count might represent disease progression rather than leukemic transformation because no blasts were found.

Recently, $CD14^+$ monocytes have been suggested to be related to marrow fibrosis in patients with PMF [6, 7]. We speculated that a counterpart of murine SatMs, namely $Ly6C^-Mac1^+F4/80^-MSR1^+CEACAM1^+$ unique monocytes, which are derived from murine bone marrow and can induce tissue fibrosis in the murine lung [8], might exist in the bone marrow of patients with PMF. According to the markers used to identify murine SatMs, their human counterparts are probably $CD14^-CD16^+CEACAM1^+MSR1^+$ cells. Therefore, we attempted to identify these counterparts by immunohistochemistry. We suspected that potential human SatMs would be located in the paratrabecular space according to the results of the immunostained serial sections. MSR1 is a commonly used marker for macrophages/monocytes, whereas CEACAM1 is expressed on granulocytes [14]. Accordingly, we thought that the most distinct characteristic of human SatMs, if they exist, would be the triple expression of CD16, CEACAM1, and MSR1. Using triple immunofluorescent staining, we observed $CD16^+CEACAM1^+MSR1^+$ triple-positive cells and CEACAM1 + $MSR1^+$ double-positive cells scattered throughout the patient's bone marrow; however, no $CD16^+CEACAM1^+MSR1^+$ triple-positive cells or $CEACAM1^+MSR1^+$ double-positive cells were observed in healthy bone marrow. According to histiocytic/monocytic markers and neutrophilic markers [13, 14], $CEACAM1^+MSR1^+$ double-positive cells are thought to be candidate SatMs. The reason why double-positive cells were not simultaneously detected with CD16 is uncertain. However, there are two possibilities: (1) the distribution of each antigen, CD16, CEACAM1, or MSR1, might be different in the cytoplasma, or (2) although $CD16^-CEACAM1^+MSR1^+$cells exist, they are currently unknown.

Overall, our data demonstrate the possible existence of human SatMs, which are thought to exhibit the following expression pattern in the bone marrow of patients with fibrotic PMF: $CD14^-CD16^+MSR1^+$Mac1 (CD11b/CD18 complex)$^+CEACAM1^+$. The triple immunostaining data suggest putative human SatMs are scattered throughout the bone marrow of the PMF patient but are not present in healthy bone marrow. These results suggest that these cells might be related to bone marrow fibrosis in this patient, although the relevance between the distribution of putative human SatMs and marrow fibrosis in fibrotic PMF is uncertain.

The strength of this study is the detection of $CD16^+MSR1^+CEACAM1^+$ cells, which may represent a human counterpart of SatMs, which are derived from murine bone marrow. No similar cell type has been previously recognized in the human tissues. In terms of the study limitations, we could not examine whether the possible human SatMs actually induced marrow fibrosis in the patient with PMF. Future studies should clarify whether human SatMs, not putative SatMs, exist in the PMF marrow and can induce marrow fibrosis in PMF, by utilizing isolated $CD14^-CD16^+CD11b^+MSR1^+CEACAM1^+$ cells from PMF bone marrow under certain conditions. However, isolating these cells from PMF patient bone marrow using flow cytometry is quite difficult because PMF must be diagnosed after histopathological examination using a biopsy specimen of the bone marrow [2], and aspiration of the marrow cells often ends up with a dry tap [12]. Furthermore, our results suggest these cells are present in very low numbers in the bone marrow of PMF.

Conclusions

We identified the putative human SatMs, which are $CD16^+CEACAM1^+MSR1^+$, in a bone marrow tissue section from a PMF patient.

Abbreviations

PMF	Primary myelofibrosis
MPN	Myeloproliferative neoplasm
ET	Essential thrombocythemia
MDS/MPN-SF3B1-T	Myelodysplastic/myeloproliferative neoplasm with SF3B1 mutation and thrombocytosis
MgK	Megakaryocyte
SatM	Segregated-nucleus-containing atypical monocyte
LDH	Lactate dehydrogenase

Hb	Hemoglobin
WBC	White blood cell

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Author contributions
SH conceptualized and investigated the case, and wrote the original draft; TO, SM, and KK resourced and clinically followed the case; MS and YN conducted the immunohistochemical work in this case; KT and YM confirmed methodology and validated the results; NM resourced and had clinically diagnosed the case; HK, NN, and AM histologically diagnosed the case; AM conceptualized, formally analyzed, wrote, and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.
The consent has been uploaded to the electronic medical chart at our hospital.

Competing interests
The authors declare that they have no competing interests.

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