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Case study

Ectopic expression of band 3 anion transport protein in colorectal cancer revealed in an autoimmune hemolytic anemia patient☆,☆☆

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Summary Cancer patients occasionally have anemia with high mean corpuscular volume in addition to iron deficiency anemia. Secondary autoimmune hemolytic anemia (AIHA) following cancer is also observed with low frequency. To date, no causal mechanisms for these disease states have been reported. Here, we present the case of an 80-year-old woman with AIHA that was resistant to prednisolone. Further examinations revealed primary adenocarcinoma of the sigmoid colon and primary squamous cell carcinoma in the right lung. After resections of these tumors, her anemia partially improved until a colon cancer–derived metastatic tumor was detected in the left lung. Immunoprecipitation of erythrocyte membrane proteins with an autoantibody followed by mass spectrometry/Western blotting identified band 3 as the target of the autoantibody. Immunohistochemical analysis revealed ectopic expression of band 3 in the colon adenocarcinoma. To our knowledge, this is the first report that identifies the cause in a case of anemia without bleeding in a cancer patient and that defines a mechanism underlying secondary AIHA following cancer progression.

1. Introduction

Secondary autoimmune hemolytic anemia (AIHA) is attributed to primary diseases, including lymphoid neoplasms, collagen diseases, and ovarian cysts. Typically, treatment of primary diseases leads to AIHA remission. Cases of AIHA secondary to cancer are rare [1].

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Band 3 protein, also known as *anion exchanger 1*, is an anion transporter that exchanges HCO_3^- and Cl^- in equal proportions [2]. It is abundantly expressed in red blood cells (RBCs) and renal tubule cells. Band 3 is one target of autoantibodies associated with idiopathic AIHA. Other targets include Rhesus group D antigen (RhD) and glycophorin A [3]. Aberrant expression of band 3 has been reported in gastrointestinal (GI) cancers, including esophageal, gastric, and colon cancers, and band 3 expression is a factor in the pathogenesis and poor prognosis of patients with GI cancers [4,5]. The relationship between AIHA onset and aberrant expression of band 3 in cancer cells remains unclear.

In this report, we identify the possible cause of an anemia with previously unknown etiology in a cancer patient by analyzing secondary AIHA following colon cancer using mass spectrometry and immunological techniques. This report provides critical information in the fields of oncology, hematology, and immunology.

2. Materials and methods

2.1. Isolation of ghosts and IgG autoantibodies

Whole blood was collected into EDTA-containing tubes, and RBCs were isolated using Ficoll-Paque PLUS in accordance with the manufacturer's instructions (cat. no. 17144002; GE, Piscataway, NJ). RBCs were processed for isolation of ghost membranes in accordance with Baker's method [6]. The IgG autoantibodies were isolated from serum using an IgG purification kit (cat. no. AP02; Dojindo Molecular Technologies, Inc, Rockville, MD). Immunoprecipitation was performed using the isolated autoantibodies with Protein G Sepharose (cat. no. IP05; Calbiochem, Darmstadt, Germany).

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

Gradient polyacrylamide gels (10%-20%) and polyvinylidene difluoride membranes were purchased from ATTO Inc (Fig. 1; cat. ET-1020L and cat. AE-6668; Tokyo, Japan). Any kD Mini-PROTEAN TGX Precast Gels and a Trans-Blot Turbo Transfer Pack were purchased from BIO-RAD Laboratories Inc (Fig. 2; cat. #456-9033 and cat #170-4156; Hercules, CA). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and immunoblotting were performed by conventional methods using the following buffers: lysis buffer (50 mmol/L Tris-HCl pH 8.0, 0.1% Tween 20, 0.1% SDS, 150 mmol/L NaCl, and 0.5 mmol/L EDTA), 2× sample buffer (0.25 mol/L Tris-HCl, pH 6.8, 20% 2-mercaptoethanol, 4% SDS, 20% sucrose, and 0.002% bromophenol blue), running buffer (25 mmol/L Tris, 0.192 mol/L glycine, and 0.1% SDS), and blocking buffer; 3% skim milk in Tween-PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L $Na_2HPO_4 \cdot 12H_2O$, 1.5 mmol/L KH_2PO_4, and 0.1% Tween 20). After incubation with the indicated antibodies, membranes were incubated with secondary antibodies. Detection was performed by enhanced chemiluminescence. Antibodies and reagents for Western blotting were commercially purchased as follows: molecular marker, Amersham (cat. no. RNP800; Piscataway, NJ) and BIO-RAD (cat. no. #1610374); peroxidase-labeled secondary anti-human IgG (H + L) antibody, Cytodiagnostics (cat. ABH-01-11; Burlington, Ontario, Canada); rabbit polyclonal anti-slc4a1 antibody, MBL (cat. BMP012; Nagoya, Aichi, Japan); horseradish peroxidase (HRP)–conjugated anti-rabbit-IgG secondary antibody, Santa Cruz Biotech (cat. SC-2004; Santa Cruz, CA); anti-human RhD antibody, Sigma-Aldrich (cat. no. sab2107753; St Louis, MO); anti-band3 antibody, MBL (cat. BMP012); and enhanced chemiluminescence substrate, Merck Millipore (cat. no. WBLUR0100; Burlington, MA).

2.3. Mass spectrometry

After staining SDS–polyacrylamide gel electrophoresis gels with Coomassie G-250 (cat. no. LC6060; Invitrogen, Carlsbad, CA), visualized protein bands were extracted from the gel. After purification of the extracted proteins, samples were analyzed by mass spectrometry (liquid chromatography ion-trap time-of-flight mass spectrometry, Shimadzu Co; Kyoto, Japan).

3. A case report

An 80-year-old woman with a history of mycoplasma pneumonia but with no history of transfusions presented with macrocytic anemia. Examination of the upper digestive tract was not performed, and no occult blood was found in her stool sample. An abdominal ultrasonogram revealed no abnormalities, but a computed tomography (CT) scan revealed a small nodule in her lower right lung.

Because her anemia worsened, she was referred to our hospital. Based on the results of her laboratory tests, including Coombs test and bone marrow aspiration, she was diagnosed with AIHA (Table). As anemia worsened (hemoglobin [Hb] 79 g/L, hematocrit [Ht] 24.6 L/L, mean corpuscular volume [MCV] 116 fL, reticulocyte [Ret] 0.17×10^{12}/L), prednisolone was initiated at a dose of 0.5 mg/kg. This treatment achieved only a partial response. We therefore suspected that a malignancy might be causing her secondary AIHA. The results of repeated tests for occult blood in stool samples were negative. A chest CT was performed to follow-up on the lesion that was previously detected in her lower right lung. Tumor growth was observed in this follow-up CT scan (Fig. 1A). Positron emission tomography (PET) revealed no metastasis of the tumor to the lymph nodes (Fig. 1B); however, fluorodeoxyglucose uptake was also observed in the sigmoid colon (Fig. 1C). Through colonoscopy, advanced cancer was confirmed (Fig. 1D), and a diagnosis of adenocarcinoma was made based on histopathological examination of the colon lesion. The lung tumor was resected and histopathologically determined to be squamous cell carcinoma. After subsequent colon cancer surgery, the patient's anemia improved slightly

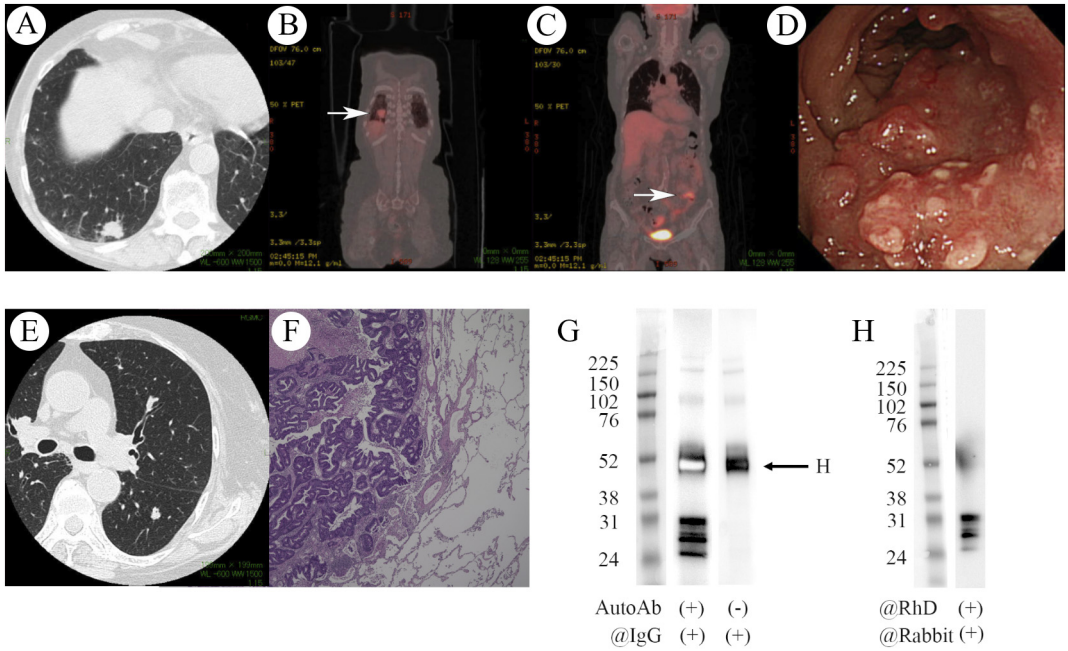

Fig. 1 Autoantibody induced in a patient with colon and lung double cancer. A and B, A squamous cell carcinoma detected in the right lung by chest CT and PET-CT examination. C, A colon adenocarcinoma detected by PET-CT examination. D, An advanced sigmoid colon adenocarcinoma without bleeding revealed by colonoscopy. E, A left lung metastatic lesion of recurrent colon adenocarcinoma. F, Hematoxylin-eosin staining of left lung metastatic lesion of colon adenocarcinoma: original magnification ×40. G, Western blotting of RBC ghost immunoprecipitates by autoantibody. Lane 1: molecular marker. Lane 2: Membrane was incubated with autoantibodies followed by HRP-conjugated anti-human IgG (H + L) secondary antibody. Lane 3: Membrane was incubated with HRP-conjugated anti-human IgG secondary antibody without autoantibody. Arrow indicates IgG heavy chain. H, Detection of similar-size bands by anti-RHD antibody. Immunoprecipitate of RBC ghost by autoantibody was subjected to Western blotting using anti-human RhD polyclonal antibody and HRP-conjugated anti-rabbit secondary antibody. Abbreviations: M, molecular marker; IP, immunoprecipitate; Ab, antibody; H, immunoglobulin heavy chain.

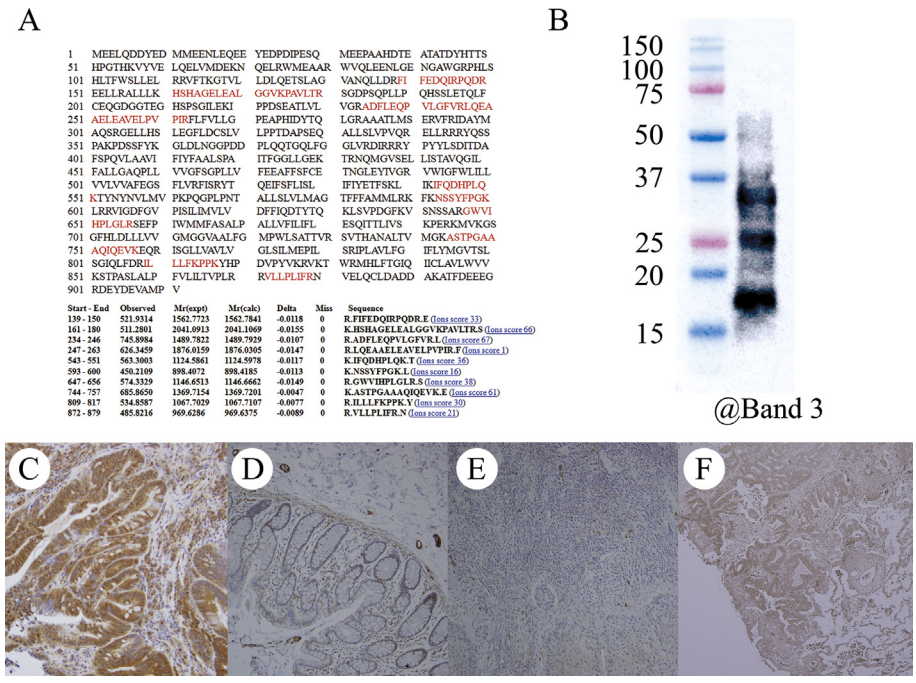

Fig. 2 Band 3 protein detected in immunoprecipitates and colon adenocarcinoma. A, Amino acid sequences detected by mass spectrometry from a 31 000-Da immunoprecipitated band. Red letters indicate detected peptides found in full-length band 3 amino acid sequence. B, Immunoprecipitates detected by recombinant anti-band 3 antibody. C-F, Histopathological staining with anti–band 3 antibody. C, Colon adenocarcinoma: ×400. D, Normal colon tissue: ×100. E, Right lung squamous cell carcinoma: ×100. F, Left lung metastatic lesion of colon adenocarcinoma: ×40.

Table Laboratory examination results at presentation

Test	Value	Unit	Test	Value	Unit
WBC	8.74	$\times 10^9$/L	AST	14	IU/L
RBC	2.85	$\times 10^{12}$/L	ALT	11	IU/L
Hb	102	g/L	LDH	148	IU/L
Ht	30.2	L/L	T.Bil	0.9	mg/dL
MCV	106	fL	D.Bil	0.1	mg/dL
Reticulocyte	0.13	$\times 10^{12}$/L	Folic acid	20.0	ng/mL
Spherocyte	+		Vitamin B12	1500	pg/mL
Direct anti-globulin test					
IgG	+		RBC-bound IgG [a]		
IgA	−		285		molecules/cell
IgM	−		(normal reference 33±13 molecules/cell)		
C3b3d	+				

Abbreviations: Ig, immunoglobulin; C, complement; AST, aspartate aminotransferasel; ALT, alanine aminotransferase; LDH, lactate dehydrogenase T. Bil, total bilirubin; D. Bil, direct bilirubin.

[a] RBC-bound IgG was measured with a previously reported method [21].

(Hb 136 g/L, Ht 42.2 L/L, MCV 99 fL, Ret 0.10 × 10^{12}/L) with low-dose prednisolone (5 mg/d). Over time, the number of reticulocytes gradually increased again, and anemia worsened 2 months postsurgery (Hb 103 g/L, Ht 32.2 L/L, MCV 105 fL, Ret 0.14 × 10^{12}/L). This indicated AIHA recurrence and lung or colon cancer relapse. The patient's chest CT scan revealed a small nodule in her left lung (Fig. 1E). This tumor was resected and histopathologically identified as adenocarcinoma, indicating colon cancer metastasis (Fig. 1F). We therefore suspected that the adenocarcinoma might have caused the AIHA. After the left lung surgery, the patient refused treatment with sufficient prednisolone to treat her AIHA and adjuvant chemotherapy for colon cancer due to fear of their adverse effects. Her AIHA was thus managed with 5 mg/d prednisolone, leaving her with moderate anemia (Hb 105 g/L, Ht 33.6 L/L, MCV 107 fL, Ret 0.13 × 10^{12}/L). The last follow-up with this patient was 3 years postsurgery, although detailed information was not available.

4. Results

After obtaining informed consent from the patient, experiments were performed to characterize the autoantibody and its target. Results of the Coombs test and flow cytometry revealed that the class of immunoglobulin that bound the patient's erythrocytes was IgG (data not shown). IgG was collected from the patient's plasma sample using Protein G Sepharose and used for immunoprecipitation and Western blotting of RBC membrane proteins. Proteins of 3 molecular weights, in the range of 24 000-30 000 Da, were detected in immunoprecipitates using the autoantibody as the primary antibody and an HRP-conjugated anti-human IgG secondary antibody (Fig. 1G). These immunoprecipitates were not detected with the secondary antibody alone, indicating that these bands were not immunoglobulins. Because the molecular weight of RhD is 31 000 Da, we suspected that one of the largest proteins detected was RhD. As expected, probing immunoprecipitates with an anti-RhD antibody as primary antibody detected similar protein bands (Fig. 1H). We hypothesized that the colon adenocarcinoma expressed a molecule that had homology with RhD antigen.

Histopathological examinations were performed using the same anti-RhD antibody. The lung cancer and normal colon tissue were not stained with the antibody (data not shown). Colon adenocarcinoma was only modestly stained. We concluded that this staining was nonspecific because this tissue stained weakly with anti-rabbit secondary antibody alone (data not shown). This suggests that the target of the patient's autoantibody was not the protein with cross-reacting epitope to RhD antigen.

To further characterize the autoantibody, three bands that were immunoprecipitated from RBC membranes with the autoantibody were extracted from the gel, fragmented, and subjected to mass spectrometry. Band 3 was detected in the immunoprecipitates, although the intact molecular weight of band 3 protein is approximately 100 000 Da. The largest band, approximately 31 000 Da, contained peptides that partially matched band 3 amino acid residues 139-263 and 541-878 and covered almost the entire sequence (Fig. 2A). Immunoblotting of the immunoprecipitates with a rabbit anti-band 3 polyclonal antibody, focusing on low–molecular weight proteins, detected 3 similar bands, although one of these was a new band at 17 000 Da rather than the band previously detected at 24 000 Da (Fig. 2B).

Proteins expressed in the patient's surgically resected colon and lung samples were analyzed immunohistochemically using the same anti–band 3 antibody used to probe immunoprecipitations. The colon adenocarcinoma stained strongly with the anti–band 3 antibody (Fig. 2C), whereas normal colon tissue and lung squamous carcinoma did not (Fig. 2D and E). The

metastatic lesion of colon adenocarcinoma found in the left lung also stained with the antibody (Fig. 2F).

5. Discussion

In the present report, we confirmed that band 3 protein was ectopically expressed in adenocarcinoma cells of the sigmoid colon and that it was targeted by autoantibodies in a patient with secondary AIHA, whose fecal immunological test result was negative.

The Coombs test is not commonly performed for patients with GI cancers because anemia is most frequently due to bleeding and improves after tumor resection. However, cancers that are associated with anemia often show high MCVs instead of iron deficiency anemia patterns. Few previous investigators have suspected that production of autoantibodies to erythrocytes may be another cause of anemia in cancer patients [1,7,8]. To date, no precise analysis of the cause of antibody production or identification of autoantibody targets has been performed.

It is possible that natural secretion of anti–band 3 autoantibody into the serum and binding to erythrocyte membranes are enhanced in many patients with colorectal cancer due to phagocytosis of cancer cells and antigen presentation by immune cells such as $\gamma\delta$ T cells in intraepithelial lymphocytes [9]. Disturbing the balance of the $\gamma\delta$ T-cell repertoire has been shown to induce autoantibody binding to hematopoietic cells [10]. In healthy individuals, naturally occurring anti–band 3 antibodies exist that remove senescent RBCs from the reticuloendothelial system [11]. In this way, immunological responses can cause high-MCV anemia that reflects the increase in reticulocytes without bleeding in cancer patients.

Two issues arose out of our protein analysis results. The first relates to the reaction between the anti-RhD antibody and the protein that is precipitated by the autoantibody (Fig. 1H). Although the reason for this reaction is unclear, a previous report supports this phenomenon [12]. Gordon et al [13] reported that an anti-C IgG autoantibody to the Rhesus blood group system appeared in a patient with adenocarcinoma. Band 3 may have been expressed in this patient's adenocarcinoma, and the anti-C antibody also reacted with band 3. In support of this explanation, we found in our current study that the anti-RhD antibody moderately reacted with colon adenocarcinoma (data not shown). The lack of RhD peptide detection in our mass spectrometry analysis of immunoprecipitated bands suggests the absence of anti-RhD autoantibodies in the serum of the present patient.

The second issue is that the band 3 fragments detected had molecular weights of 17 000-30 000 Da, whereas the putative molecular weight of full-length band 3 is 100 000 Da (Figs. 1G and H and 2B). Baker's method for preparing RBC protein membranes, erythrocyte "ghosts," does not cleave band 3 [6]. We also confirmed that our reducing conditions, using 2-mercaptoethanol, did not cleave band 3 (data not shown). This result agrees with that of a previous report, which analyzed senescent cell antigens related to band 3 [14]. Proteolytic fragments of band 3 are recognized by physiologic IgG autoantibodies [15]. As residual leukocyte debris after preparation of erythrocyte "ghosts" has proteolytic activity, it is possible that this debris caused the fragmentation of band 3 during storage [16]. In addition, caspase-3, a member of the cysteine–aspartic acid protease family, might be responsible for our observation because parts of the N-terminal cytoplasmic and transmembrane domains of band 3 are known to be cleaved by caspase-3 [17]. Caspase-3 fragments of band 3 possess autoantibody recognition sites. The amino acid residues 160-878 of band 3 that were detected by mass spectrometry in the current study also possess antigenicity [18,19]. As caspase-3 is expressed in colon cancer and is considered to be a poor prognostic factor for patients, autoantibody binding to band 3 could increase owing to cancer-related stress [20].

Autoantibody binding to erythrocyte membranes of patients with AIHA is measurable even in those with Coombs-negative AIHA [21]. The amount of RBC–bound IgG in the present case was higher than normal reference levels (Table) and was detectable by flow cytometry (data not shown). Therefore, the cancer-related anti–band 3 autoantibody can potentially be quantified by flow cytometry and might be useful for screening of colorectal cancers that substitute fecal immunological test [22]. Considering the follow-up duration of the current case after surgery, screening by flow cytometry might improve survival of colorectal cancer patients even in advanced stages.

In conclusion, the autoimmunity induced by ectopic expression of band 3 in patients with colon adenocarcinoma correlates with development of secondary AIHA and causes anemia without bleeding, possibly due to phagocytosis of autoantibody-bound RBCs by macrophages.

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