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Original article

Title

Band 3 ectopic expression in colorectal cancer induces an increase in erythrocyte membrane-bound IgG and may cause immune-related anemia

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Running head

RBC membrane-bound IgG increases in CRC

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Abstract

Autoimmune hemolytic anemia (AIHA) is a rare comorbidity in colorectal cancer (CRC) and has an unknown etiology. Previously, we described an AIHA case secondary to CRC with ectopic band 3 expression. Herein, we investigated ectopic band 3 expression and erythrocyte membrane-bound IgG in a CRC cohort. Between September 2016 and August 2018, 50 patients with CRC and 26 healthy controls were enrolled in the present study. The expression of band 3 and *SLC4A1* mRNA was observed in 97% of CRC surgical specimens. Although clinical AIHA was not observed in any patient with CRC, a direct antiglobulin test was positive in 10 of the patients in the CRC group ($p=0.01$). Flow cytometry revealed significantly increased erythrocyte membrane-bound IgG among patients with CRC compared to healthy controls (mean $\pm$ standard deviation; 38.8 ± 14.7 vs. 29.9 ± 15.6 , $p=0.012$). Normocytic anemia was observed, including in cases negative for fecal occult blood, suggesting a shortened erythrocyte life-span due to increased membrane-bound IgG. Immunoprecipitation revealed increased anti-band 3 autoantibodies in patients' sera. Mouse experiments recapitulated this phenomenon. We also confirmed that band 3 expression is controlled by 5'AMP-activated protein kinase under hypoxic conditions. These findings increase our understanding of the etiology of cancer-related anemia.

Key words

Colorectal cancer, Band 3/Anion exchanger 1, Erythrocyte membrane-bound IgG,

5'AMP-activated protein kinase, Autoimmune hemolytic anemia

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Introduction

Quality of life (QOL) is important in the management of patients with cancer [1]. Anemia causes symptoms such as general weakness and is also responsible for a reduction in performance status and QOL in patients with this disease [2, 3].

Cancer-related anemia is commonly observed in patients with many types of cancers. In colorectal cancer (CRC), the cause of anemia is usually chronic bleeding [4]. However, we often observe that patients with CRC who have a negative result in a fecal occult blood (FOB) test have normocytic anemia with a high reticulocyte count prior to cancer treatment.

It is known that autoimmune hemolytic anemia (AIHA) rarely occurs as a paraneoplastic syndrome [5]. In AIHA, IgG-type autoantibodies are produced mainly against erythrocyte membranes resulting in hemolysis. One of the major antigens recognized by such autoantibodies is band 3 protein [6]. Band 3 is also known as anion exchanger 1 (encoded *SLC4A1*) and functions as an anion transporter for chloride and bicarbonate [7]; its expression in normal tissue is restricted to the erythrocyte membrane and renal tubules [8].

We have previously reported the ectopic expression of band 3 in a case of CRC with AIHA and suggested that band 3 expression in CRC may stimulate production of autoantibodies that

1 bind to erythrocyte membranes [9]. Other groups have also reported the ectopic expression of
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4 2 band 3 in digestive tract cancers [10, 11]. Ectopically expressed band 3 may increase the
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7 3 amount of erythrocyte membrane-bound IgG in patients with CRC, even if AIHA is not
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10 4 clinically exhibited. Since it is proposed that erythrocytes that bind a high amount of
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13 5 anti-band 3, a naturally occurring antibody, are phagocytosed by the mononuclear phagocyte
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16 6 system leading to a shortening of erythrocyte lifespan, this may be another mechanism of
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19 7 anemia in patients with CRC who show no apparent bleeding [12-14]. However, a prospective
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22 8 and definitive study investigating the relationship between band 3 expression and AIHA in
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25 9 CRC is lacking. Therefore, we conducted a prospective observational study to investigate the
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29 10 relationship between band 3 expression and erythrocyte membrane-bound IgG in patients
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32 11 with CRC. Moreover, we also conducted studies in a mouse transplantation model and
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35 12 investigated the regulation of band 3 expression using human CRC cell lines.
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42 14 **Materials and Methods**

45 15 **Patients and healthy controls**

48 16 Patients were enrolled between September 2016 and August 2018. The inclusion criteria
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51 17 covered patients diagnosed with histologically confirmed CRC, were over 20 years of age and
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54 18 had operable lesions. Patients who had a past history of an AIHA, and those undergoing
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57 19 systemic steroid treatment, were excluded.
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1 The inclusion criteria for healthy controls were participants aged over 20 years who were
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4 2 not receiving any medical treatment. Those who had a history of CRC were excluded. All
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7 3 participants provided written, informed consent and all study protocols were approved by the
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10 4 Ethics Committee of Kobe University Graduate School of Medicine (permission number:
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13 5 160054). Blood specimens were taken from patients before surgery. In the case of healthy
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16 6 controls, a blood specimen was also collected.
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23 8 **Direct antiglobulin test**

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26 9 A direct antiglobulin test was performed using a column method (Ortho Clinical Diagnostics,
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29 10 Linden, NJ, USA). Results were then evaluated by transfusion department technicians,
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32 11 blinded to patient information, using an Ortho-vision analyzer (Ortho Clinical Diagnostics).
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39 13 **Immunohistochemistry (IHC) and evaluation of staining**

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42 14 Formalin-fixed paraffin-embedded (FFPE) tissues of patients' surgical specimens were
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45 15 prepared. After deparaffinization, BLOXALL blocking solution (Vector Laboratories, Inc.,
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48 16 Burlingame, CA, USA) and Blocking One Histo (Nacalai Tesque, Kyoto, Japan) were used to
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51 17 prevent non-specific binding. Anti-band 3 antibody (MBL, Nagoya, Japan, BMP012) was
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54 18 used as a primary antibody. After incubation using an ImmPRESS kit (Vector Laboratories)
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57 19 for the anti-rabbit secondary antibody, ImmPACT DAB substrate (Vector Laboratories) was
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1 used for color development.

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3 **Messenger RNA *in situ* hybridization**

4 Serial sections of FFPE blocks were used. Using an RNA scope probe (Advanced Cell
5 Diagnostics, Newark, NJ, USA) that hybridized to the mRNA of *SLC4A1*, experiments were
6 conducted according to the manufacturer's protocol. After hybridization, counter staining was
7 carried out using hematoxylin and ammonia solution.

9 **Flow cytometry analysis**

10 One-percent erythrocyte suspensions were incubated with either anti-human IgG antibody
11 (DAKO, Carpinteria, CA, USA; Cat. No. F020202-2) or isotype control (Abcam, Cambridge,
12 UK; Cat. No. ab37406) conjugated to fluorescein isothiocyanate (FITC) and analyzed using a
13 FACS-Verse flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA).
14 Human IgG-type anti-human Diego-b (Dib) antibody, kindly provided by Japan Red Cross
15 Society, Osaka, Japan, was used for experiments with human CRC cell lines. Viable cells
16 were gated after staining with 7-aminoactinomycin D (Becton Dickinson and Company). The
17 difference in mean fluorescence intensity (MFI) was analyzed using FlowJo software v10.5.3
18 (Tomy Digital Biology, Tokyo, Japan).

Immunoprecipitation

Erythrocytes were lysed with radioimmunoprecipitation (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-24948) and hemoglobin was depleted using HemoVoid (Biotech Support Group, NJ, USA, Cat. No. HVK-10). Immunoglobulin IgG was purified by using IgG Purification Kit-G (Dojindo, Kumamoto, Japan; Cat. No. AP02). The concentration of IgG was measured by using Nanodrop 2000 (Thermo Fisher Scientific). For immunoprecipitation 10 µg of the erythrocyte protein solution was incubated with 30 µg of purified IgG and 25 µl of protein G Plus/Protein A Agarose Suspension (EMD Millipore Corp. CA, USA; Cat. No. IP05) at 4 °C, overnight with gentle agitation. After washing with RIPA buffer, the agarose was resuspended with sample buffer (Bio-Rad, Hercules, CA, USA; Cat. No. #1610747) and boiled at 95 °C for 8 minutes. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 5 µL of each sample was loaded on a 7.5% polyacrylamide gel (Bio-Rad; Cat. No.4561023). After transfer and blocking with 5% skim milk, a polyvinylidene fluoride membrane (Bio-Rad, Cat. No. 1704156) was incubated with anti-human band 3 antibody (MBL) or anti-mouse band 3 antibody (Proteintech, Rosemont, IL, USA; Cat. No. 28131-1-AP). For the detection of IgG, the membrane was incubated with horse radish peroxidase (HRP) conjugated rabbit anti-human polyclonal antibody (Bethyl Laboratories. Inc, Montgomery, TX, USA; Cat. No. A80-118P) at a 1:10,000 dilution or anti-mouse IgG (Cell Signaling Tech., Tokyo, Japan; Cat.

No. #7076).

Cell line culture

The human colon adenocarcinoma cell line, HCT116, and mouse colon adenocarcinoma cell line, Colon-26, were obtained from RIKEN Cell Bank (Tsukuba, Japan). Mouse cell lines, NHOS, UV-BAL-5.4G and WEHI-3 were also provided by RIKEN Cell Bank. The human colon adenocarcinoma cell line, SW480, was obtained from the American Type Culture Collection (Manassas, VA, USA). For the induction of hypoxic conditions, cobalt chloride hexahydrate (CoCl_2 ; Nacalai Tesque) was used at the indicated concentrations.

Western blot

Western blotting was performed using a conventional method. Anti-hypoxia inducible factor (HIF) 1- α antibody (Cell Signaling Technology Japan; Cat. No. #36169), monoclonal anti-band 3 antibody (Abcam; Cat. No. ab108414), polyclonal anti-band 3 antibody (MBL), anti-5'AMP activated protein kinase (AMPK) antibody (Cell Signaling Technology Japan; Cat. No. #2793S), and anti-phospho-AMPK antibody (Cell Signaling Technology Japan; Cat. No. #2535S) were applied as primary antibodies at a 1:1,000 dilution. HRP-conjugated anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody at a dilution of 1:3,000. ECL Prime Western Blotting Detection Reagents (GE Healthcare

Japan, Tokyo, Japan) was used to detect the signal. Subsequently, using the same membrane, primary and secondary antibodies were detached with a stripping buffer (Nacalai Tesque), and anti- β actin antibody (Sigma–Aldrich, St. Louis, MI, USA) was used as an internal loading control. The Band 3 protein band was measured by densitometry and compared with β -actin.

Quantitative real-time PCR for *SLC4A1* mRNA

RNA was extracted from cultured cells using a Nucleo Spin RNA kit (TaKaRa Bio, Shiga, Japan) and cDNA was prepared using reverse transcriptase (TaKaRa Bio). Quantitative real-time PCR was carried out using cyber green (TOYOBO, Osaka, Japan) method and target gene *SLC4A1* primer (TaKaRa Bio). β 2-microglobulin was used as an internal control.

Animal studies

Male BALB/c mice (4 weeks of age) were purchased from Japan SLC, Inc., Hamamatsu, Japan. Two million Colon-26 and WEHI-3 cells expressing band 3 protein were injected subcutaneously and blood was collected from the tail vein 2 weeks later. Erythrocyte membrane-bound IgG was measured by flow cytometry using anti-mouse IgG antibody (Thermo Fisher, Waltham, MA, USA; Cat. No. A11001). The animal study was approved by the Institutional Animal Care and Use Committee (permission number: P160302-R1) and carried out according to Kobe University Animal Experimental Regulations.

Statistical analysis

All results are expressed as mean $\pm$ standard deviation. Significant differences were analyzed using a two-tailed *t*-test, Fisher's exact test or one-way analysis of variance followed by Tukey pairwise comparison. The analyses were performed using Prism software v8.0.0 (Graph Pad, San Diego, CA, USA). The significance level was set at $p < 0.05$.

Results

Participant characteristics

All participant characteristics are shown in Table 1. The number of stage 4 patients was low because they were not targeted for surgery. Only five patients received neo-adjuvant chemotherapy. Most cases of anemia seen in patients with CRC were normocytic. The haptoglobin level was significantly higher in patients with CRC compared to healthy controls, suggesting that clinical hemolysis was not occurring, and reflected inflammation by cancer ($p = 0.01$). However, direct antiglobulin test results were positive in 10 patients and negative in healthy controls ($p = 0.01$). Even in patients with FOB negative CRC, normocytic anemia was observed, with the number of reticulocytes tending to be higher than in healthy controls (Table 2). In some FOB positive cases, normocytic anemia may be confused by the co-existence of microcytic anemia and an increase in reticulocytes. Moreover, similar results

1 were observed in neo-adjuvant chemotherapy naïve patients with FOB negative CRC,
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4 2 implying an additional mechanism for the onset of anemia (Table 3).
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10 4 **IHC for band 3 and *SLC4A1* mRNA *in situ* hybridization in CRC surgical specimens**

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13 5 The band 3 protein was expressed in tissues of patients with CRC (Fig. 1A, C) but not in
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16 6 normal tissue as determined by IHC (Fig. 1B, D); 97% of patients with CRC showed band 3
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19 7 protein expression. Band 3 expression was also confirmed with an anti-band 3 monoclonal
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22 8 antibody (data not shown). *SLC4A1* mRNA detected with a red probe were only observed
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26 9 intracellularly in CRC (Fig. 1E) but not normal tissue (Fig. 1F), supporting the idea of the
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29 10 protein being expressed in tumor cells. These results showed that band 3 was ectopically
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32 11 expressed at both protein and mRNA levels in CRC tissue.
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39 13 **Comparison of erythrocyte membrane-bound IgG and serum IgG between CRC**

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42 14 **patients and healthy controls**

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45 15 The MFI was higher among patients with CRC (38.8 ± 14.7) compared to healthy controls
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48 16 (29.9 ± 15.6 ; $p = 0.012$; Fig. 2A). To confirm whether the IgG targeting band 3 increased in
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51 17 patients' sera, immunoprecipitation was performed using hemoglobin-depleted erythrocyte
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54 18 protein and IgG collected from healthy controls' and patients' sera. Although the amount of
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58 19 IgG was the same for healthy controls and CRC patients, the patients' IgG more efficiently
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precipitated band 3 compared to that of healthy controls, indicating that the ratio of band 3 specific IgG was increased in CRC patients' sera (Fig. 2B, C). Taken together, the autoantibody level recognizing band 3 was increased in CRC patients' sera and bound to erythrocytes (Fig. 2A, B). In a subset analysis, the MFI did not depend on age, FOB status or stage (Fig. 2D).

Increase in erythrocyte membrane-bound IgG and anti-band 3 antibody in a mouse model transplanted with mouse malignant cell lines

In BALB/c mice, erythrocytes obtained from animals with tumors established with the CRC cell line, Colon-26, also showed an increase in erythrocyte membrane-bound IgG as compared with healthy control mice (Fig. 3A). When other BALB/c background cell lines were analyzed, these also expressed band 3. Of these, the hematopoietic WEHI-3 cell line expressed only 70-kDa band 3 (Fig. 3B). To confirm anti-band 3 antibody production, WEHI-3 cells were transplanted subcutaneously. An immunoprecipitation experiment using IgG purified from mice sera revealed that an IgG fraction recognizing band 3 was increased in samples from mice with transplanted WEHI-3 cells compared to those from normal mice (Fig. 3C). Interestingly, this IgG fraction recognized a 70-kDa band 3 that was the same size as that expressed in the WEHI-3 cell line (Fig. 3B, C). These results support the idea that the ectopic expression of band 3 in patients with CRC is associated with an increase in erythrocyte

1 membrane-bound IgG.

2

3 **Band 3 protein expression is negatively regulated by phosphorylation of AMPK in**
4 **human CRC cell lines**

5 Erythropoietin and erythropoietin receptors are induced by HIF-1 α in cancer cells [15].

6 AMPK is activated under hypoxic conditions and controls the expression of band 3 during
7 erythrocyte maturation [16]. We therefore investigated the effect of hypoxic conditions on the
8 expression of p-AMPK and band 3 in human CRC cell lines. In order to mimic the hypoxic
9 environment of tumors, cells were stimulated with CoCl₂, which inhibits HIF-1 α
10 decomposition. To elucidate regulation, HCT116 and SW480 cell lines were selected because
11 they show different responses to CoCl₂ stimulation [17].

12 The transcription of *SLC4A1* mRNA in HCT116 cells did not change significantly in
13 response to CoCl₂ stimulation at indicated concentration (Fig. 4A). On the other hand, the
14 protein expression of HIF-1 α and p-AMPK increased by CoCl₂ stimulation. Although
15 100-kDa band 3 protein expression did not change, 75-kDa protein was suppressed in
16 accordance with upregulation p-AMPK in HCT116 cells (Fig. 4B, C). In contrast, SW480
17 cells showed an intense response to 200 nmol/L CoCl₂, resulting in the 50-fold upregulation
18 of *SLC4A1* mRNA (Fig. 4D). Compared with HCT116 cells, the upregulation of HIF-1 α and
19 p-AMPK by CoCl₂ was moderate. However, upregulation of the 100-kDa band 3 was

1 observed in correlation with an increase in HIF-1 α and *SLC4A1* mRNA (Fig. 4E, F). From
2 these two cell line experiments, it was found that p-AMPK induced by HIF-1 α correlated with
3 suppression of the 75-kDa band 3 in HCT116 cells, while expression of HIF-1 α correlated
4 with the 100-kDa band 3 in SW480 cells. In total, band 3 protein expression in HCT116 cells
5 was slightly decreased, while that of SW480 cells was increased by CoCl₂ stimulation.

6 Similar results were observed by flow cytometry. Both cell lines were stained with
7 anti-human Diego-b (Dib) antibody that reacts with an antigen located in the seventh
8 extracellular loop of band 3. HCT116 cells showed no increase in band 3 expression with
9 CoCl₂ treatment, while SW480 cells showed upregulated band 3 expression (Fig. 4E).

10 Taken together, band 3 expression is controlled by a balance between *SLC4A1* mRNA and
11 p-AMPK levels under hypoxic conditions depending on the cellular context.

13 Discussion

14 Herein, we made the novel observations that band 3 is frequently expressed in CRC tissue
15 and that erythrocyte membrane-bound IgG is increased in patients with CRC.

16 Band 3 staining in the current study was detected in up to 97% of patients with CRC. Alrefai
17 et al. found that *SLC4A1* mRNA and band 3 protein were not detectable in any regions of the
18 normal intestine using PCR and western blotting, respectively [18]. Our findings, based on the
19 use of *SLC4A1* mRNA *in situ* hybridization, strengthen the evidence for the protein

1 expression of band 3 in CRC. Aberrant expression of *SLC4A1*, a mesodermal gene, in
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4 2 epithelial cells may be the result of abnormal methylation and histone modifications in the
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7 3 *SLC4A1* promotor region in CRC [19-21]. We also performed *in vivo* experiments and found
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10 4 that erythrocyte membrane-bound IgG and serum anti-band 3 antibody were increased when
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13 5 tumor cells expressing band 3 were introduced in mice (Fig. 3A, C). This data supports the
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16 6 conclusion that the production of erythrocyte membrane-bound IgG is stimulated by band 3
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19 7 protein expression in CRC.
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23 8 Although CRC cases showed no apparent hemolytic findings, at direct antiglobulin test was
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26 9 positive in 20% of cases in the current study. This indicates the existence of silent red blood
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29 10 cell autoantibodies in patients with CRC. Such autoantibodies, which do not induce clinical
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32 11 hemolysis under both physiological and pathological conditions, have been reported by
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35 12 Mauro et al. [22]. Because the column method is more sensitive than the tube method for
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38 13 detecting antiglobulin, this may explain the higher positive rate of the direct antiglobulin test
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41 14 compared to the results of a previous report [23, 24]. Occasionally, healthy people also test
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44 15 positive in a direct antiglobulin test, but the frequency, at about 0.01%, is very low [25, 26]. It
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47 16 is known that some medications are able to cause drug-induced AIHA [27]. However, patients
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50 17 with a positive antiglobulin test result in the current study had no history of using such
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53 18 medications. This suggests that CRC may be solely responsible for the positive results of the
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56 19 direct antiglobulin tests.
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We found that increased erythrocyte-bound IgG was also detectable by flow cytometry as well as by the direct antiglobulin test in patients with CRC (Fig. 2A). One of the reasons for this observation may depend on a change in binding affinity of autoantibodies to the erythrocyte membrane under the oxidative stress induced by cancer. Briefly, when erythrocytes undergo oxidative stress, band 3 proteins form aggregates, and the binding affinity of the anti-band 3 antibody increases [28, 29]. Compared to our primary case that showed erythrocytes bound IgG at 285 molecules per cell as measured by immunoradiometric assay [9, 30], the current cohort of patients were considered to have lower numbers of molecules, presumably at a similar level to non-AIHA patients with antiglobulin negative hemolytic anemia as described in a previous report [31]. Although the amount of IgG antibodies is known to correlate with the strength of direct or indirect antiglobulin tests [26], CRC patients with increased erythrocyte membrane-bound IgG in the current study showed only moderate anemia compared to AIHA. As the IgG bound to erythrocytes consists of both IgG₁ and IgG₃, a higher amount of IgG isotype may affect the phagocytosis of erythrocytes by autologous macrophages [26]. In the current study, we observed that FOB-negative cases, especially the neo-adjuvant chemotherapy naïve cohort, showed normocytic anemia and a higher reticulocyte count than healthy controls (Tables 2, 3). These observations suggest that anemia due to increased production of anti-band 3 antibodies occurs even in an early stage of CRC without gastrointestinal bleeding; this notion is critical for an early diagnosis of CRC

[32].

Band 3 is the most abundant protein present in the erythrocyte membrane [33]. Since the band 3 null phenotype is extremely rare and corresponding erythrocytes are thus not available [34], a direct target analysis of induced autoantibody is not possible by either direct or indirect antiglobulin test. In our previous case report, we demonstrated, using mass spectrometry, that a protein abundantly immunoprecipitated by the autoantibody was band 3 [9]. In the current study, immunoprecipitation results revealed that anti-band 3 antibody was higher in the IgG fraction of CRC patients' sera compared to that of healthy controls; a band 3-expressing cell line also showed increased erythrocyte bound IgG in a mouse model (Fig. 2B, C and Fig. 3). These findings clearly demonstrate the mechanism of autoimmune disease at least of AIHA.

In vitro experiments using human CRC cell lines revealed that the protein expression of band 3 was regulated by AMPK under hypoxic conditions in a cellular context-dependent manner. These results are consistent with a previous report showing that a decreased p-AMPK level induced band 3 expression in the erythroid cell maturation process, and that GSK621, an AMPK activator, suppressed band 3 expression [16]. AMPK and HIF-1 α are activated by hypoxia, a condition under which tumor cells usually reside [35]. However, the response to hypoxia differs depending on cancer cell subtype [17], with the reason is still unclear. The 75-kDa band that was observed in this experiment was smaller than the normal molecular weight of erythrocyte band 3 and was thought to be an isoform identical to that expressed in

1 kidney cells [36]. Mouse kidney band 3, 70-kDa protein, is smaller than that of rat [37]. Since
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4 kidney band 3 utilizes a unique transcription initiation site, p-AMPK may affect 75-kDa band
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7 3 expression by binding to the kidney band 3 promoter, while HIF-1 α binds to the 100-kDa
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10 erythrocyte band 3 promoter.
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13 The limitations of this study were that the number of cases studied was small and that we
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16 could not enroll healthy controls that were age-matched to patients with CRC. Therefore,
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19 differences in ages between patients with CRC and healthy controls may have affected the
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22 amount of erythrocyte-bound IgG measured. However, among patients with CRC, a
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25 significant increase in MFI for those over 65 years of age was not observed (Fig. 2D).
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29 In conclusion, the clinical significance of erythrocyte membrane-bound IgG in patients with
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32 CRC may be as a major etiology of cancer-related anemia; this suggests that the mechanism
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35 may be boosted by the use of immune checkpoint inhibitors [38].
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39 40 41 42 **Conflict of interest**

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46
47
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49
50
51 Ltd.; and Chugai Pharmaceutical Co., Ltd.
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Author Contributions

AK collected the data, performed experiments, analyzed the results and drafted the initial manuscript. SK designed the study, performed experiments and proofread the manuscript. KK and IH collected the data. TY performed pathological investigations. HM, YS, YK collected the data. TK designed the study and reviewed the manuscript. HM reviewed the manuscript. This manuscript was approved by all authors.

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Table 1: Characteristics of participants

Table 2: Characteristics of CRC patients according to FOB

Table 3: The anemia status of a FOB negative cohort according to treatment with NAC

Fig. 1

Expression of band 3 protein and *SLC4A1* mRNA in CRC tissues

1 A, C, E: Colorectal cancer (CRC) surgical specimens. B, D, F: normal colon tissues. A, B:

2 Hematoxylin–eosin (H–E) staining.

3 C, E: In CRC tissue, band 3 protein and *SLC4A1* mRNA expression was detected by
4 immunohistochemistry and *in situ* hybridization, respectively. The arrow in E indicates the
5 red dot of the *SLC4A1* mRNA hybridization probe.

6 D, F: Band 3 protein and *SLC4A1* mRNA expression, respectively, were not observed in
7 normal colon tissue.

8 The bar at the lower right of each figure represents 100 μ m.

9 Arrowheads in C and D indicate red blood cells.

10 Abbreviations: T, tumor; S, stroma; I, intestinal gland.

11
12 **Fig. 2**

13 Comparison of erythrocyte membrane-bound IgG and serum IgG between healthy controls
14 and patients with CRC

15 A: Distribution of MFI values obtained from flow cytometry in healthy controls and patients
16 with CRC (all participants). * $p < 0.05$ (t -test).

17 B: Immunoprecipitation by serum IgG. Hemoglobin-depleted erythrocyte protein was
18 immunoprecipitated using 10 μ g of IgG purified from healthy controls and patients with the
19 indicated MFI value.

1 C: Quantification by densitometry. The band 3 signal was adjusted by applied IgG. $^{**}p <$
2
3
4 2 0.05 (t-test).
5
6

7 3 D: MFI obtained from flow cytometry were compared for each subcategory of CRC. A
8
9
10 4 statistically significant difference in the MFI depending on, age, FOB status, and clinical
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12
13 5 stage, was not found.
14
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16 6 Horizontal lines in each graph indicate average values at the center and standard deviations at
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19 7 the top and bottom.
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22 8 Abbreviations: MFI, mean fluorescence intensity; CTLs, healthy controls ;CRCs, colorectal
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25 9 cancer patients ; MW, molecular weight ; FOB, fecal occult blood.
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32 11 **Fig. 3**
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35 12 IgG production in mice transplanted with a mouse CRC cell line
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39 13 A: In BALB/c mice, erythrocyte membrane-bound IgG levels in samples from mice with and
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42 14 without CRC tumors were evaluated by flow cytometry.
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45 15 Horizontal lines in each dot plot indicate the average MFI at the center and standard
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48 16 deviations at the top and bottom, respectively. $^{*}p < 0.05$ (t-test)
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51 17 Similar results were obtained from three independent experiments.
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54 18 B: Mouse malignant cell lines expressing two band 3 proteins of different molecular weight.
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58 19 C: Immunoprecipitation of IgG from sera of mice with WEHI-3 transplants compared to sera
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1 from normal mice. Arrow indicates detected protein bands at 70-kDa.

2 Abbreviations: MFI, mean fluorescence intensity; MW, molecular weight.

3

4 **Fig. 4**

5 Regulation of band 3 expression by AMP activated protein kinase

6 A: Comparison of the expression level of *SLC4A1* mRNA as determined by quantitative
7 PCR in control and HCT116 cells cultured under normal conditions or treated with 100 or 200
8 nmol/L CoCl₂ for 24 hours. Error bars represent the standard deviation. The control used was
9 normal colon mRNA.

10 B: Western blot results of protein samples from HCT116 cells cultured under normal
11 conditions and after treatment with 100 or 200 nmol/L CoCl₂ for 24 hours. Arrows indicate
12 band 3 proteins.

13 C: The intensities of 75-kDa and 100-kDa bands detected by anti-band 3 antibody in a
14 western blot were measured by densitometry and compared to that of the corresponding
15 β -actin band.

16 D, E, F: The same experiments as shown in A–C were performed using SW480 cells. * $p <$
17 0.05 (one-way ANOVA followed by Tukey pairwise comparison).

18 G: The results of flow cytometry, using anti-Dib antibody, of human CRC cell lines are
19 shown. In each histogram panel, red is the control antibody and blue is the anti-Dib antibody.

1 The results obtained by culturing each cell line under normal conditions and after treatment
2
3
4 2 with 100 or 200 nmol/L CoCl₂ for 72 hours are shown. The percentage shown in each panel is
5
6
7 3 the positive Dib rate compared to the control.
8
9
10 4 Abbreviations: CoCl₂, cobalt chloride; HIF-1 α , hypoxia inducible factor-1 α ; AMPK, 5'AMP
11
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13 5 activated protein kinase; Dib, Diego-b.
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Table 1: Characteristics of participants

	CRC patients	CRC patients under 65 years	Healthy controls	<i>P</i> value
	(N = 50)	(N = 12)	(N = 26)	
Age, years, median (range)	72 (38–85)	57.5 (38–62)	44.5 (30–62)	
Gender, F/M	22/28	5/7	14/12	
CRC clinical stage, n (%)				
1	10 (20)	0 (0)	N/A	
2	15 (30)	4 (33)	N/A	
3	18 (36)	6 (50)	N/A	
4	8 (16)	2 (17)	N/A	
^a Received neo-adjuvant chemotherapy n (%)	5 (10)	2 (17)	N/A	
Hb (g/dL)				
(ref: 13.5-17) Mean ± SD	12±1.82	12.2±1.83	13.4±1.59	.01 ^b
Mean corpuscular volume (MCV) (fl)				
(ref: 84-99) Mean ± SD	88.3±7.0	89.1±6.2	92.6±6.1	.01 ^b
Reticulocytes (/μL)				
(ref: 36,000-114,000) Mean ± SD	80,382±82,333	57,333±16,816	48,165±12,944	.05 ^b
Haptoglobin (mg/dL)				
(ref: 83-209) Mean ± SD	131.4±75.5	143.2±99.9	89.5±44.1	.01 ^b
IgG (mg/dL)				
(ref: 870-1700) Mean ± SD	1205±281.3	1217±312.5	1168±232.5	.57 ^b
Direct antiglobulin test positive, n (%)	10 (20)	4 (33)	0	.01 ^c
Clinically AIHA	0	0	0	

^a Neo-adjuvant chemotherapy regimen: FOLFOX + bevacizumab, FOLFOXIRI + bevacizumab, FOLFOX + cetuximab, UFT + LV

^b Statistical significance between CRC patients and healthy control groups by *t*-test.

^c Statistical significance between CRC patients and healthy control groups by Fisher's exact test.

Abbreviations: AIHA, autoimmune hemolytic anemia; CRC, colorectal carcinoma; F, female; FOLFOX, 5-fluorouracil, leucovorin and oxaliplatin; FOLFOXIRI, 5-fluorouracil, leucovorin, oxaliplatin and irinotecan; Hb, hemoglobin; LV, leucovorin; M, male; N/A, not applicable; ref, reference value; SD, standard deviation; UFT, tegafur/uracil

Table 2: Characteristics of CRC patients according to FOB

	FOB positive N = 24	FOB negative N = 26
Age, median (range)	69.5 (54–84)	76.5 (56–84)
Gender, F/M	7/17	15/11
CRC clinical stage 1/2/3/4	4/7/6/7	6/8/12/0
Hb (g/dL) Mean $\pm$ SD	12.4 $\pm$ 2.1	11.5 $\pm$ 1.25
MCV (fl) Mean $\pm$ SD	89.26 $\pm$ 6.48	87.25 $\pm$ 7.41
Reticulocytes (/ μ L) Mean $\pm$ SD	93,853 $\pm$ 102,833	65,789 $\pm$ 47,508
Direct antiglobulin test positive	4	6

Abbreviations: CRC, colorectal carcinoma; FOB, fecal occult blood; Hb, hemoglobin; MCV, mean corpuscular volume; SD, standard deviation

Table 3. The anemia status of an FOB negative cohort according to treatment with NAC

	NAC (+) N = 4	NAC (-) N = 22
Hb (g/dL) Mean $\pm$ SD	11.7 $\pm$ 0.69	11.5 $\pm$ 1.34
MCV (fl) Mean $\pm$ SD	92.75 $\pm$ 2.94	86.15 $\pm$ 7.54
Reticulocyte (/ μ L) Mean $\pm$ SD	57,250 $\pm$ 12,049	67,487 $\pm$ 51,594
Direct antiglobulin test positive	1	5

Abbreviations: FOB, fecal occult blood; Hb, hemoglobin; MCV, mean corpuscular volume; NAC, neo-adjuvant chemotherapy; SD, standard deviation

Fig 1.

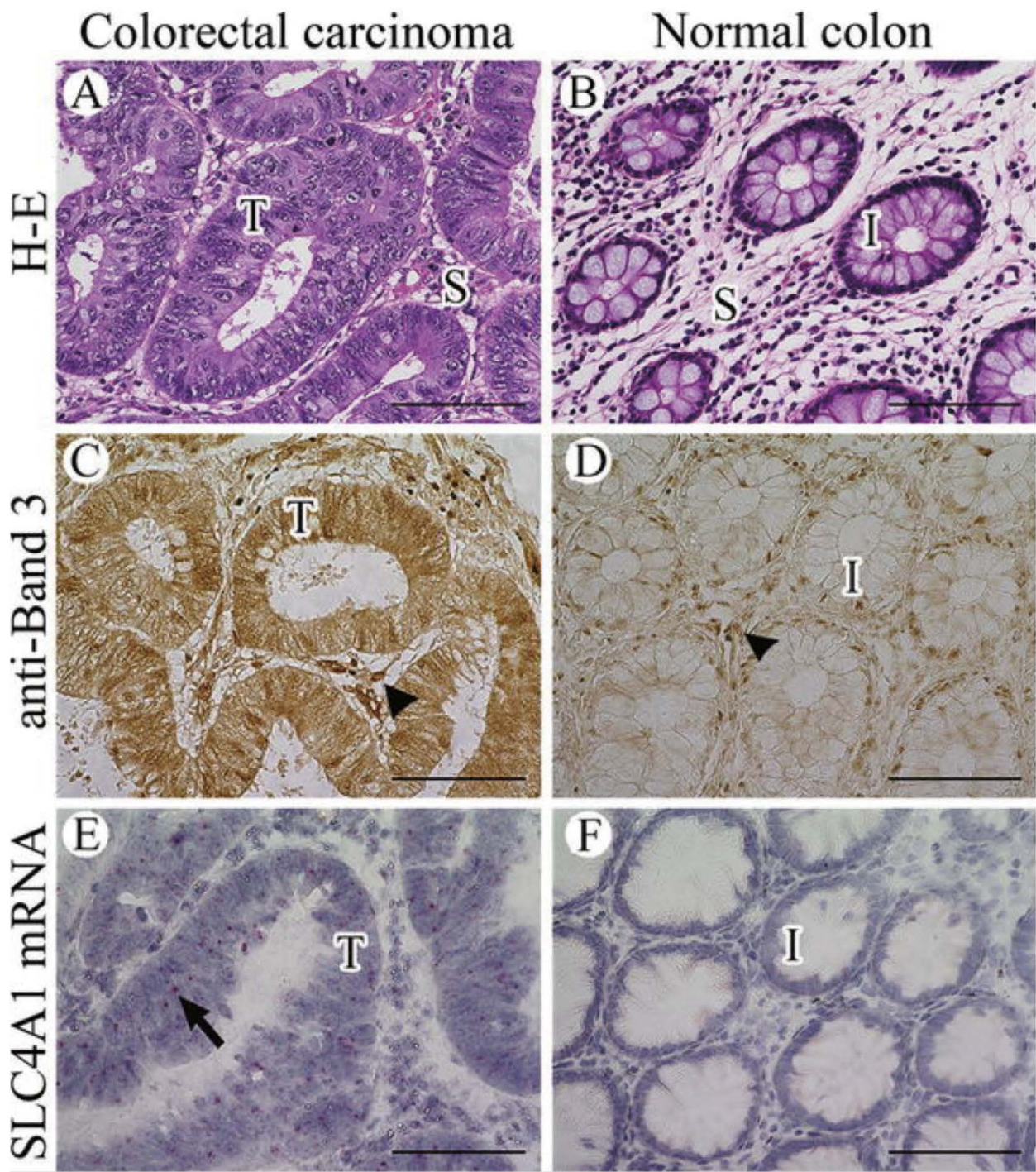
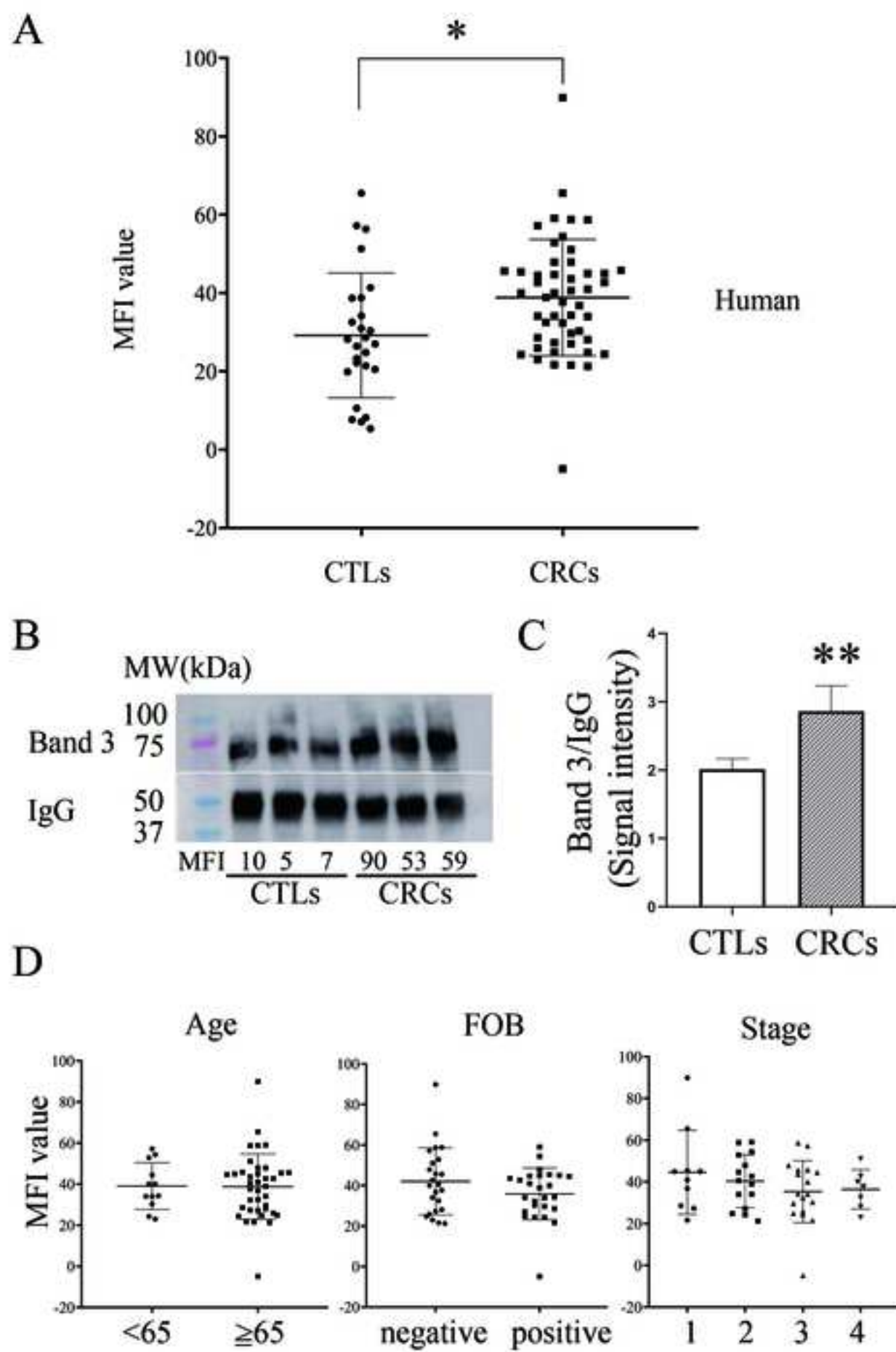
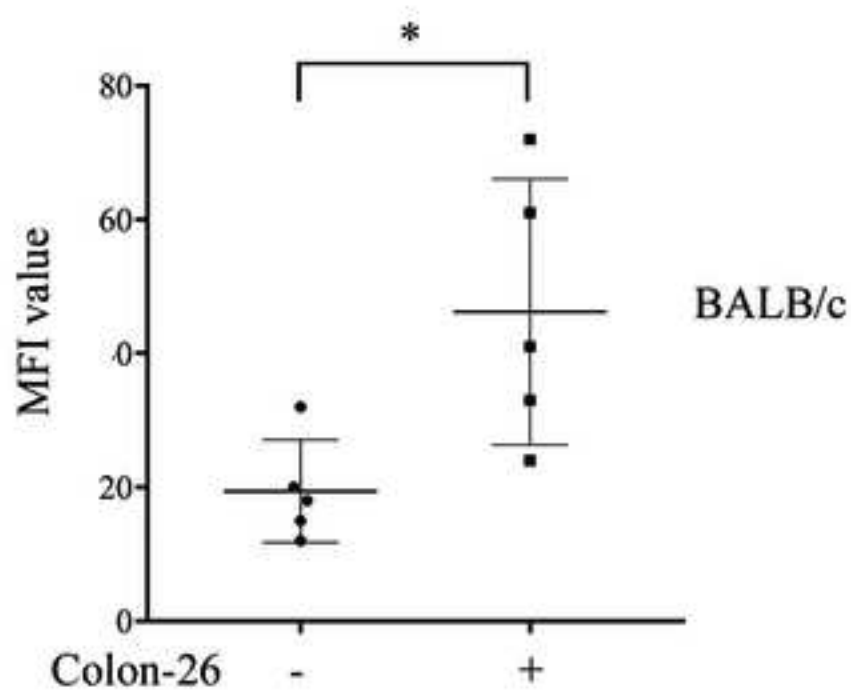


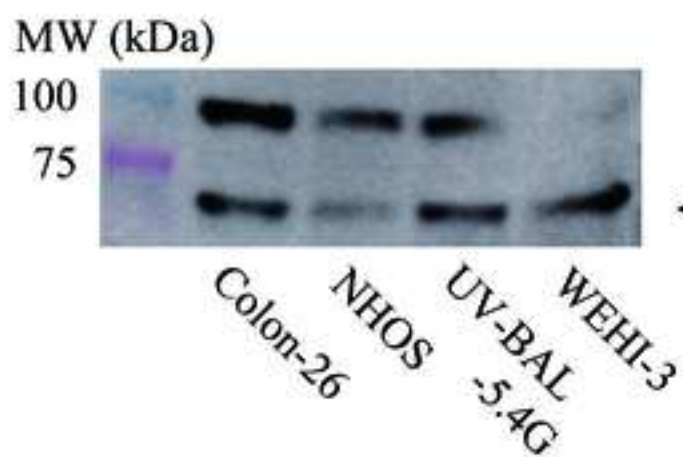
Fig 2



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B



C

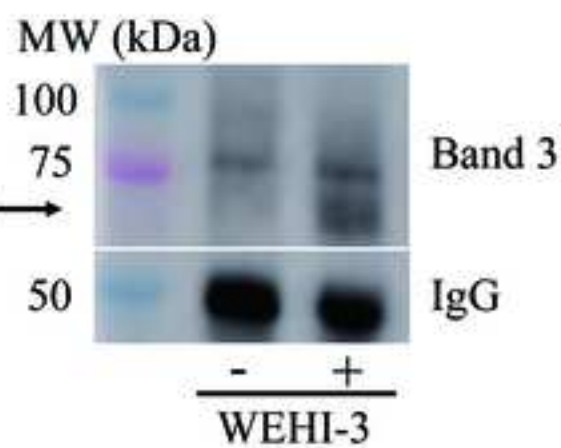


Fig 4

