Is anti-HMGB1 Antibody a Potential Characteristic Autoantibody for Sjögren's Syndrome?

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Research article

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Abstract

BACKGROUND: Sjögren's syndrome (SS) is a common chronic inflammatory autoimmune disease, affects about 0.33% to 0.77% population in China. The positive for antinuclear antibodies (ANA) is one of the key features of SS, which show typical granular pattern in indirect immunofluorescent antibody test (IIFT). About 70% SS patients have the anti-SS-A and/or SS-B antibodies, which indicates other autoantibodies in SS patients.

METHODS: The anti-HMGB1 antibody in 93 SS patients and 96 healthy controls were investigated with in-house built ELISA and immunoblotting; and the location of HMGB1 and fluorescent pattern of anti-HMGB1 antibody were investigated with IIFT. The contribution of anti-HMGB1 antibody in ANA-IF with Cas9-induce HMGB1 knockout cells were also detected.

RESULTS: The anti-HMGB1 antibody in SS patients (9.96±5.55 VS 4.9±1.4 RU/ml RU/ml compared to healthy controls) increased. With ROC curve analysis, when taking 8 RU/ml as the cutoff value, the sensitivity, specificity, and the area under the curve were 64.5%, 96.9% and 0.83, respectively. A total of 18 patients (20.7%) with granular pattern in ANA-IF test were anti-HMGB1 antibody positive only. With commercial antibody, the fluorescent pattern of anti-HMGB1 antibody showed typical granular pattern. The serum from ANA-IF (+), SS-A (-), and SS-B (-) SS patients showed typical granular pattern in wildtype B16 cells, but no fluorescence in HMGB1 knockout B16 cells.

CONCLUSIONS: Anti-HMGB1 antibody may be one of the characteristic autoantibodies of SS except anti-SS-A and SS-B. The detection of anti-HMGB1 antibody can provide more laboratory evidence for clinical diagnosis of SS.

Background

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease characterized by invasion of exocrine glands. Its clinical manifestations include symptoms caused by dryness of the glands and symptoms caused by involvement of the extraglandular system(1, 2). In China, the incidence of SS is between 0.33% and 0.77%, which is the second highest prevalence of autoimmune disease after rheumatoid arthritis (RA), and the male to female incidence rate is about 1: 9 to 1: 19(3). The pathogenesis of SS has not been fully elucidated. The positive for antinuclear antibodies (ANA) is one of the key features of SS, which show typical granular pattern in indirect immunofluorescent antibody test (IIFT). In the clinical laboratory, 70% of the SS patients can show anti-SS-A antibody, and about 45% of them can show anti-SS-B antibody. Nucleoprotein and nucleic acid are important sources of autoantigens. At present, it is known that SS-A is composed of protein antigens of 52 kD and 60 kD combined with cytoplasmic RNA species, and SS-B is composed of a 48-kD protein combined with RNA species. Partial confirmed SS patients show positive for ANA in IIFT, while negative for SS-A/SS-B in antibody test; which indicates SS patients have other autoantibodies. According to literature review and our preliminary researches(4–6), High mobility group box 1 (HMGB1) played a key role in inflammation-

related immune regulation, and other studies have shown that anti-HMGB1 autoantibody can be detected in a variety of autoimmune diseases. In this article, the possibility of HMGB1 being one of SS autoantigens was systematically investigated.

Materials And Methods

SOURCES OF SPECIMEN

A total of 93 patients diagnosed as SS according to the SS diagnostic criteria of Chinese Association of Rheumatology were included in the SS group (7), and another 96 healthy persons were recruited into the control group. The demographical data, e.g. age and gender, were collected. The serum samples were collected and stored at -80 °C. This study used the remaining samples from the clinical laboratory and exempt informed consent, and was approved by the Medical Ethics Committee of Changhai Hospital.

IN-HOUSE ELISA TEST OF ANTI-HMGB1 ANTIBODY

The procedure of serum anti-HMGB1 antibody test was according to reference(8) with minor modification: Maxisorp polystyrene 96-wells plates were coated with 50 µL per well of rHMGB1 (R&D Systems, Minneapolis, USA) at 1 µg/ml in PBS and incubated overnight at 4 °C. After one wash, plates were blocked with Blocker Casein (Thermo, Rockford, USA) for one hours. Serum samples, diluted 1:50 with the sample buffer, were added in duplicate (100 µl/well) and incubated for two hours at room temperature. After five washes, 100 µl HRP-conjugated rabbit anti-human IgG (Euroimmun, Lubeck, Germany) was added to each well and incubated for 30 minutes at room temperature. After washing, bound antibodies were detected using 3,3',5,5'-tetramethylbenzidine dihydrochloride/hydrogen peroxide (TMB/H_2O_2). The reaction was stopped with 0.5 M sulphuric acid and the absorbance was measured at 450 nm using a microplate-spectrophotometer (Thermo MK3, Rockford, USA). Anti-HMGB1 antibody levels were expressed in relative units.

INDIRECT IMMUNOFLUORESCENCE TEST: IIFT

There were three different IIFTs in this study. Antinuclear antibody immunofluorescence kit (Euroimmun, Lubeck, Germany. Item No. FC 1510) was used to perform the ANA-IF test, and the test took fixed Hep2 cells and monkey liver slices as the detection matrix. The procedure was according to the manufacturer's instructions.

Anti-HMGB1 antibody fluorescence pattern study used the same fixed Hep2 cells and monkey liver slices as those in ANA-IF test. The anti-HMGB1 antibody (AB79823, Abcam) and isotype control (AB172730, Abcam) diluted at 1:100 were used as the primary antibodies. The secondary antibody was alex488-goat-anti-rabbit IgG antibody (AB150077, Abcam) diluted at 1:200. The detection procedure was the same as above.

In order to study the effect of HMGB1 knockout on the serum ANA-IF pattern of SS patients, mouse melanoma cell lines (Wild type B16 and HMGB1 knockout type $B16^{HMGB1-}$) fixed on slides were used as

the matrix to detect the fluorescence patterns of antinuclear antibody in SS patients. The secondary antibody used FITC-goat anti-human IgG antibody (Euroimmun, Lubeck, Germany). The detection procedure was the same as above.

IMMUNOBLOTTING TEST

Anti-SS-A and anti-SS-B antibodies were detected with Euroline ANA profile kits (Euroimmun, Lubeck, Germany) on an automatic EUROBlotone instrument according to manufacturer's protocol. Anti-HMGB1 antibodies were further confirmed by an in-house built immunoblotting: Nitrocellulose membrane was coated with three bands of different amounts of rHMGB1 protein (0.001, 0.01, and 0.1 μg) as well as a control band. The strips were incubated with 15 μl serum for 2 hours at room temperature. After 3 washes, ALP-conjugated goat anti-human IgG (Euroimmun, lubeck, Germany) was added to each lane and incubated for 30 minutes at room temperature. After washing, bound antibodies were detected using nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). The bands in the strip were detected with a scanner and analyzed with EUROLineScan.

Statistical analysis

All data were expressed as $\bar{X}\pm SD$. Student *t* test was used for comparison between two groups. The ROC curve was used to evaluate the diagnostic efficacy of anti-HMGB1 antibodies in SS with Graphpad® Prism 6. $P < 0.05$ indicates statistical significance.

Results

Anti-HMGB1 autobodies in the serum of SS patients were significantly increased

The SS patients aged 23 to 80 (median, 52 years old) (Table 1). Among them, 66.7% tested positive for SS-A antibodies, while 40.9% tested positive for SS-B antibodies. The results of anti-HMGB1 ELISA showed the content of anti-HMGB1 autobodies in SS patients (9.96 ± 5.55 RU/ml) were significantly higher than those of the control group (4.9 ± 1.4 RU/ml) (Fig. 1a, supplFig. 1). In ROC curve analysis, when taking 8RU/ml as the cutoff value, the sensitivity and specificity were 64.5% and96.9% respectively, and the area under the curve was 0.83 (95% confidence interval: 0.76–0.90) (Fig. 1b).

Table 1
Clinical characteristics and serum anti-HMGB1 in SS and healthy controls.

	SS	Healthy Control
Gender(M/F)	5/88	53/43
Age	52(23–80)	52(15–84)
anti-SS-A	67.7%(63/93)	-
anti-SS-B	39.8%(37/93)	-
Anti-HMGB1	9.96 ± 5.55	4.9 ± 1.4

Detection of related autoantibodies in SS patients with ANA-IF positive

In the SS group, 87 from 93 SS patients were positive for ANA-IF, appearing the typical granular pattern with or without other fluorescence modes. The results of immunoblotting detecting anti-SS-A, anti-SS B, and anti-HMGB1 antibodies were shown in Fig. 2. Sixty-one patients (70.1%) were positive for anti-HMGB1, among whom: 25 patients (28.7%) were also detected anti-SS-A and anti-SS-B, 18 patients (20.7%) were also detected anti-SS-A, and 18 patients (20.7%) were only detected anti-HMGB1 positive. Among those patients with negative for anti-HMGB1 (26, 29.9%), 20 patients (23.0%) were detected anti-SS-A antibodies, 12 patients (13.8%) were detected anti-SS-B antibodies, and 6 patients (6.9%) were detected none.

The anti-HMGB1 antibody showed a typical granular fluorescence pattern

In the 24 ANA-IF positive cases without the detections of SS-A and SS-B, 18 cases were detected positive for anti-HMGB1 antibody. Commercial anti-HMGB1 antibody was used to study the fluorescence pattern of anti-HMGB1 antibody. The test took fixed Hep2 cells and monkey liver slices as the detection matrix. The detection results were shown in Fig. 3. The fluorescence of anti-HMGB1 antibody presented the typical granular fluorescence pattern of SS.

Some ANA-IF positive in SS patients came from anti-HMGB1 autoantibodies was confirmed by using HMGB1 knock outing cell line $B16^{HMGB1-}$

In order to further investigating the existing of anti-HMGB1 antibodies, we constructed a Cas9-induced HMGB1 knockout B16 cell line ($B16^{HMGB1-}$) in our lab (see supplementary methods, and SupplFig.2). Taking the Wild Type Mouse Melanoma Cell Line (B16) and HMGB1 knockout type $B16^{HMGB1-}$ as the detection matrix, the serum of the SS patients with ANA-IF presented typical granular fluorescence pattern reran the IIFT. The detection results were shown in Fig. 4. ANA(+), SS-A(+), and SS-B(±) serum still showed typical granular fluorescence pattern in $B16^{HMGB1-}$; while ANA (+ or ±), SS-A (-), and SS-B (-) serum presented typical granular fluorescence pattern in wild type B16, and there was no fluorescence in

$B16^{HMGB1-}$, which confirmed that ANA-IF fluorescence came from the anti-HMGB1 autoantibodies in the patient.

Discussion

HMGB1 is a highly conserved non-histone nuclear protein that is abundantly expressed in most eukaryotic cells (10^6 molecules/cell). Composed of 215 amino acids, HMGB1 in the nucleus can regulate the stability of nucleosomes, participate in the recombination, replication, repair, and transcription of DNA, and can be actively or passively released under physiological and pathological conditions to exert an inflammatory cytokine-like role. In the nucleus, the HMGB1 protein generally binds to the minor groove of the DNA double helix on the nucleosome, and the nucleosome and its components, such as nucleosomes, histones, DNA, etc., are a large source of autoantigens(9, 10). Based on these, it is inferred that HMGB1 may also cause an autoimmune response, produce autoantibodies, and cause autoimmune diseases. A large number of studies, including our previous basic work, have shown that HMGB1 plays a role in the occurrence and development of infectious diseases and autoimmune diseases. It, therefore, is an important point in the regulation of immune homeostasis, which may be an important target for the diagnosis and treatment of related diseases.

Laboratory indicators play a key role in the diagnosis and efficacy monitoring of autoimmune diseases. Indirect fluorescent ANA screening and detection of related autoantibodies can provide important evidences for the diagnosis and differential diagnosis of autoimmune diseases(1). In SS, ANA shows a typical granular fluorescence pattern. The presence of multiple autoantibodies in patients with SS is one of the characteristics of the disease. Among these patients, anti-SS-A autoantibody can be detected in about 70% of the patients, and SS-B autoantibody can be detected in 45% of the patients. In some ANA-positive patients, both anti-SS-A and SS-B antibodies are negative, indicating that there are some autoantibodies with other characteristic in SS patients. Through in-house built ELISA and immunoblotting, we found that the serum anti-HMGB1 antibody level in SS patients increased. Taking 8 RU/ml as the cut-off value, the sensitivity and specificity of diagnosing SS reached 64.5% and96.9%, respectively, and the area under the ROC curve was 0.83, which had potential clinical application value.

The nuclear components are abnormally recognized by the body's immune system and produce autoantibodies, which is an important pathogenesis of autoimmune diseases. Whether HMGB1, as a non-histone nuclear protein, can cause the body's autoimmune response and lead to pathological changes is one of the hotspots of research. Many studies have shown that anti-HMGB1 antibodies can be detected in patients with systemic lupus erythematosus (SLE) (8, 11–13), and it is related to the severity of the disease. A variety of autoantibodies can appear in patients with autoimmune diseases, which have certain disease specificities, such as anti-ds-DNA antibodies and anti-Sm antibodies in SLE patients. There are also some antibodies that can be presented in multiple autoimmune diseases, for example, anti-ss-DNA (single-stranded DNA) antibodies can be detected in SLE, mixed connective tissue disease (MCTD), and polymyositis/dermatomyositis.

In this study, it was found through immunoblotting detection that 61 (70.1%) of 87 patients with ANA-IF exhibiting a typical granular fluorescence pattern were positive for anti-HMGB1, among whom 43 patients (49.4%) were positive for anti-SS-A, and 20.7% (18) were positive for anti-HMGB1 alone, suggesting that anti-HMGB1 antibody was one of the sources of fluorescence in the ANA-IF experiment. Then commercial anti-HMGB1 antibody was used to study the typical fluorescence pattern of anti-HMGB1 antibody. Fixed Hep-2 and monkey liver slices were used as the matrix, and the anti-HMGB1 antibody presented a typical granular fluorescence pattern, which was consistent with our hypothesis. Some studies have shown that the anti-HMGB1 antibody presented a diffuse cytoplasmic staining pattern, which is believed to be caused by activation of tumor cells, and then HMGB1 exits the nucleus and enters the cytoplasm(12). Our experimental results showed that the fluorescence pattern of anti-HMGB1 antibody in HEP-2 showed a typical granular pattern, and the fluorescence in the cytoplasm was very weak. The fluorescence in the monkey liver slices also showed a granular pattern. Taking the fact that HMGB1 is a nuclear protein into consider, we believe that granular fluorescence pattern is the actual pattern for anti-HMGB1 antibody.

In order to further confirm that anti-HMGB1 antibody is one of the characteristic autoantibodies of SS, we used Cas9 technique to construct a HMGB1 knockout mouse melanoma cell line $B16^{HMGB1-}$ to repeat the ANA-IF experiment. The results showed that ANA (+ or ±), SS-A (-), and SS-B (-) serums showed typical granular fluorescence pattern in wild-type B16, but there was no fluorescence in $B16^{HMGB1-}$, confirming that ANA-IF fluorescence came from the anti-HMGB1 autoantibodies in the patients.

In conclusion, the results of this study show that anti-HMGB1 antibody may be one of the characteristic autoantibodies of SS. The detection of anti-HMGB1 antibody can provide laboratory evidence for clinical diagnosis of SS, and further improve the accuracy of SS diagnosis based on the existing detection indicators. Further research on the role of HMGB1 in the pathogenesis of SS may lead to the development of new drugs or treatment strategies for the treatment of SS.

Abbreviations

SS
Sjögren's syndrome
HMGB1
High mobility group box 1
ANA
antinuclear antibodies
IIFT
indirect immunofluorescent antibody test

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Changhai Hospital. The need for patient consent was waived due to this study used the remaining samples from the clinical laboratory and the absence of personally identifiable data in the report.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Conflict of interests

All authors declare that they have no conflict of interests.

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Authors' contributions

M.C and Y.Q. contributed to the research idea and study design. M.C., Y.Z., M.X., and R.Z. contributed to the data acquisition. M.C., M.X., L.J., L.L., and Y.Q. contributed to the data interpretation. Y.H. and L.L. performed the statistical analysis. Y.Z. and Y.Q. prepared the manuscript. All authors approved the final submission.

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Figures

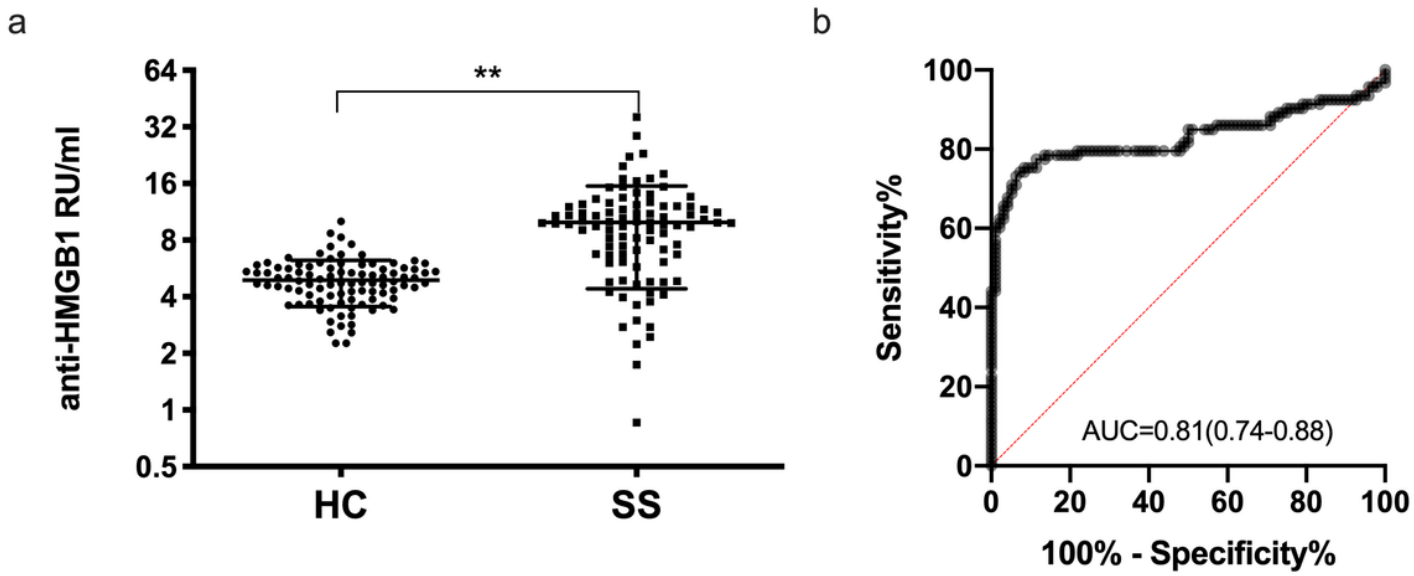
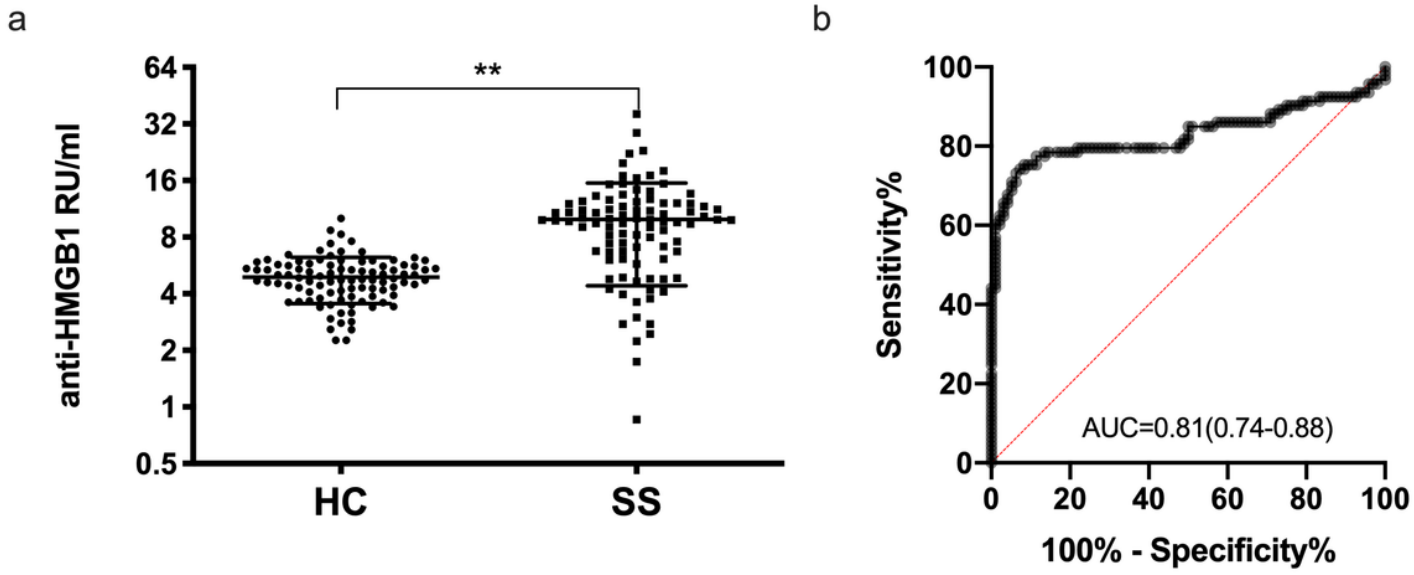


Figure 1

Serum anti-HMGB1 antibody in SS patients a) ELISA results showed that the anti-HMGB1 antibody were 4.9±1.4RU/ml and 9.96±5.55RU/ml in healthy controls and SS patients, respectively. The comparison between the healthy controls and SS patients was tested by Student's t-test. ** represents P<0.01. b) ROC curve analysis was performed to calculate the optimal cutoff value of anti-HMGB1 antibodies in diagnosis of SS. When taking 8RU/ml as the cut-off value, and the sensitivity and specificity were 64.5% and96.9% respectively, and the AUC was 0.83 (95% confidence interval: 0.76-0.90).



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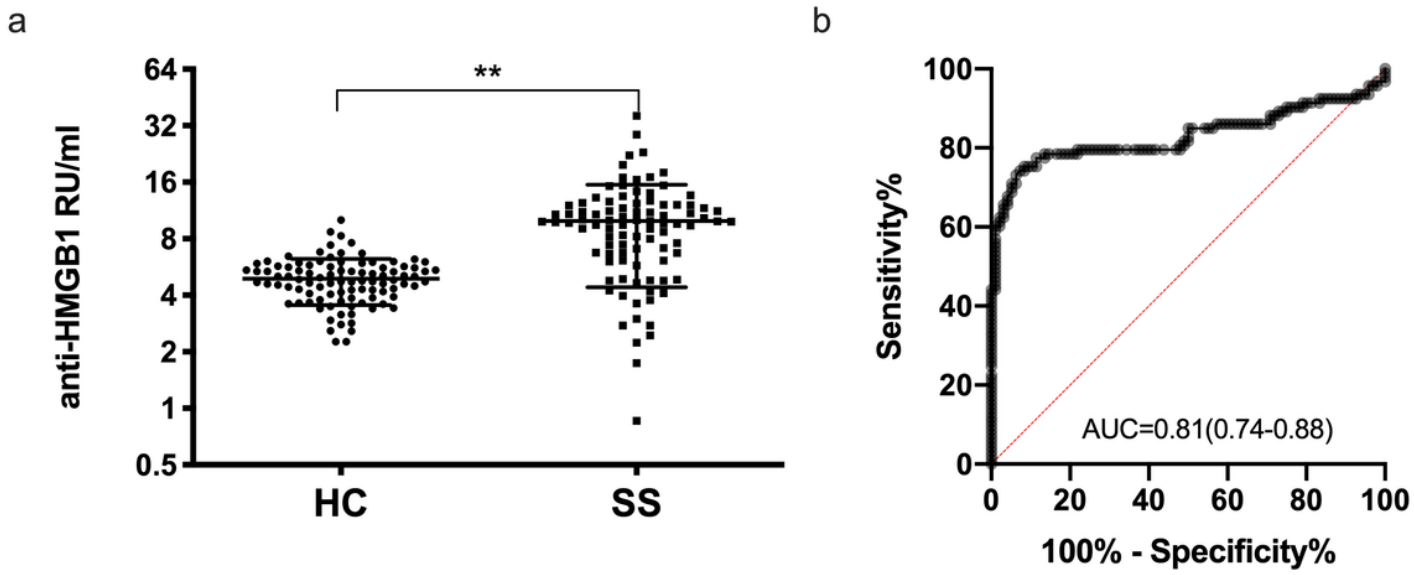

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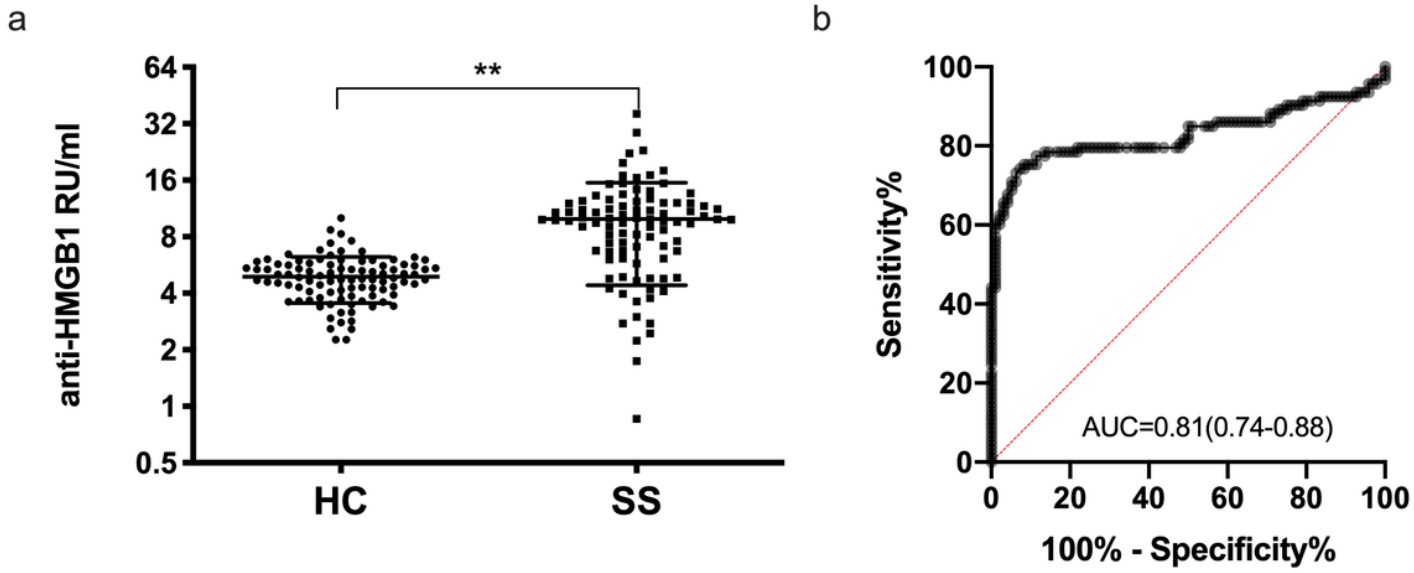

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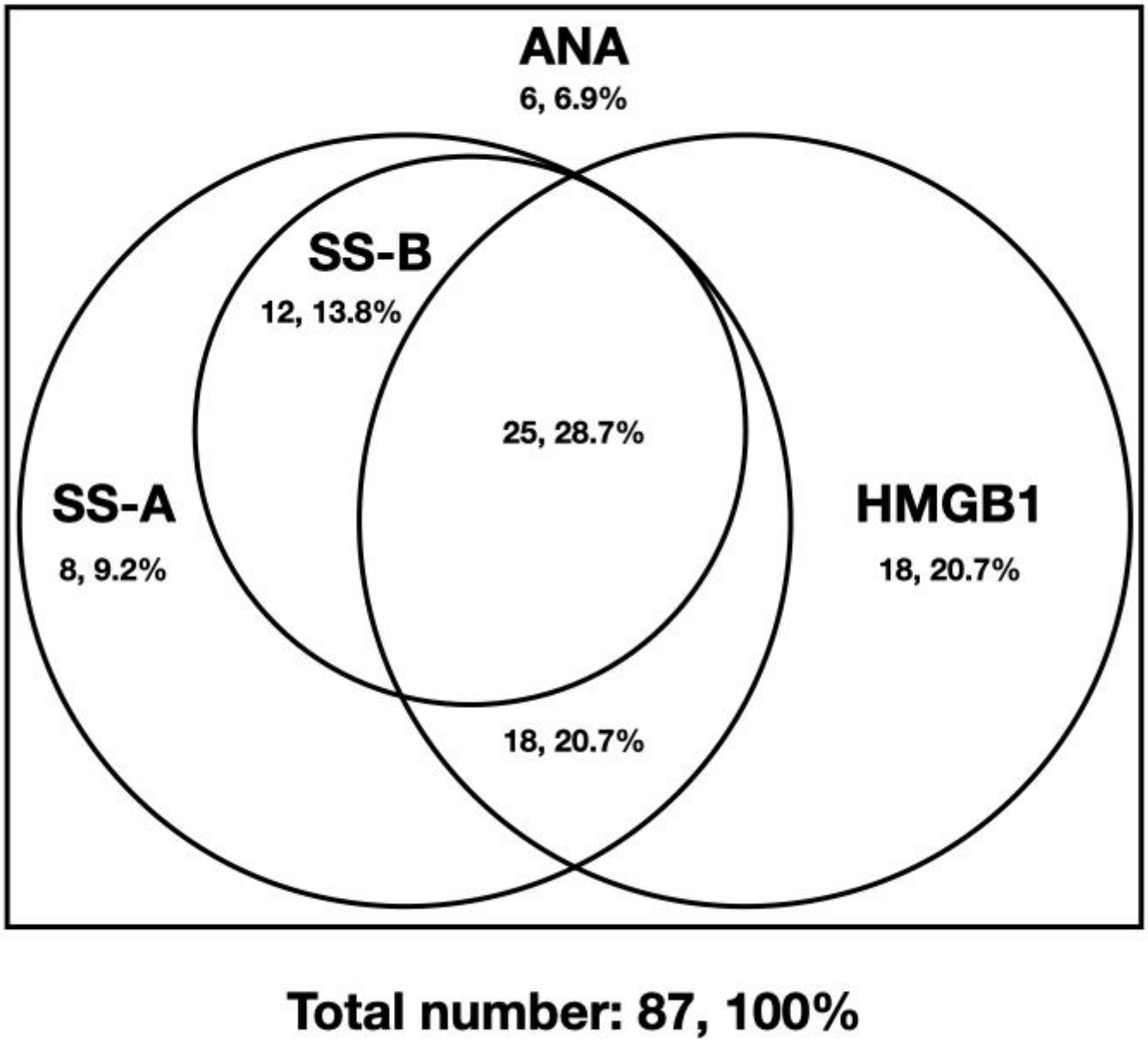
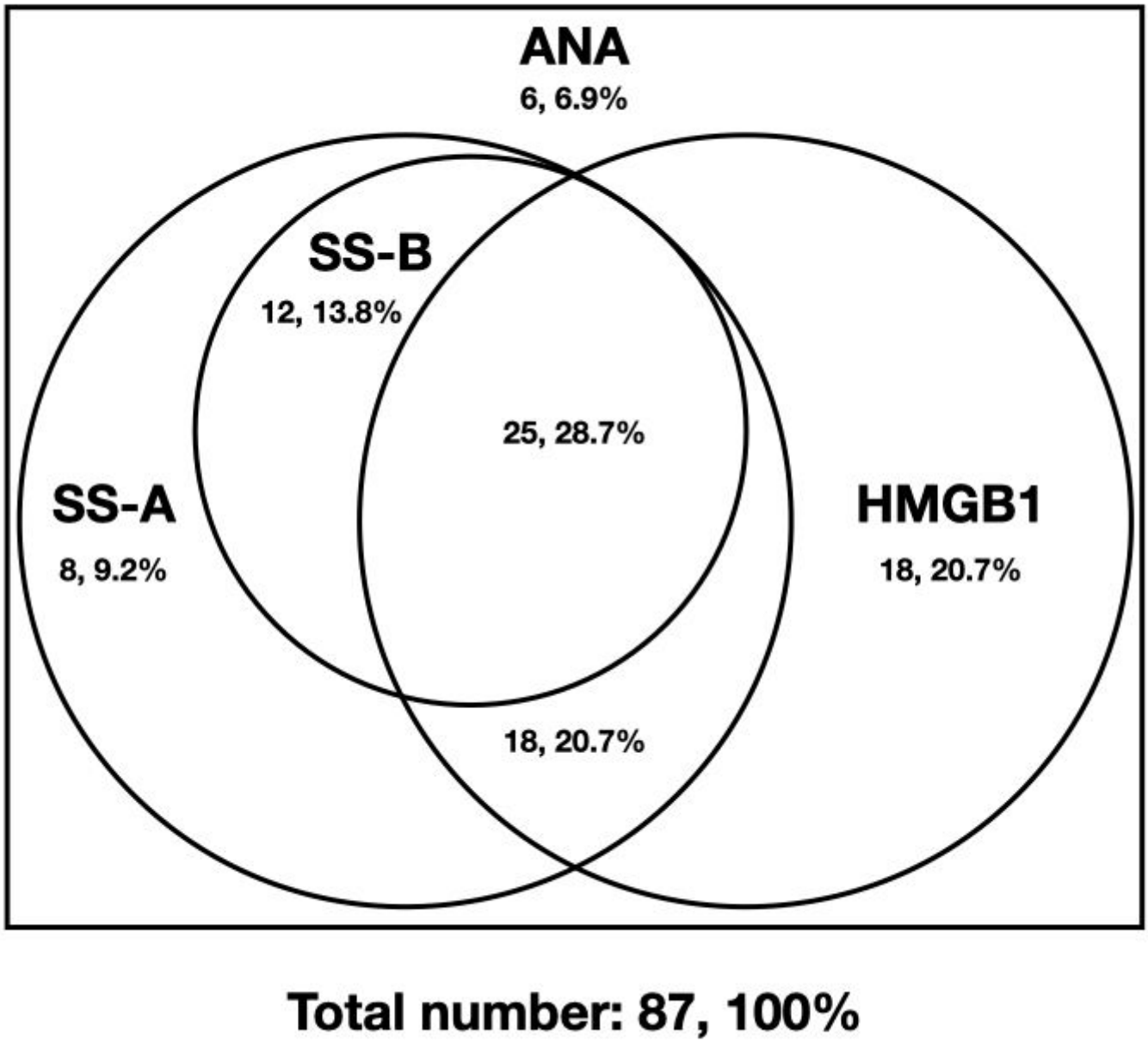


Figure 2

The overlap of anti-HMGB1, anti-SS-A, and anti-SS-B in ANA-IF positive SS patients



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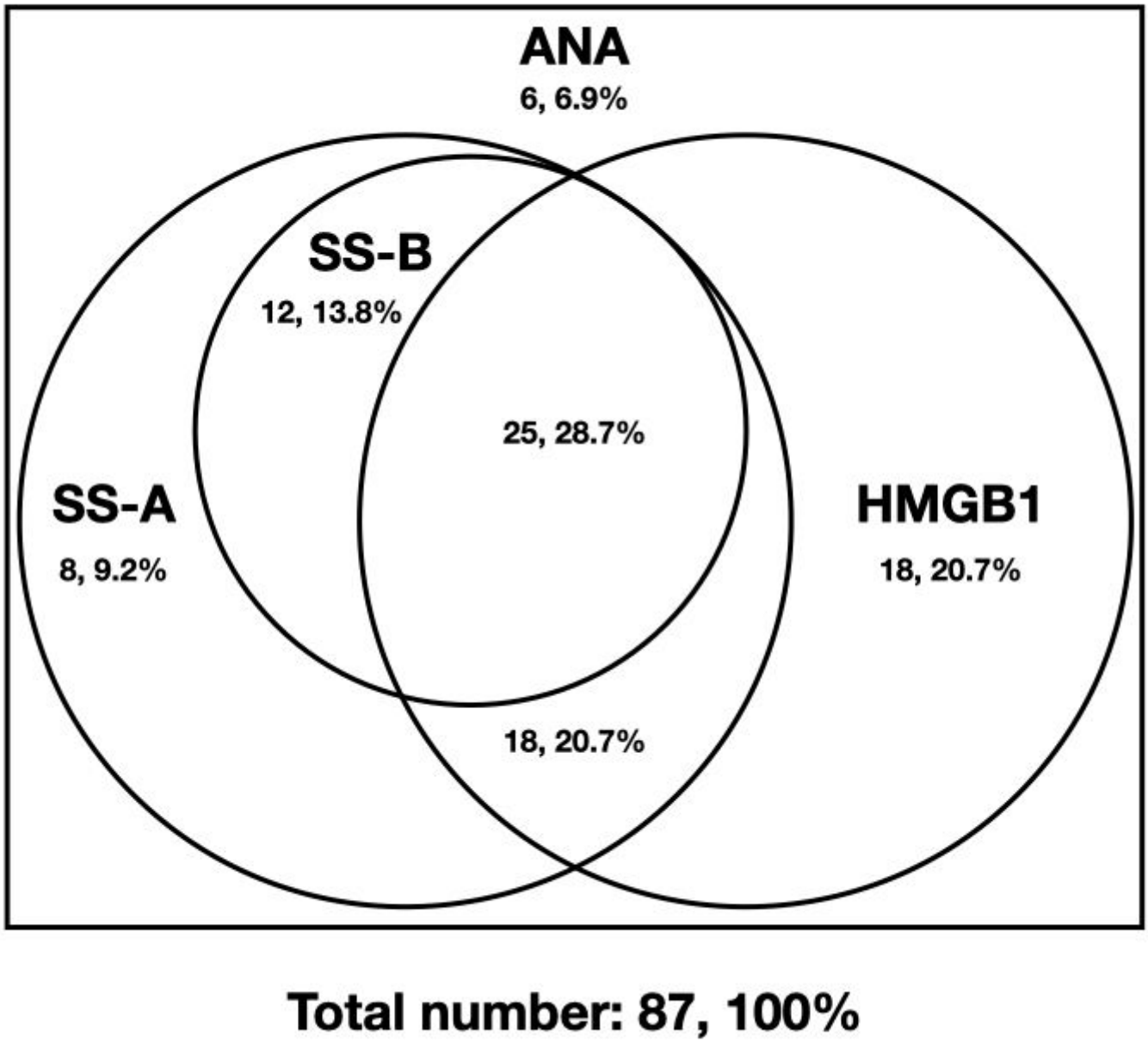

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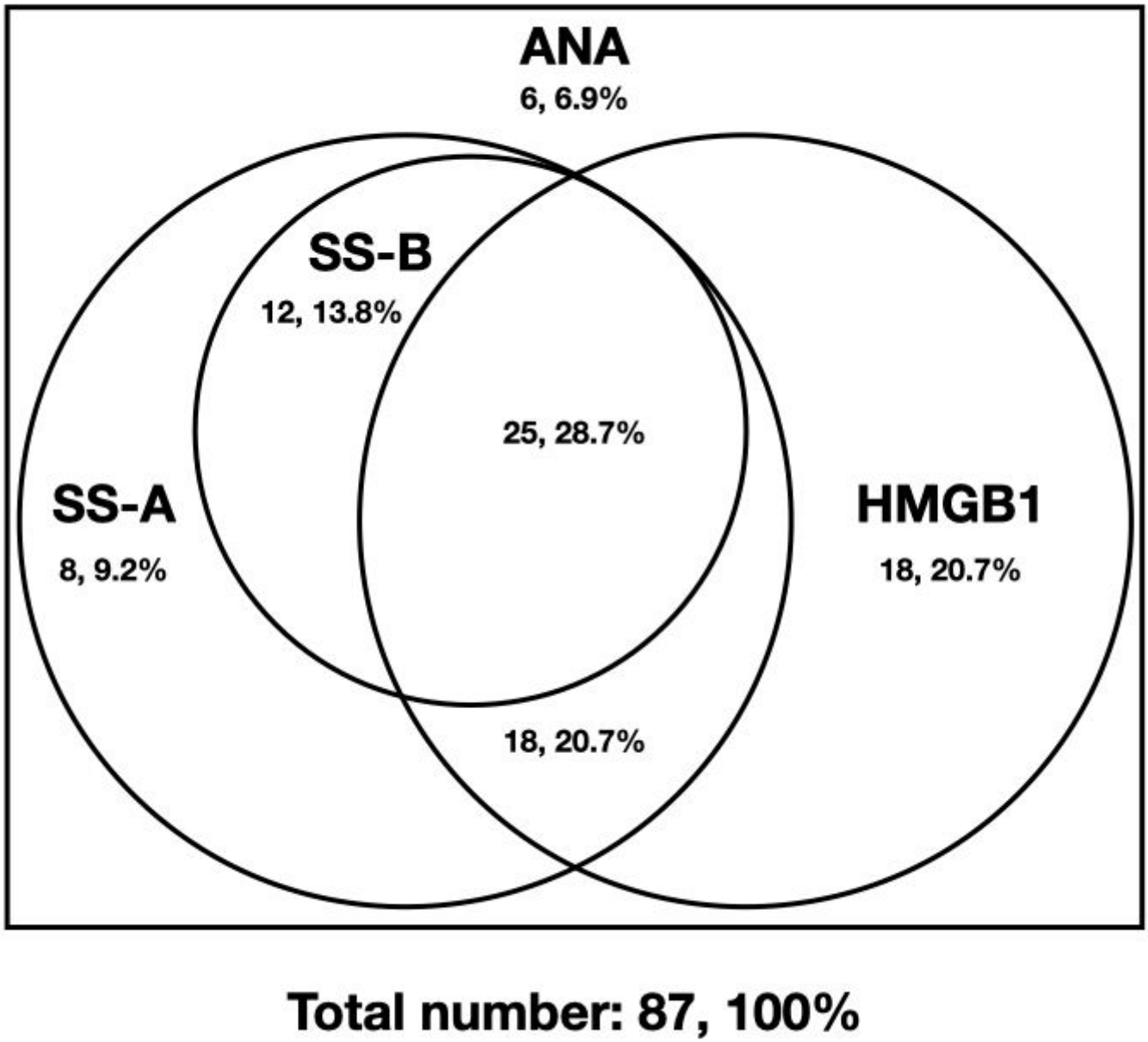

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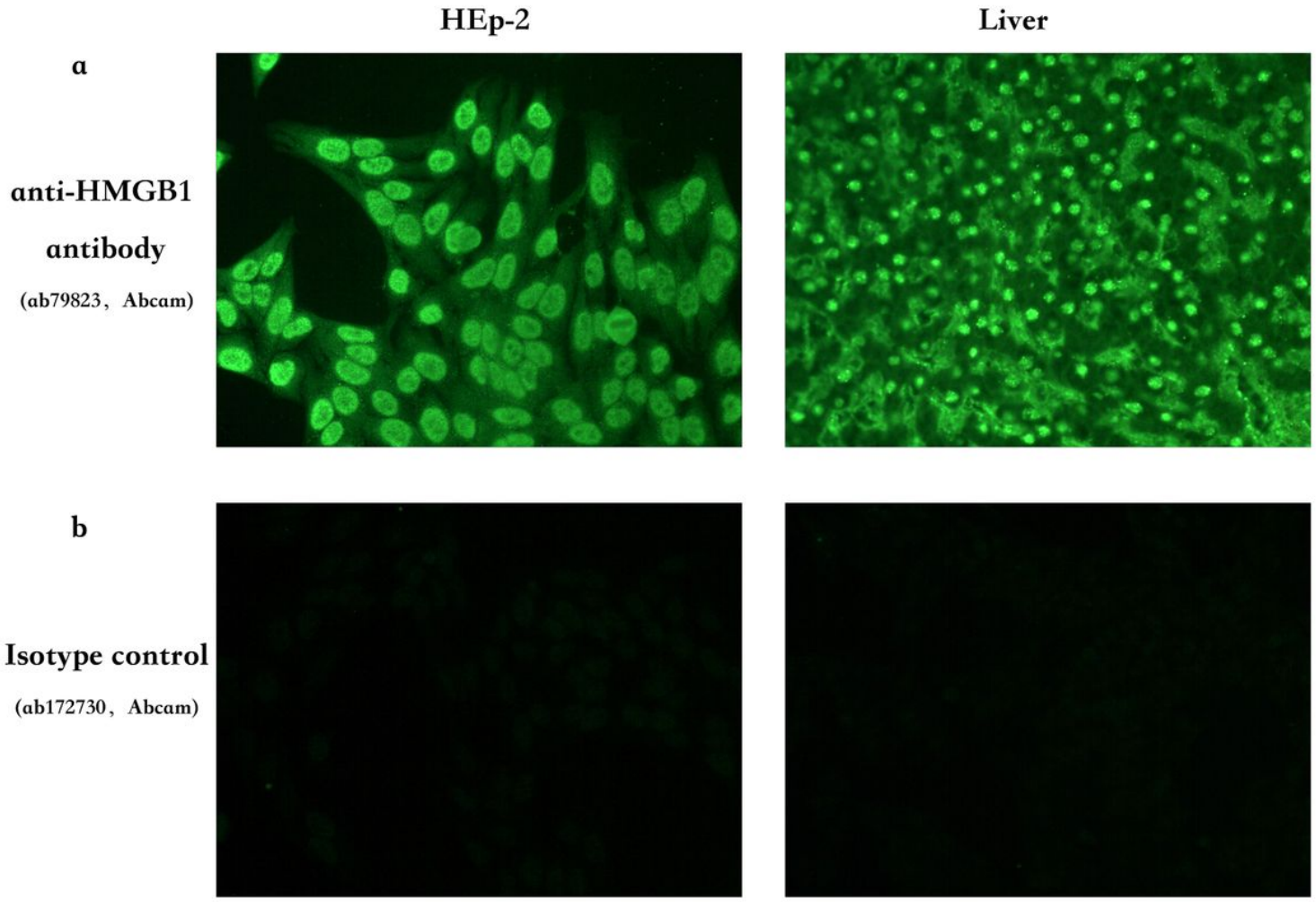
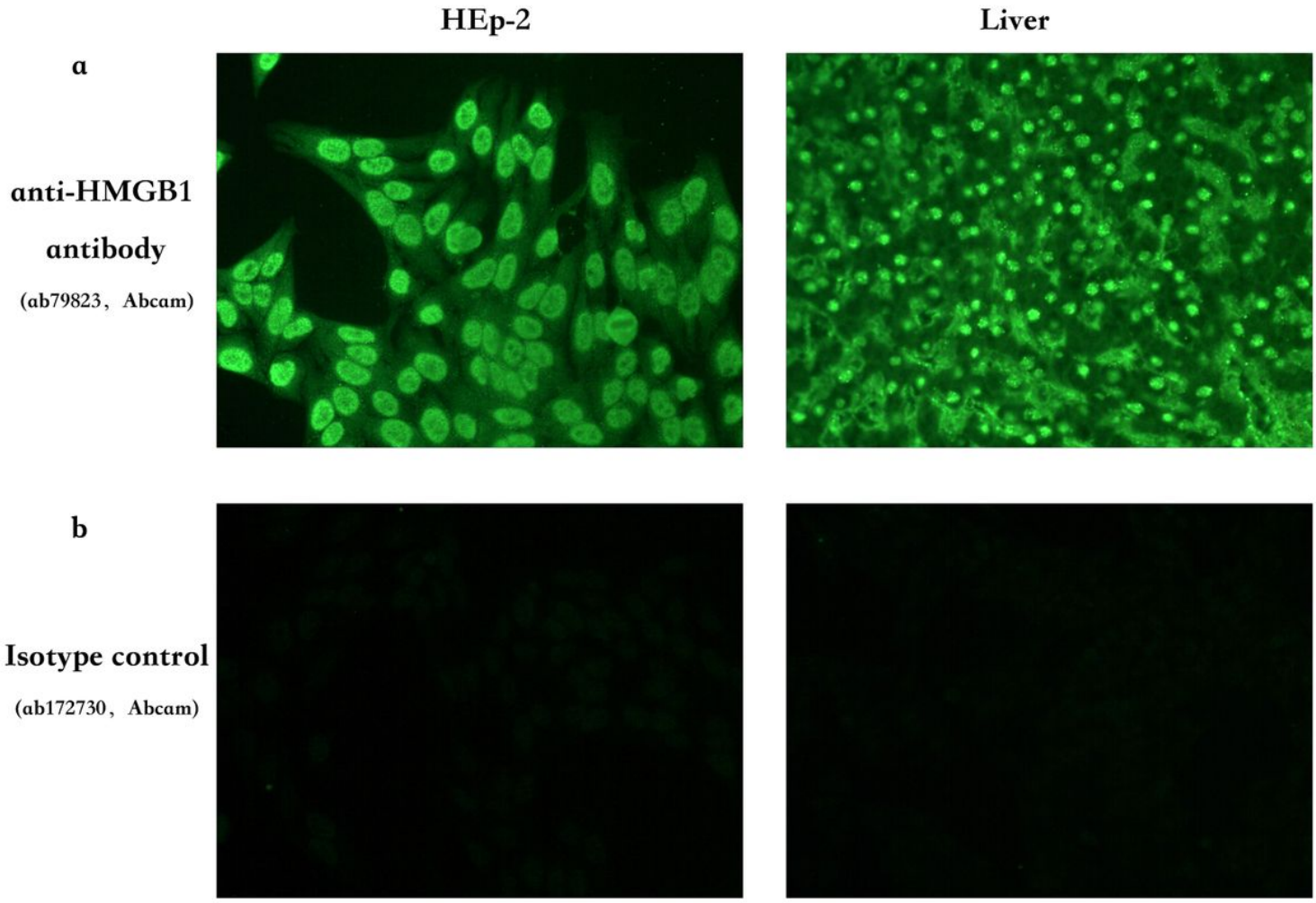


Figure 3

Fluorescence pattern of anti-HMGB1 antibody Anti-HMGB1 detected with IIFT a) Commercial anti-HMGB1 antibody was used to determine the location of HMGB1 and the typical fluorescence patter in fixed Hep-2 cells and monkey liver slice. b) Isotype control antibody was used to rule out the nonspecific fluorescence.



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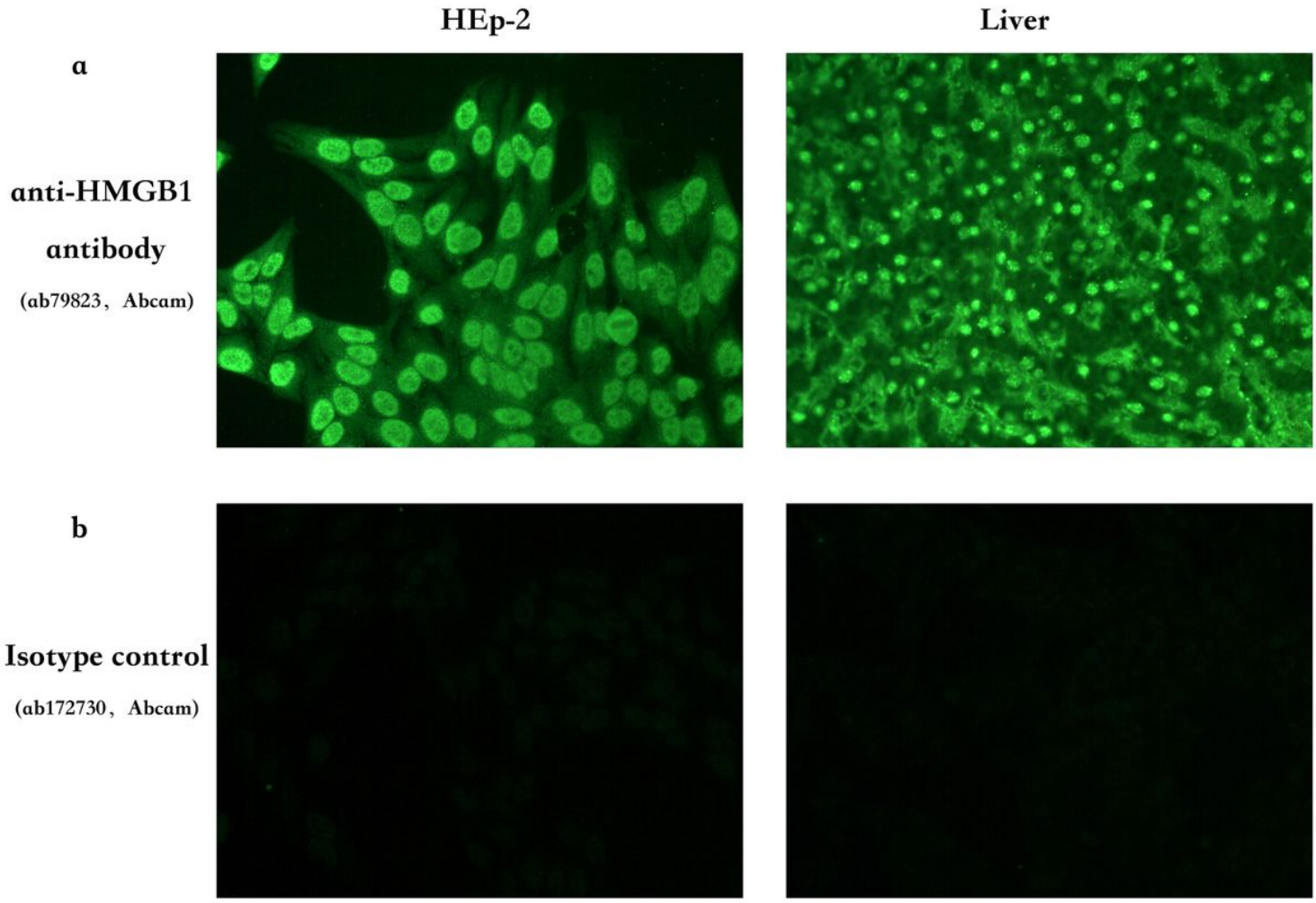

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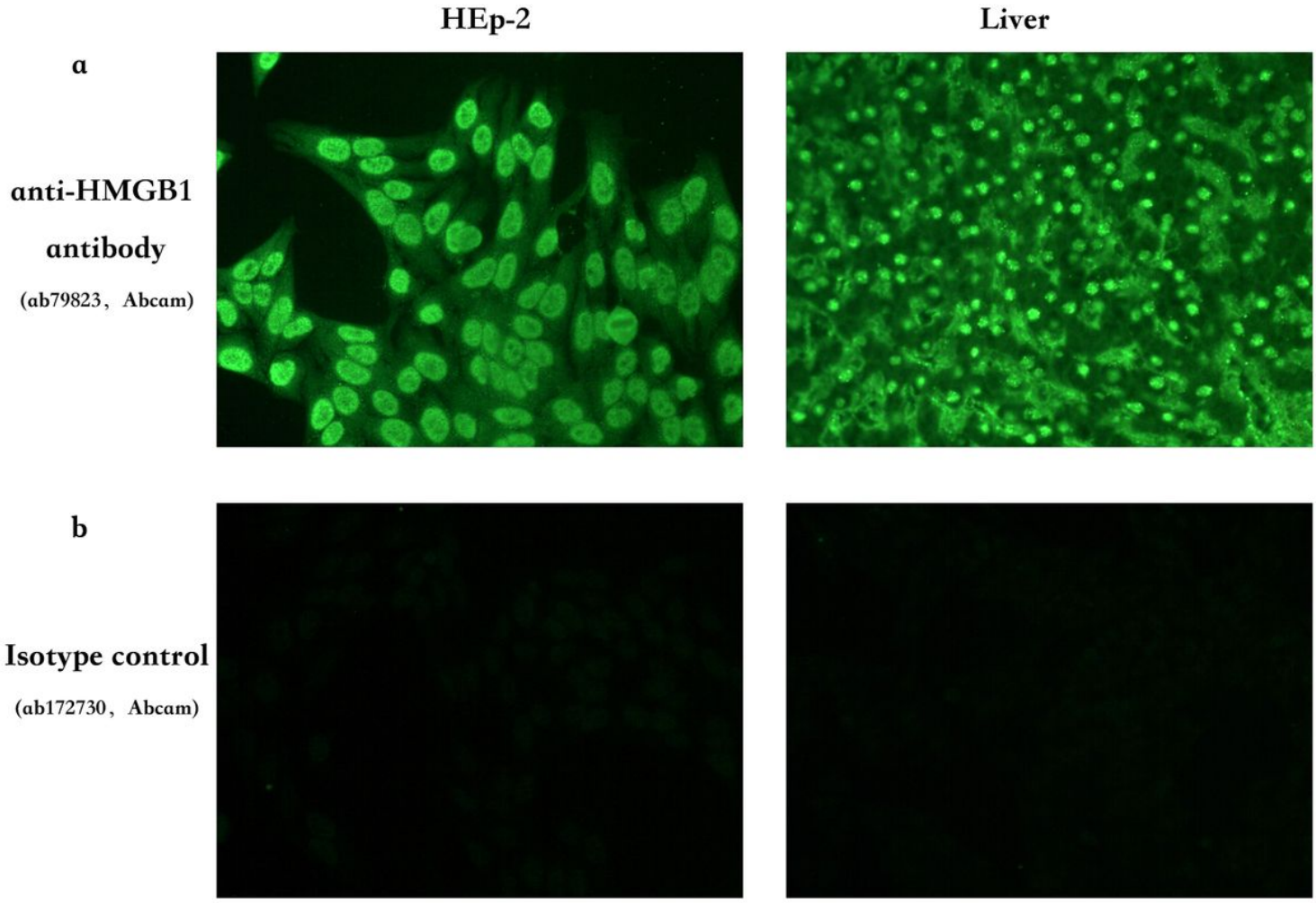

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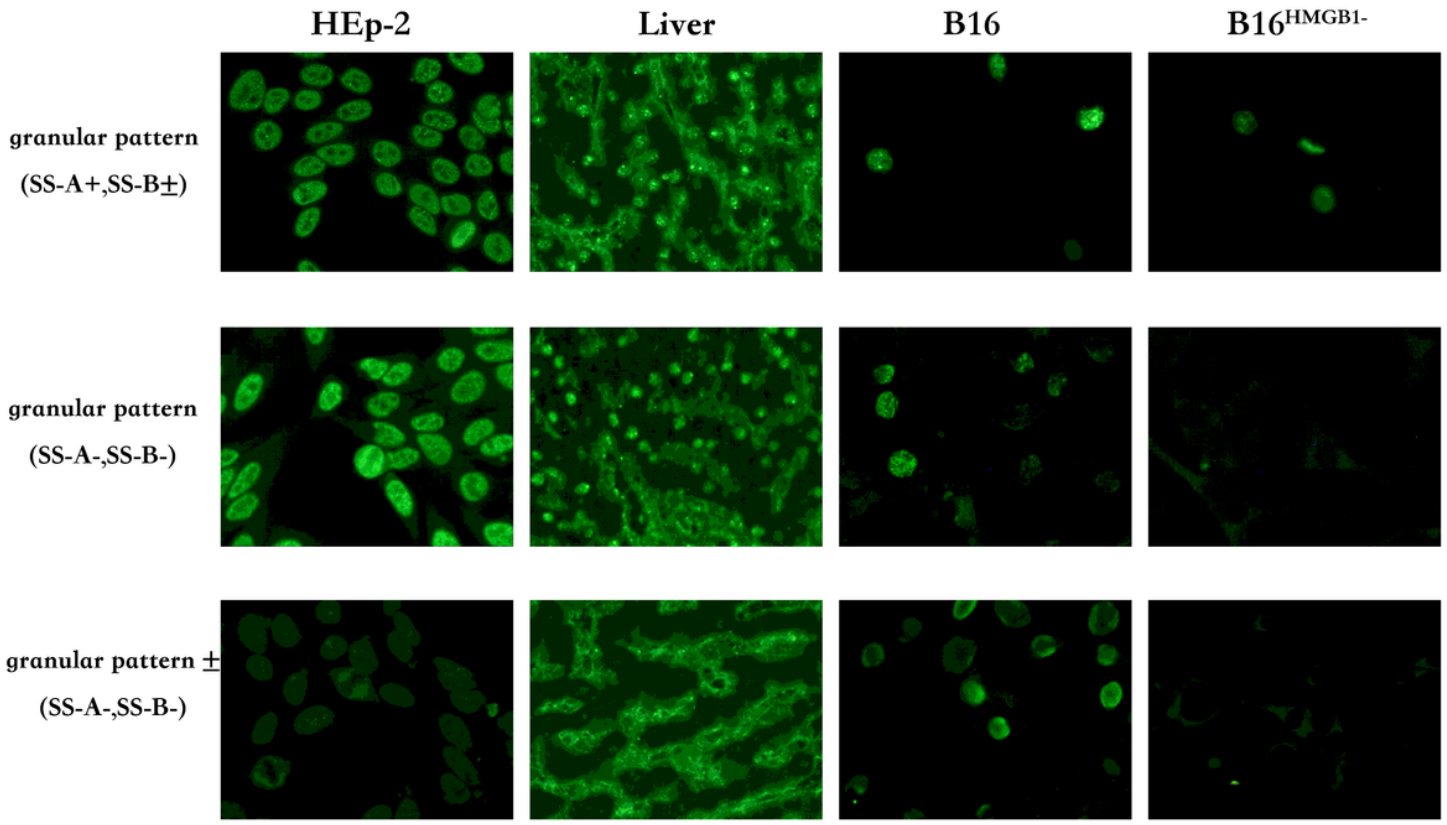
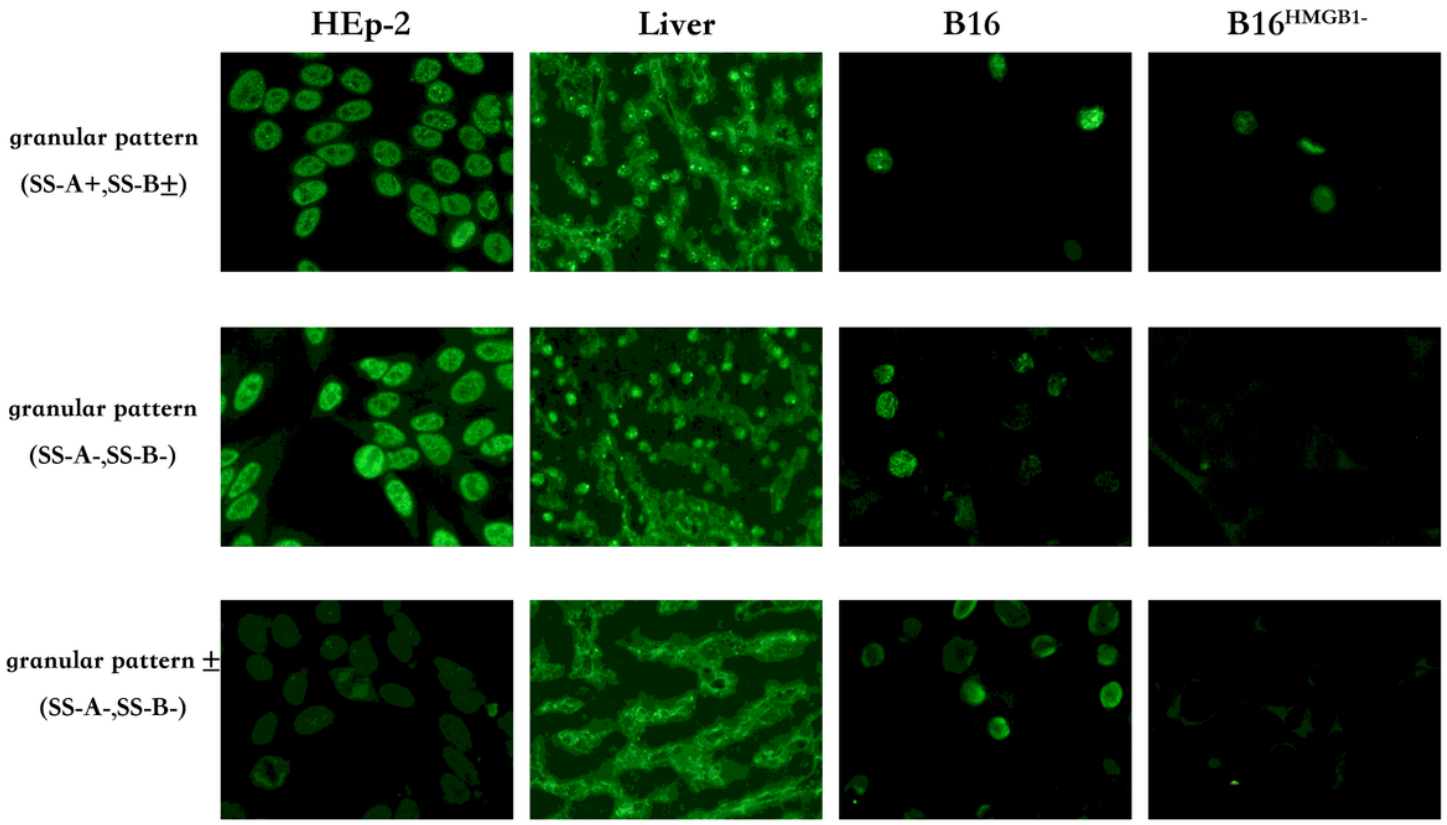


Figure 4

ANA-IF test with HMGB1 knockout cells in SS patients HMGB1 knockout and wildtype cells were pre-fixed on slides as the matrix to study the anti-HMGB1 antibody's contribution to ANA-IF, especially in ANA-IF positive, SS-A and SS-B negative patients. Three representative results were shown, Upper: ANA (+), (SS-A+, SS-B±), there are typical granular fluorescence pattern in both B16 and B16HMGB1- cells. Middle: ANA (+), (SS-A-, SS-B-), there is typical granular fluorescence pattern in B16 cells, while no fluorescence in B16HMGB1- cells. Down: ANA (±), (SS-A-, SS-B-), there is typical granular fluorescence pattern in B16 cells, while no fluorescence in B16HMGB1- cells.



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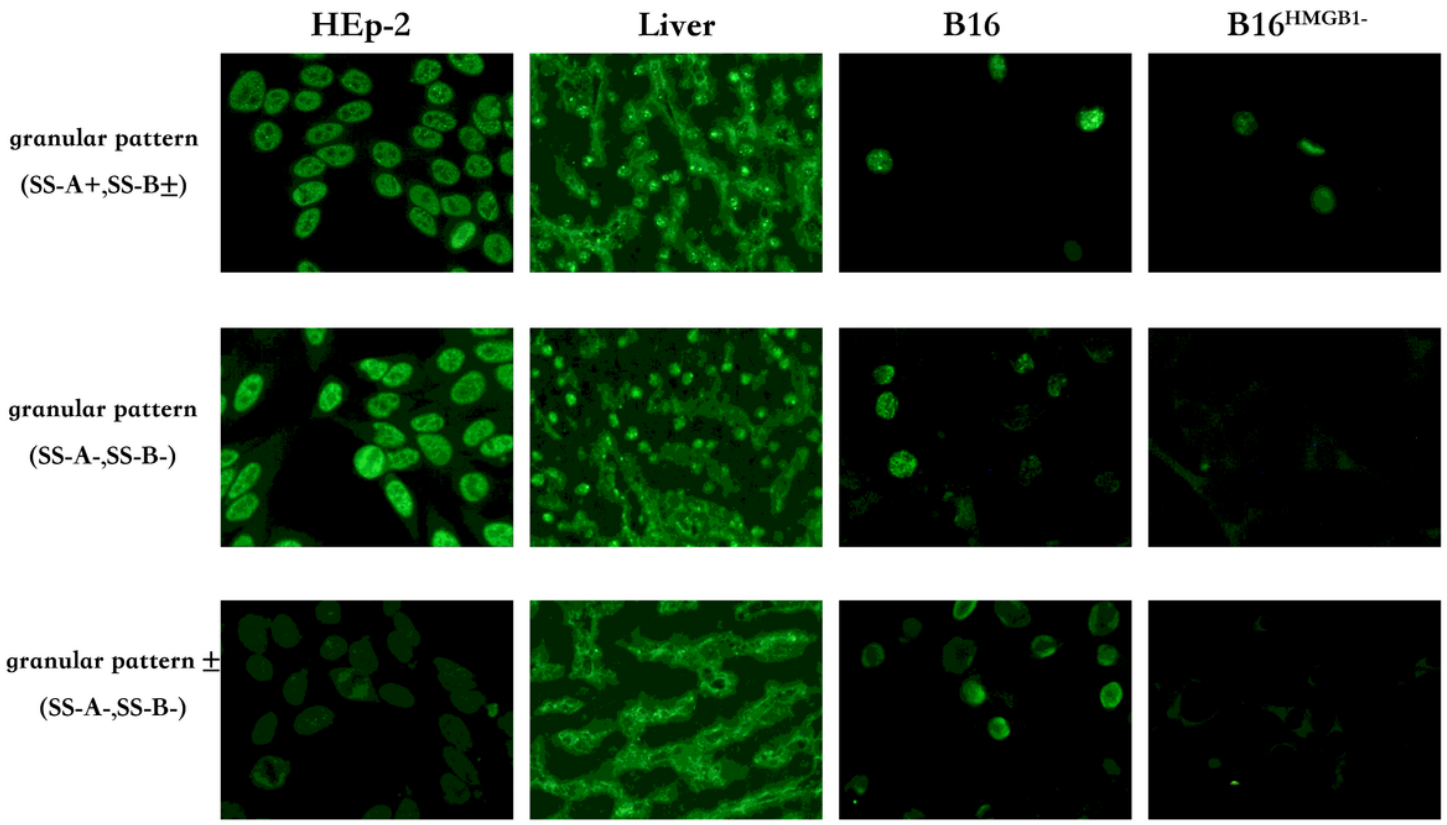

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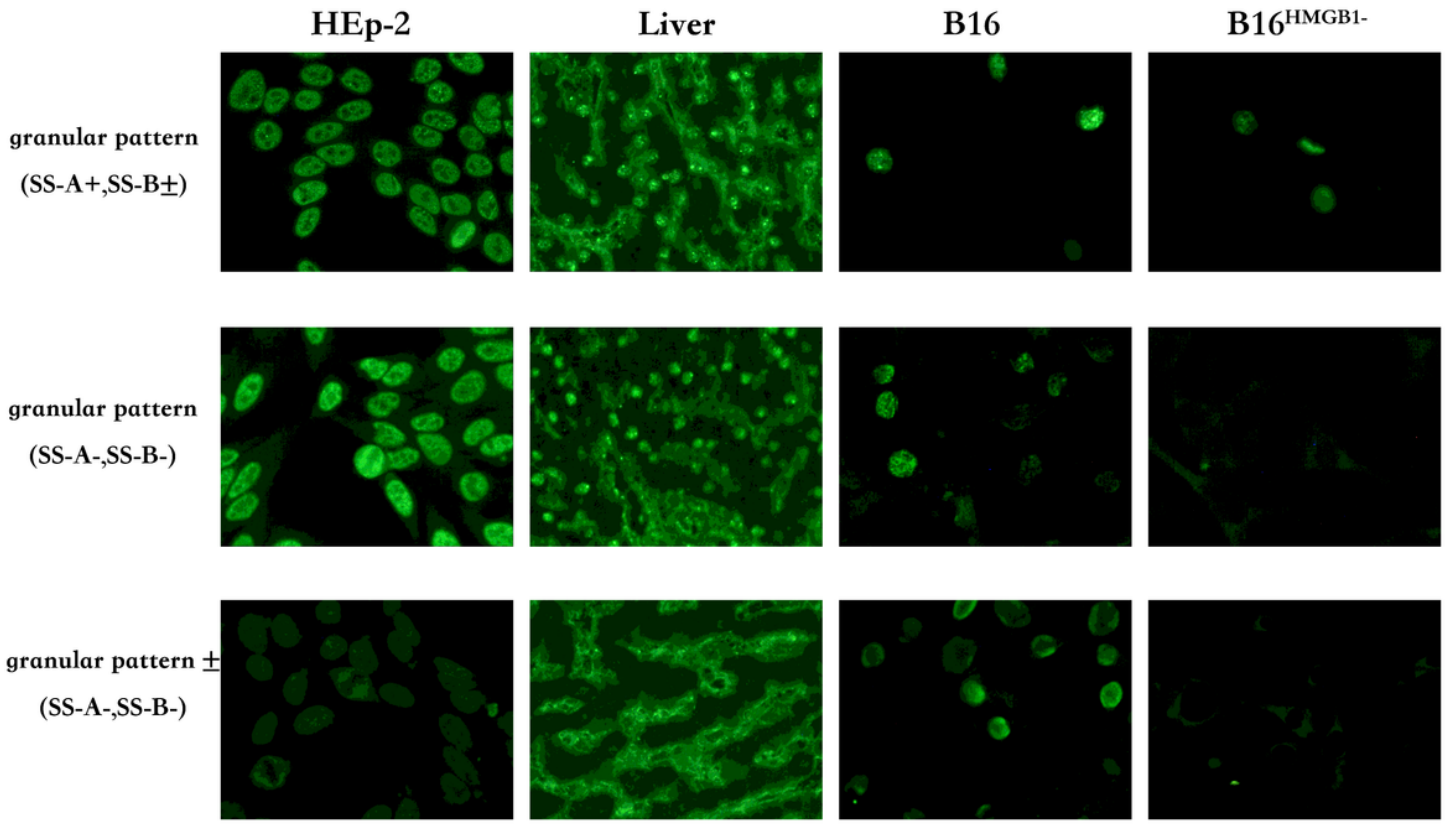

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Supplementary Files

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- Supplementarymethods.docx
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- supplfigure1.jpg
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- supplfigure1.jpg
- supplfigure2.jpg
- supplfigure2.jpg
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