# Adenosine Receptor A2B Antagonist Inhibits the Metastasis of Gastric Cancer Cells and Enhances the Efficacy of Cisplatin

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## Abstract

Adenosine receptors play a key role in cancer progression. This study investigated the effect of the adenosine A2B receptor (ADORA2B) on epithelial–mesenchymal transition (EMT) markers and cell metastasis of gastric cancer (GC) cells. Public databases were used to investigate the specificity of ADORA2B expression in GC tissue. We used immunohistochemistry and immunofluorescence to detect ADORA2B expression in GC tissue, paracancerous tissue, and metastatic greater omental tissue. AGS and HGC-27 GC cells were selected. The effect of ADORA2B on the invasion and migration of GC cells was examined using cell scratch and transwell assays. The effect of ADORA2B on the expression of EMT marker proteins (β-catenin, N-cadherin, and vimentin) in GC cells was measured by cellular immunohistochemistry, immunofluorescence, and Western blot. The effects of an ADORA2B inhibitor combined with cisplatin on EMT markers in GC cells were further explored. The expression levels of ADORA2B in GC tissue, metastatic greater omental tissue, and lymphatic metastasis tissue were significantly higher than those in paracancerous tissue, and ADORA2B was associated with lymph node metastasis and invasion. ADORA2B significantly regulated the invasion and migration ability of GC cells and the expression levels of EMT marker proteins. The combination of an ADORA2B antagonist (PSB-603) and cisplatin had a more significant effect on reversing the expression of EMT marker proteins. ADORA2B was overexpressed in GC tissue, metastatic greater omental tissue, and metastatic lymph node tissue. ADORA2B regulated the expression of EMT marker proteins in GC cells and affected GC cell metastasis. Antagonizing ADORA2B expression increased the efficacy of cisplatin treatment.

## Introduction

Gastric cancer (GC) metastasis is still an unavoidable problem in the treatment of GC. Chemotherapeutic drug resistance in the tumor microenvironment is a substantial issue in targeted therapy for GC metastasis.[1] Exploring the key factors that regulate GC metastasis is necessary to achieve targeted therapy for GC and improve the patient survival rate.

The role of energy metabolism in the tumor microenvironment in tumor progression cannot be ignored. As a key factor

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in energy metabolism, adenosine (ADO) is involved in the proliferation and metastasis of many cancers. ADO receptor (ADOR) also plays an indispensable role in cancer metastasis. In particular, ADO A2B receptor (ADORA2B) plays an important role insignaling.[2] In lung cancer studies, bioinformatics-related websites have shown that ADORA2B is overexpressed and amplified in lung adenocarcinoma and that high expression indicates a poor prognosis for individuals with lung adenocarcinoma.[3] In a tumor immunity study, inhibition of ADO-producing enzymes and ADO A2A or A2B receptor stimulated antitumor immunity and limited tumor progression, and inhibition of ADORA2B significantly enhanced antitumor immunity.[4] A study on breast cancer and head and neck squamous cell carcinoma suggested that ADORA2B promotes cell growth, migration, and angiogenesis.[5]

The targeting role of ADORA2B in cancer progression has been observed in many cancers.[3, 6, 7] However, the regulatory effect of ADORA2B on GC cell metastasis and epithelial–mesenchymal transition (EMT) markers is unclear. To elucidate whether ADORA2B can be used as a GC therapeutic target, the present study described the regulation of ADORA2B on GC cell metastasis and EMT marker proteins and evaluated the effect of combined cisplatin treatment. Therefore, we used GC patient specimens and two GC cell lines to conduct a combined study to reveal the possibility of ADORA2B becoming a therapeutic target for GC metastasis.

## Materials and Methods

### Cell Culture and Chemical

GC cell line (AGS : TCHu232 and HGC-27 : TCHu 22 cells) was purchased from National Collection of Authenticated Cell Cultural, China (https://www.cellbank.org.cn/). According to the manufacture's instruction, cell line was grown in RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) at 37°C and 5% $CO_2$.

### Specimen of Human Pathological Tissue

Clinical specimens of 75 patients with low differentiated GC were collected from the Department of Pathology, Ningxia Hui People's Hospital. None of the patients had a history of chemotherapy or radiotherapy (n = 3). The project was approved by the Ethics Committee of Ningxia Hui People's Hospital (approval no. 2020-NZR-100, approval time: October 30, 2020). Each patient signed a written informed consent form.

### Cell Viability Assay-Cell Counting Kit-8

GC cells (AGS and HGC-27) were seeded in a 96-well plates (cell density controlled at $1 \times 10^4$ cells/well) and cultured in a 37°C incubator until the cells reached 80% confluence. Different concentrations of ADORA2B antagonist (PSB-603 : 5-200µM) and agonist (BAY 60-6583 : 5-200µM) were added into the Wells, respectively. After incubation for 24h, 10µ LCCK 8 solution was added and incubated at 37°C for 3h. The absorbance at 450nm was measured using a microplate reader.

### Transwell Assay

The cells were first cultured for 24h serum free. Then the logarithmic growing cells were inoculated into the upper chamber, and the cells were cultured for 4h to intervene in different groups of cells. A 600µL complete medium containing 10% FBS was added to the lower chamber. After 24h, the culture medium in the upper chamber was discarded, then fixed with 4% paraformaldehyde for 15min, stained with 0.1% crystal violet for 15min, wiped the cells in the upper chamber with cotton swabs, and observed the number of cells in the lower chamber under a microscope.

### Wound-Healing Scratch Assay

The cells were cultured in 6-well plates, and when cell confluence was above 90%, the central area of cell growth was lined with a 200µL pipette tip. Images were collected under a microscope (0h), followed by intervention of different groups of cells, and images were collected under an inverted microscope (24h) after incubation for 24h. Cell mobility was analyzed by image-Pro Plus software.

### Immunohistochemistry and Immunofluorescence

For GC tissue, paracancer tissue, and greater omentum metastasis tissue, paraffin sections were firstly dewaxed, antigen repair, peroxidase block, and sheep serum block. Then choose the primary antibody, ADORA2B (1:100, ER1903-44, HUABIO, China), β-catenin (1:200, ab32572, Abcam, USA), N-cadherin (1:200, ab18203, Abcam, USA), vimentin (1:100, 10366-1-AP, Proteintech, China) and E-cadherin (1:200, ab40772, Abcam, USA) incubated overnight in a 4°C humidor. For immunohistochemical detection, secondary antibody (PV6000, ZSGB-BIO, China) was added and incubated at 37°C for 20min. Then DAB was performed. For immunofluorescence, sections were cleaned and goat antirabbit antibody combined with secondary antibody Alexa 488 was incubated for 1h. The nuclei were restained with DAPI.

### Western Blot Assay

The total protein extraction kit was purchased from KeyGENBioTECH (Jiangsu, China) and the protein quantification kit by BCA method was purchased from LEAGENE (Beijing, China). The target proteins of different molecular weights were separated by 10% polyacrylamide gel electrophoresis and transferred to the solid phase carrier PVDF membrane. Incubated primary antibody ADORA2B (1:1000, ER1903-44, HUABIO, China) ,β-catenin (1:1000, ab32572, Abcam, USA) ,N-cadherin (1:2000, ab18203, Abcam, USA) ,vimentin (1:2000, 10366-1-AP, Proteintech, China), and E-cadherin

(1:2000, ab40772, Abcam, USA) overnight at 4°C and incubated horseradish peroxidase labeled rabbit secondary antibody (1:5000, Zb-2301, ZSGB-BIO, China) for 1h. After 3 times of TBST washing, the protein bands were displayed by ECL luminescence solution and autoradiography. Finally, the gray values were analyzed by ImageJ software.

## Statistical Analysis

Data were expressed as mean ± standard error. T-test was used for comparison of the mean of 2 samples, one-way analysis of variance was used for comparison of the mean of multiple samples, and $P < .05$ was considered statistically significant (*$P < .05$; **$P < .01$; ***$P < .001$). The experiments for each group were repeated at least 3 times.

## Results

1. **The expression level of ADORA2B in GC tissue, paracancerous tissue, metastatic greater omental tissue, and metastatic lymph node tissue**

The expression levels of ADORA2B in GC tissue, metastatic greater omental tissue, and metastatic lymph node tissue were significantly higher than those in paracancerous tissue ($P < .01$), and the expression level of ADORA2B in metastatic greater omental tissue was significantly higher than that in GC tissue ($P < .001$). ADORA2B expression in GC tissue and metastatic greater omental tissue was mainly localized in the cytoplasm, with a small amount in the nucleus. However, in
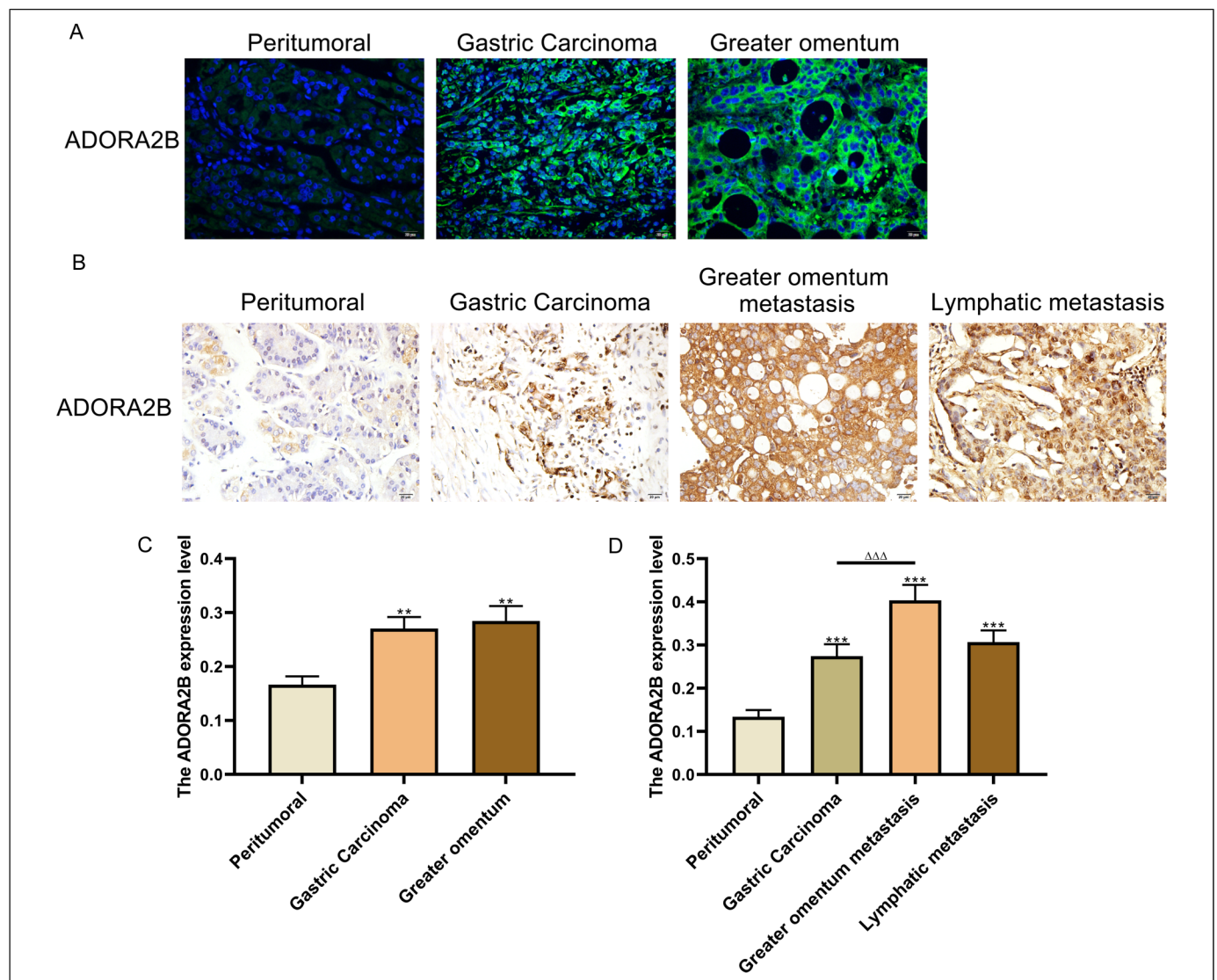


**Figure 1.** ADORA2B presents high expression in both GC tissue, greater omental metastasis tissue, and lymph node metastasis tissue than in paracancerous tissue. (A) and (C) IF analysis of ADORA2B expression in paracancerous, gastric carcinoma, and greater omental tissue. (B) and (D) IHC analyses of ADORA2B expression in paracancerous, gastric carcinoma, and greater omental metastasis and lymphatic metastasis tissue. Bar = 20μm, 400×, **$P < .01$, ***$P < .001$. Abbreviations: ADORA2B, adenosine A2B receptor; GC, gastric cancer; IF, immunofluorescence; IHC, immunohistochemistry.

**Table 1.** ADORA2B Expression in Normal Tissue and Gastric Cancer Tissue.

| Groups | n | − | + | PR (%) |
|--------|---|---|---|--------|
| | | | ADORA2B expression | |
| Normal | 75 | 71 | 4 | 5.3 |
| Cancer | 75 | 18 | 57 | **76.0**[①] |

[①]$P < .05$.
Abbreviations: ADORA2B, adenosine A2B receptor; PR, positive rate.

**Table 2.** Relationship Between Expression of ADORA2B Protein and Clinicopathological Features of Gastric Cancer.

| Clinicopathological features | n | − | + | + + | + + + | PR (%) | *P* value |
|------------------------------|---|---|---|-----|-------|--------|-----------|
| | | | | Expression ADORA2B | | | |
| **Age** | | | | | | | |
| ≤50 | 32 | 12 | 5 | 9 | 6 | **62.5** | **.091** |
| >50 | 43 | 6 | 14 | 14 | 9 | **86.0** | |
| **Size** | | | | | | | |
| <3 | 29 | 8 | 6 | 11 | 4 | **72.4** | **.497** |
| ≥3 | 46 | 10 | 13 | 12 | 11 | **78.3** | |
| **Lymph node metastasis** | | | | | | | |
| − | 53 | 14 | 16 | 10 | 13 | **73.6** | **.007*** |
| + | 22 | 4 | 3 | 13 | 2 | **81.8** | |
| **Distant metastasis** | | | | | | | |
| − | 66 | 16 | 17 | 19 | 14 | **75.8** | **.780** |
| + | 9 | 2 | 2 | 4 | 1 | **77.8** | |
| **Clinical stage** | | | | | | | |
| I | 35 | 12 | 8 | 11 | 4 | **65.7** | **.140** |
| II-III | 40 | 6 | 11 | 12 | 11 | **85.0** | |
| **Vascular infiltration** | | | | | | | |
| − | 33 | 8 | 11 | 10 | 4 | **75.8** | **.345** |
| + | 42 | 10 | 8 | 13 | 11 | **76.2** | |
| **Nerve invasion** | | | | | | | |
| − | 62 | 17 | 18 | 14 | 13 | **72.6** | **.009*** |
| + | 13 | 1 | 1 | 9 | 2 | **92.3** | |
| **Differentiation** | | | | | | | |
| Well-differentiated | 12 | 2 | 5 | 3 | 2 | **83.3** | **.309** |
| Moderately differentiated | 45 | 14 | 10 | 11 | 10 | **68.9** | |
| Poorly differentiated | 18 | 2 | 4 | 9 | 3 | **88.9** | |

*$P < .05$.
Abbreviations: ADORA2B, adenosine A2B receptor; PR, positive rate.

metastatic lymph node tissue, ADORA2B expression in the nucleus was significantly increased ($P < .001$) (Figure 1). Analysis of the relationship between ADORA2B and the clinicopathological characteristics of GC patients indicated that ADORA2B was closely associated with invasion and lymph node metastasis (Tables 1 and 2).

### 2. Effects of ADORA2B agonists and antagonists on cell migration and invasion

The effects of different concentrations of an ADORA2B agonist (BAY 60-6583) and antagonist (PSB-603) on the proliferation of 2 GC cell lines were screened by Cell Counting Kit-8 (CCK8). The results showed a dose-dependent effect. Based on the results, 10 μM was selected for both the agonist and antagonist in subsequent experiments (Figure 2 A-D).

We demonstrated by transwell assay that the ADORA2B agonist (BAY 60-6583) enhanced the number of invading GC cells, and the antagonist (PSB-603) significantly reduced the number of invading GC cells (Figure 2 E-H, $P < .01$).

Cell scratch assays indicated that BAY 60-6583 promoted the migration ability of GC cells, while PSB-603 inhibited the migration ability of GC cells (Figure 2 I-L, $P < .01$).
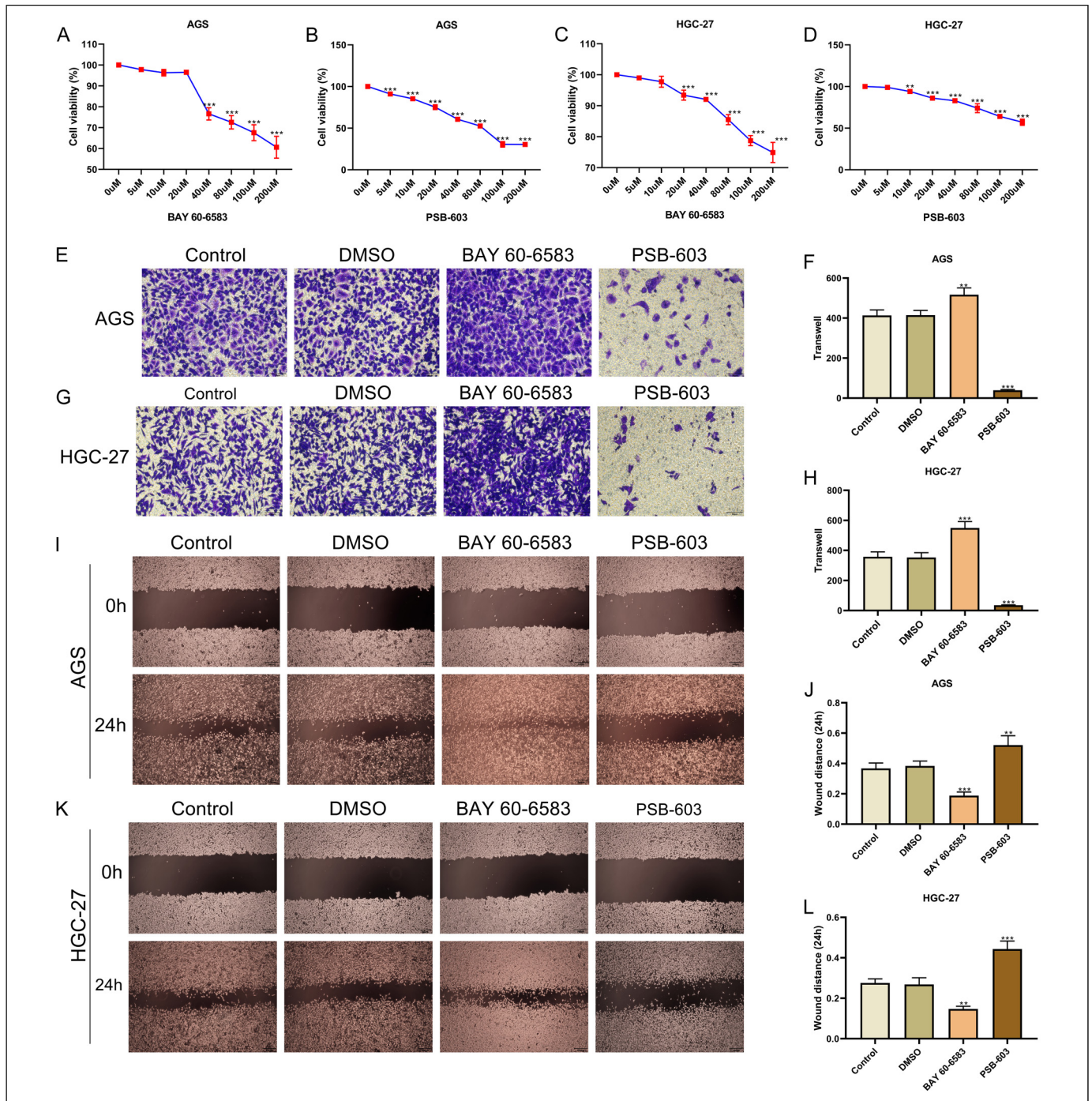
**Figure 2.** The invasion and migration of GC cells were affected by ADORA2B agonists and antagonists. (A)–(D) The effects of different concentrations of an ADORA2B agonist and antagonist on the proliferation of two GC cell lines were screened by CCK8. (E)–(H) The effects on the invasion and migration of GC cells subjected to ADORA2B agonists and antagonists were examined by transwell assay; bar = 50μm, 200×. (I)–(L) The effects on the invasion and migration of GC cells subjected to ADORA2B agonists and antagonists were examined by Wound-Healing Scratch assay. Bar = 200μm, 4×, **$P < .01$, ***$P < .001$. Abbreviations: ADORA2B, adenosine A2B receptor; CCK8, Cell Counting Kit-8; GC, gastric cancer.

### 3. Effect of ADORA2B on the expression of EMT marker proteins

ADORA2B agonists significantly upregulated the expression of ADORA2B, β-catenin, N-cadherin, and vimentin and decreased the expression of E-cadherin; antagonists significantly reduced the expression of ADORA2B, β-catenin, N-cadherin, and vimentin and upregulated the expression of E-cadherin, indicating that ADORA2B regulates the expression of EMT marker proteins (Figures 3 and 4).
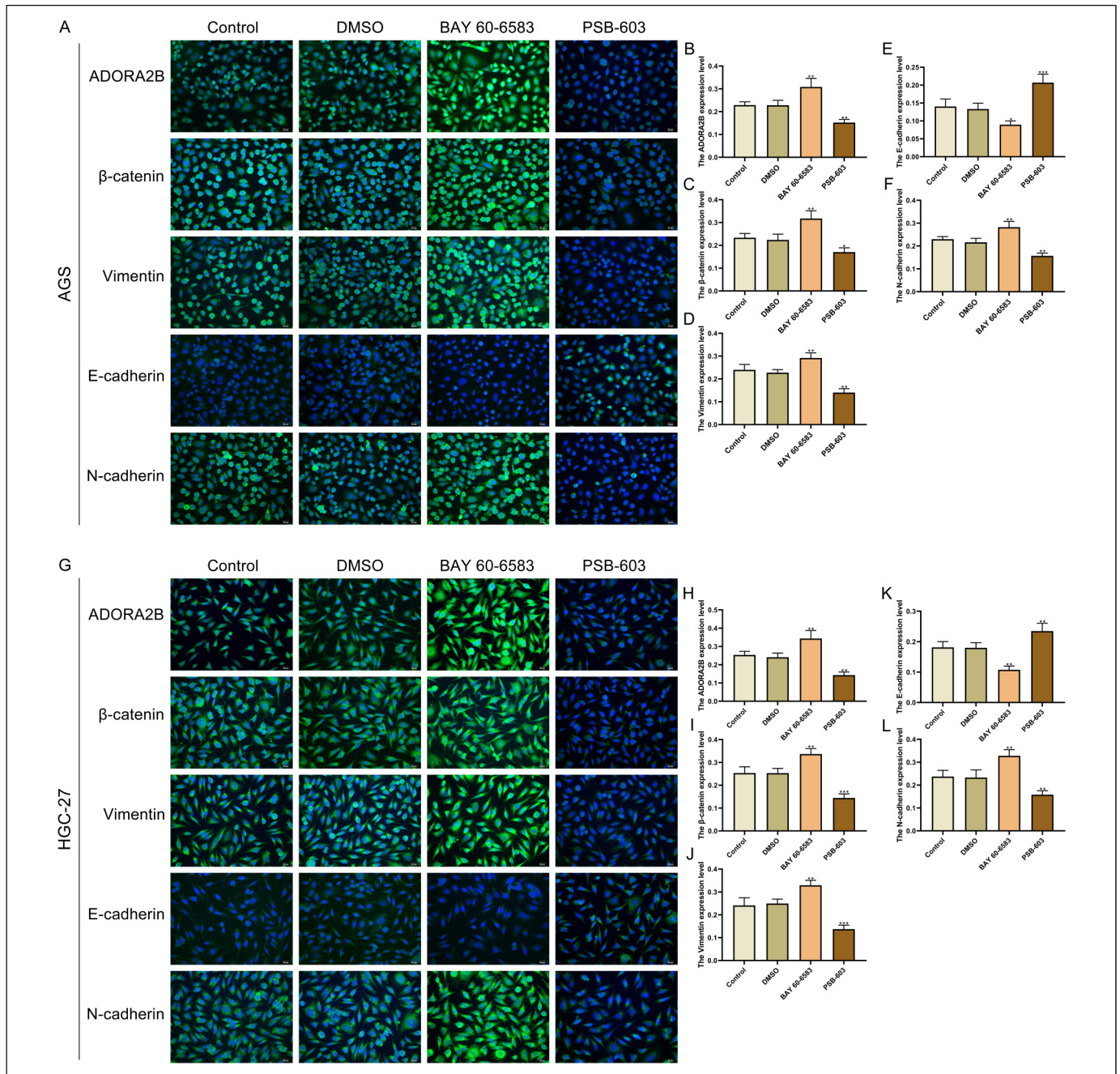
**Figure 3.** If analyses of ADORA2B regulated the expression of EMT marker proteins. (A and G) IF staining analysis the expression of ADORA2B, β-catenin, N-cadherin, vimentin, and E-cadherin in GC cells (AGS and HGC-27), under the regulation of ADORA2B agonists and antagonists. Quantification of the expression of (B and H) ADORA2B, (C and I) β-catenin, (D and J) vimentin, (E and K) E-cadherin, and (F and L) N-cadherin in GC cells (AGS and HGC-27) under the regulation of ADORA2B agonists and antagonists. Bar = 20μm, 400×, **$P < .01$, ***$P < .001$. Abbreviations: ADORA2B, adenosine A2B receptor; EMT, epithelial–mesenchymal transition; GC, gastric cancer; IF, immunofluorescence.

### 4. ADORA2B antagonist treatment enhanced the anticancer effect of cisplatin

ADORA2B agonist treatment resulted in an increase in the grayscale values of ADORA2B, β-catenin, N-cadherin, and vimentin; PSB-603 treatment resulted in significant reductions in the grayscale values of β-catenin, N-cadherin, and vimentin, indicating that ADORA2B may regulate the expression of EMT marker proteins (Figure 5 A-J). The combined application of antagonists and cisplatin resulted in a more significant reduction in EMT marker protein expression than that observed with cisplatin alone, further indicating that in addition to its independent function, ADORA2B enhances the anticancer effect of cisplatin (Figure 6 A-J).
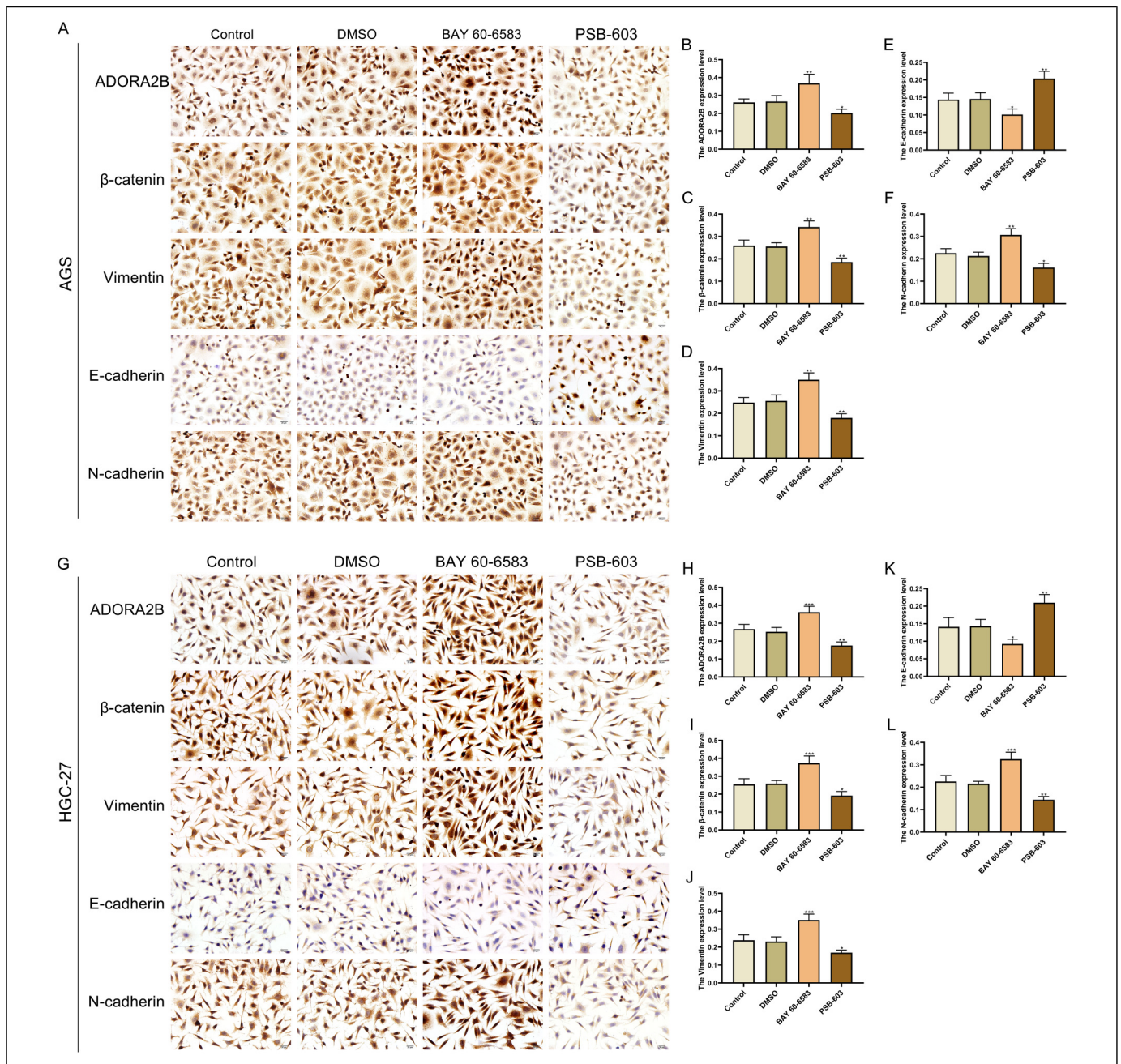
**Figure 4.** IHC analyses of ADORA2B regulated the expression of EMT marker proteins. (A and G) IHC detected the expression of ADORA2B, β-catenin, N-cadherin, vimentin, and E-cadherin in GC cells (AGS and HGC-27), under the regulation of ADORA2B agonists and antagonists. Quantification of the expression of (B and H) ADORA2B, (C and I) β-catenin, (D and J) vimentin, (E and K) E-cadherin, and (F and L) N-cadherin in GC cells (AGS and HGC-27), under the regulation of ADORA2B agonists and antagonists. Bar = 20μm, 400×, *$P<$.05, **$P<$.01, ***$P<$.001. Abbreviations: ADORA2B, adenosine A2B receptor; EMT, epithelial–mesenchymal transition; GC, gastric cancer; IHC, immunohistochemistry.

## Discussion

Energy metabolism in the tumor microenvironment is an important factor in cancer development and is also the top priority when treating cancer metastasis. In recent years, many cancer patients have benefited from the development of targeted drugs, and survival rates have significantly improved, but cancer cell metastasis is still a challenge that must be overcome.[8] Exploring the role of energy metabolism effector factors in the process of cancer development and whether they can serve as targets is particularly important. ADO is a key effector factor in energy metabolism and is involved in the progression of many cancers, indicating the importance of ADO in cancer development.[9] We first verified
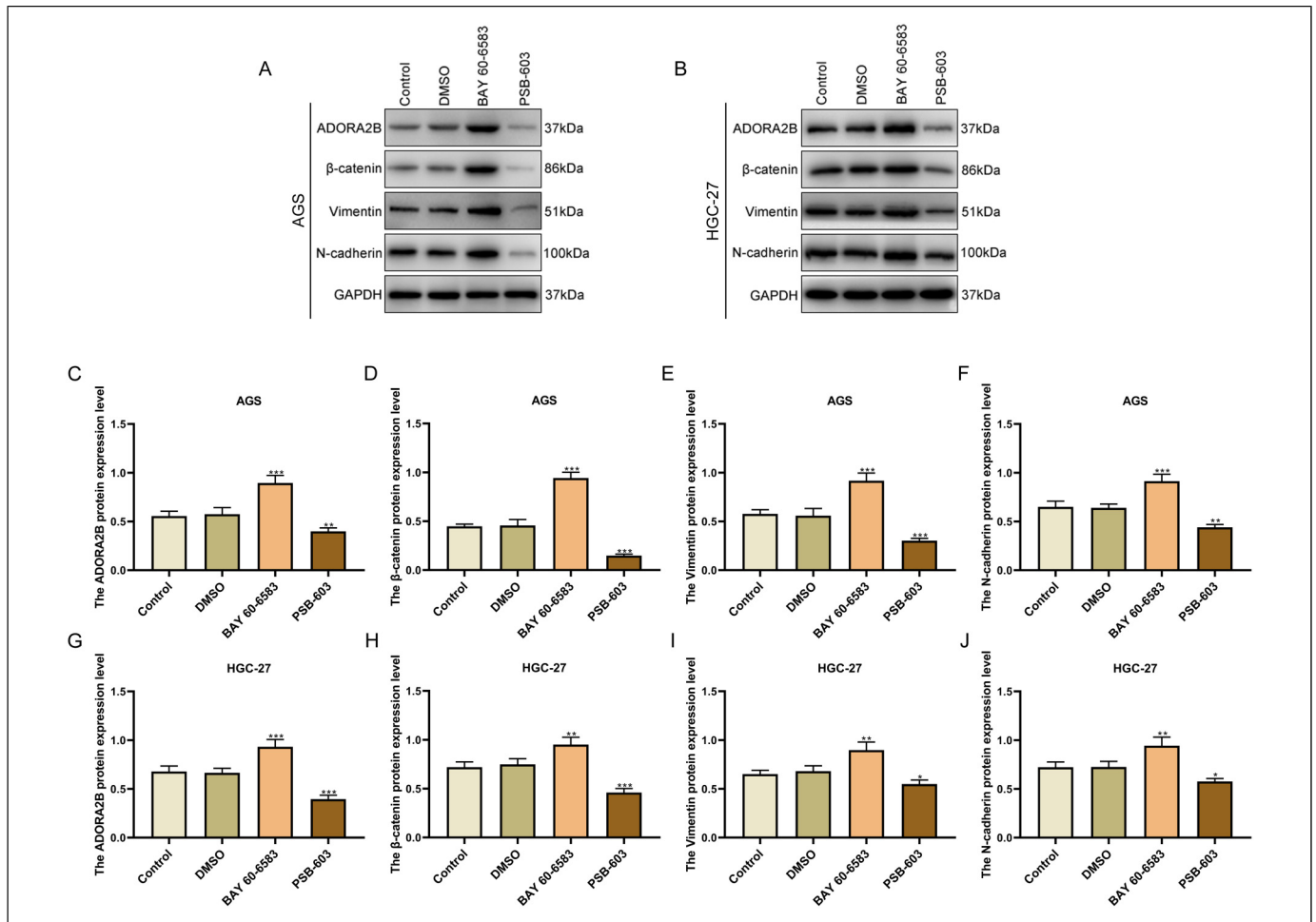
**Figure 5.** ADORA2B agonists and antagonists regulate the expression of EMT marker proteins. (A, C, and F) Western blotting assay analyses the expression of ADORA2B, β-catenin, vimentin, and N-cadherin in AGS under treatment of ADORA2B agonists and antagonists. (B, and G–J) Western blotting assay analyses the expression of ADORA2B, β-catenin, vimentin, and N-cadherin in HGC-27 under treatment of ADORA2B agonists and antagonists. *$P < .05$, **$P < .01$, ***$P < .001$. Abbreviations: ADORA2B, adenosine A2B receptor; EMT, epithelial–mesenchymal transition.

the relationship between ADORA2B expression and clinical pathology in GC tissue and metastatic tissue. In vitro studies of GC cells demonstrated that ADORA2B regulated the invasion and migration abilities of GC cells and affected the expression levels of EMT marker proteins. The potential of ADORA2B as a therapeutic target for GC metastasis was further elucidated.

ADORA2B is considered to have two-sided effects. On the one hand, tumor cells themselves express ADOR and directly regulate tumor cell activity; on the other hand, ADO has an inhibitory effect on immune cells. The above functions mainly depend on the type of ADOR, the tumor itself, and the environment.[10] In addition to the difference in the role of ADOR in energy metabolism, there are also differences in various research fields. The antagonism of ADORA1 induces apoptosis in breast cancer and colon cancer cells, and an activator of ADORA2A induces apoptosis in colon cancer and liver cancer cells.[11] In a bladder urothelial carcinoma (BUC) study,

the ADORA2B antagonist MRS1754 inhibited the progression of BUC through the mitogen-activated protein kinase pathway.[12] In melanoma, 5'-methylthioadenosine affects the proliferation and invasion of melanoma cells through ADORA2B.[13] Studies in oral squamous cell carcinoma have demonstrated that ADORA2B regulates cell proliferation by activating HIF-1α expression.[14] This study preliminarily verified the significance of ADORA2B expression in GC tissue. The expression level of ADORA2B was significantly higher in metastatic greater omental tissue than in GC tissue. We speculate that this difference may be related to the enhanced metastatic ability of cancer cells and higher pro-oncogenic factor expression under hypoxic conditions, as shown in a previous study.[15] Interestingly, the number of cells overexpressing ADORA2B in the nucleus was significantly increased in the metastatic lymph node tissue, further indicating that ADORA2B might participate in an immune mechanism in the process of GC metastasis, which we will explore in the
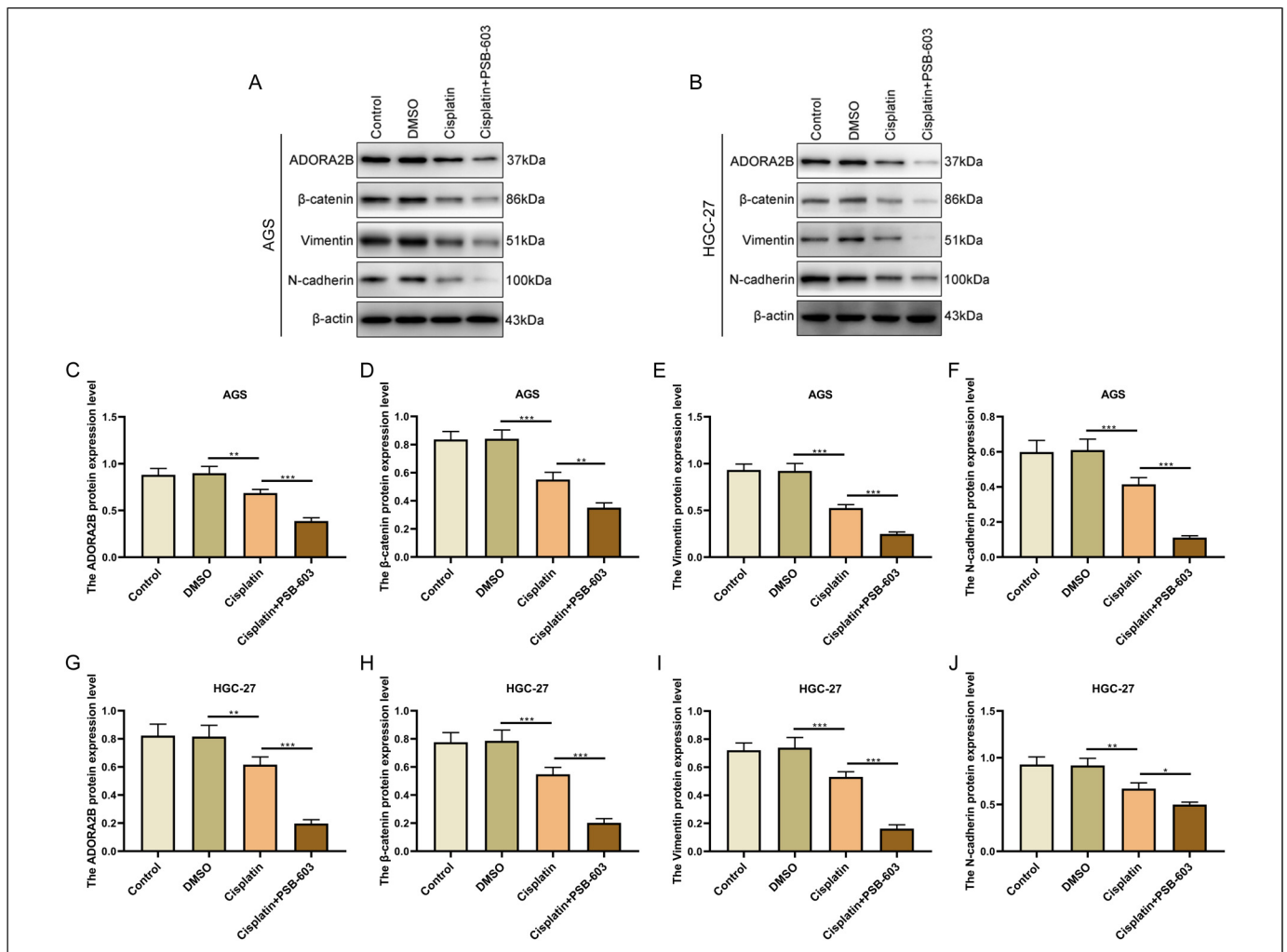
**Figure 6.** The combined application of antagonists and cisplatin resulted in reduction in EMT marker proteins expression. (A and C–F) Western blotting assay analyses the expression of ADORA2B, β-catenin, vimentin, and N-cadherin in AGS under treatment of ADORA2B antagonists and combined application of antagonists and cisplatin. (B and G–J) Western blotting assay analyses the expression of ADORA2B, β-catenin, vimentin, and N-cadherin in HGC-27 under treatment of ADORA2B antagonists and combined application of antagonists and cisplatin. $*P<.05$, $**P<.01$, $***P<.001$. Abbreviations: ADORA2B, adenosine A2B receptor; EMT, epithelial–mesenchymal transition.

future. Notably, in metastatic lymph node tissues, excessive ADORA2B expression in the nucleus increased significantly, further suggesting that ADORA2A may participate in GC to transfer the immune mechanism in the process, but due to the limited sample size, which did not allow identification of correlations with vascular invasion and distant metastasis, we will expand the sample size in the future for further exploration. In summary, we demonstrated the critical role and clinical value of ADORA2B in the process of GC metastasis.

ADORA2B promoted the invasion and migration of breast cancer cells and head and neck squamous cell carcinoma and was associated with a poor patient prognosis.[5, 16] In this study, the application of ADORA2B agonists significantly promoted the invasion and migration abilities of GC cells, and the invasion and migration abilities of GC cells was significantly weakened after antagonizing ADORA2B expression. In addition, the results were consistent with the findings of

Campos-Contreras et al, that is, ADORA2B regulates the migration of ovarian cancer cells. The above results indicate that ADORA2B regulates the invasion and migration abilities of GC cells and is important in the regulatory mechanism of GC cell metastasis.

EMT is a relatively well-elucidated metastasis mechanism in cancer research. Studies have found that the most critical link in the occurrence of distant metastasis from the primary tumor is the occurrence of EMT in tumor cells. This situation also involves the transformation of suspension cells into adherent cells with EMT characteristics.[17] In tumor studies, the expression levels of EMT markers indirectly reflect the ability of cancer to metastasize.[18] Many cancer studies have demonstrated that β-catenin, N-cadherin, and vimentin are involved in the metastasis of cancer cells, including GC.[19, 20] In this study, ADORA2B agonists and antagonists effectively regulated the expression levels of β-catenin, N-cadherin, and

vimentin. Additionally, unlike that in GC tissue, ADORA2B expression was elevated in both the nucleus and the cytoplasm of metastatic cells. However, after treatment with antagonists, ADORA2B expression was significantly reduced, especially in the nucleus. Based on the results of the in vitro cell experiments, we speculate that the phenomenon of higher ADORA2B expression in metastatic tissue than in primary GC tissues has considerable clinical significance (clerical error). In terms of treatment, the combination of antagonists and cisplatin significantly increased the antitumor effect of cisplatin, fully demonstrating the clinical value of ADORA2B in GC metastasis and treatment. However, in ovarian cancer research, the application of an ADORA2B antagonist can promote the migration of ovarian cancer cells (SKOV-3), and conversely, an inducer can reduce the migration of ovarian cancer cells,[2] which is contradictory to our results. We believe that this finding is likely related to the levels of estrogen and progesterone, which will be further verified in the future. This experiment has been verified only in GC cell lines and not in in vivo experiments. In subsequent studies, we will use animal experiments to further verify the role of ADORA2B in GC metastasis.

In summary, ADORA2B is overexpressed in GC tissue, metastatic greater omental tissue, and metastatic lymph node tissue, and its expression is more prominent in metastatic tissue. ADORA2B affects GC cell metastasis by regulating the expression of EMT marker proteins in GC cells. The combination of an ADORA2B antagonist and cisplatin can enhance therapeutic effects.

## Conclusion

ADORA2B is a key marker in the pathological diagnosis and metastasis mechanism of GC. ADORA2B plays a role in promoting carcinogenesis in the development of GC, and may become a therapeutic target for GC.

### Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Ethical Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Ningxia Hui People's Hospital (No.2020-NZR-100, approval time: October 30, 2020). Each patient signed a written informed consent form.

### Funding

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