Allograft inflammatory factor-1 is an independent prognostic indicator that regulates β-catenin in gastric cancer

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Abstract. Previous studies have revealed that expression of allograft inflammatory factor-1 (AIF-1) protein appears to be increased in malignancies and is correlated with a poorer prognosis in cervical cancer, while its role in gastric cancer has not been reported. We analyzed the expression of AIF-1 in 78 cancer lesions and the corresponding non-cancerous tissues by immunohistochemistry. In contrast with other cancers, we found that AIF-1 protein levels were significantly decreased in 53 of the 78 (67.9%) gastric cancer tissues when compared with the matched normal tissues. This was further confirmed using 7 pairs of fresh gastric cancer tissues and matched adjacent normal tissues. Low tumoral AIF-1 expression was significantly correlated with less favorable clinicopathological characteristics, as well as with reduced overall survival ($P<0.001$) in the gastric cancer patients. Furthermore, knockdown of AIF-1 obviously increased proliferation, migration and β-catenin expression in BGC-823 and SGC-7901 gastric cancer cells. Taken together, for the first time, we provide evidence that the level of AIF-1 expression may serve as a protective prognostic indicator for gastric cancer.

Introduction

Gastric cancer is second only to lung cancer as the leading cause of cancer-related deaths worldwide. Although the overall incidence of gastric cancer has declined, it remains high in Asian countries (1). The 5-year survival rate for patients with gastric cancer is only ~20%. The high mortality rates of patients with gastric cancer are known to be associated with metastatic spread of cancer cells from the stomach to common sites such as the liver and peritoneum (2). Metastasis is the result of several sequential steps including proliferation, invasion, detachment of tumor cells, migration into lymph nodes and blood vessels, adhesion and survival in the circulation, and extravasation into the target organ, where, again, proliferation occurs. These are also the key elements influencing clinical treatment and prognosis (3). Therefore, understanding of the molecular and genetic mechanisms involved in gastric cancer progression may provide us with novel biomarkers and highlight potential avenues of investigation for targeted therapies.

The allograft inflammatory factor-1 (AIF-1) is a 17-kDa interferon (IFN)-γ-inducible Ca^{2+}-binding EF-hand protein that is encoded within the human leukocyte antigen (HLA) class III genomic region on chromosome 6p21.3, which is known for clusters of genes involved in the inflammatory response (4). AIF-1 is closely associated with cardiac allograft vasculopathy (5), rheumatoid arthritis (6), inflammatory skin disorders (7) and systemic sclerosis (8). It has been reported that AIF-1 may promote breast cancer proliferation through activation of the NF-κB/cyclin D1 pathway (9) and breast cancer cell migration by upregulation of TNF-α-mediated activation of the p38 MAPK signaling pathway (10). In addition, AIF-1 may play a significant role in the pathophysiology and progression of hemangiomas (11). However, it has not yet been reported whether AIF-1 is also involved in the development of gastric cancer.

In the present study, to elucidate the potential role of AIF-1 expression in gastric cancer, we evaluated AIF-1 staining in 78 primary gastric cancer biopsies and matched non-cancerous gastric tissues using tissue microarray (TMA) technology and immunohistochemistry. In addition, we investigated the effects of AIF-1 on the proliferation and migration of the gastric cancer cell lines BGC-823 and SGC-7901 *in vitro*.

Materials and methods

Patients and specimens. The study cohort included 103 patients who underwent radical gastrectomy at Nantong Cancer Hospital from May 1, 1990 to June 1, 1995. A TMA including whole gastric cancer samples and matched non-

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cancerous gastric mucosa was constructed. Due to missing data in processing, the cohort included 103 patients but 20 samples were omitted, and 5 samples were lost during antigen retrieval or without tumor cells in the core. Finally 78 paired tissues were evaluated for AIF-1 expression. Written informed consent was obtained from each patient prior to tissue acquisition. Institutional approval was acquired from the Ethics Review Board of Nanjing Medical University prior to the present study. Detailed clinicopathological information was obtained from the medical records of the hospital. The histological types of gastric cancer were classified according to Lauren (12) and staged according to the tumor-node-metastasis (TNM) guidelines (13). Only confirmed intestinal, diffuse and mixed types were included.

TMA construction and immunohistochemistry. The gastric cancer TMAs were constructed as previously described (14). Duplicate 1.0-mm diameter cores of tissue from each sample were punched from the paraffin tumor block and corresponding non-tumoral tissues in the cohort. As a tissue control, the biopsies of normal gastric epithelium tissues were inserted in the four angles and the center of each slide.

A standard protocol was used for the immunostaining of the TMAs, as described in our previous study (14). The polyclonal rabbit anti-AIF-1 antibody (1:200 dilution; Abgent Technology, San Diego, CA, USA) was used. The omission of the primary antibody served as the negative control. The staining scores of the tissue controls in each microarray slide were pre-evaluated as quality control for the immunostaining.

TMA slides were de-waxed at 55˚C for 20 min followed by three 5-min washes with xylene. Rehydration of tissues was performed by 5-min washes in 100, 95 and 80% ethanol and distilled water, respectively. Antigen retrieval was performed by heating the samples at 95˚C for 30 min in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity of the tissue was blocked by incubation in 3% hydrogen peroxide for 30 min. After a 30-min blocking with Universal Blocking serum (Dako Diagnostics, Carpinteria, CA, USA), the sections were incubated with the anti-AIF-1 antibody at 4˚C overnight. The sections were then incubated for 30 min each with a biotin-labeled secondary antibody and then streptavidin-peroxidase (Dako Diagnostics). The samples were developed using 3,3'-diaminobenzidine substrate and counterstained with hematoxylin. Dehydration was then performed following a standard procedure, and the slides were sealed with coverslips.

Evaluation of immunohistochemistry. By applying a semi-quantitative immunoreactivity score (IRS) in the cohort, staining of AIF-1 in the tissues was scored independently by 2 pathologists blinded to the clinical data, as reported elsewhere (15). Category A categorized the intensity of immunostaining as 0-3 (0, negative; 1, weak; 2, moderate and 3, strong). Category B categorized the percentage of immunoreactive cells as 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). Multiplication of category A and B resulted in an IRS ranging from 0 to 12 for each tumor or non-tumor sample.

The optimum cutoff value of IRS was obtained by receiver-operator characteristic (ROC) analysis. The area under the curve (AUC) at different cutoff values of AIF-1 IRS for overall survival (OS) at 1, 3 and 5 years was calculated. The optimum value of cutoff points for AIF-1 IRS was 3 since the predictive value of this cutoff point for death was optimal. Therefore, samples with IRS 0-3 and IRS 4-12 were classified as having low and high AIF-1 expression, respectively, in the tumors.

Cell culture. For the *in vitro* experiments, BGC-823 and SGC-7901 human gastric cancer cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL Life Technologies, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Tianhang Biological Technology, Hangzhou, China). All cells were maintained in a 5% CO_2 atmosphere at 37˚C.

siRNA and cell growth assay. Control siRNA or AIF-1 siRNA (both from Ruibo Biotechnology, Guangzhou, China) was transfected using Lipofectamine 2000 (Invitrogen Life Technologies) according to manufacturer's instructions. For measurement of cell growth, a colorimetric water-soluble tetrazolium salt assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) was used to assess the number of viable cells at various time points following transfection.

Western blotting. Western blotting was carried out as previously described (14). Polyclonal rabbit anti-AIF-1 antibody (1:500 dilution; Abgent Technology, San Diego, CA, USA), monoclonal rabbit anti-β-catenin antibody (1:1,000 dilution; Epitomics, Burlingame, CA, USA), monoclonal mouse anti-tubulin antibody (1:2,000 dilution) and mouse anti-β-actin antibody (1:2,000 dilution) (both from Beyotime Biotechnology, Nantong, China) were used as the primary antibody. Immunoreactive bands were detected with a Phototope®-HRP Western Blot Detection kit (Cell Signaling Technology, Inc., Beverly, MA, USA). AIF-1 protein bands on the blots were measured by ImageJ software (version 1.44, National Institutes of Health, USA), after normalization to the corresponding tubulin level.

Transwell migration assay. Transwell migration assay was carried out in a 24-well modified 2-chamber plate (Corning, Tewksbury, MA, USA). The upper surface consisted of 6.5-mm diameter filters with 8-μm pore size. The transfectants ($2x10^4$ cells/well) were transferred into the upper chamber. After 12 h of incubation, the migrated cells on the lower surface of filters were fixed with 95% methanol and stained with crystal violet staining solution (Beyotime Biotechnology), and stained cell nuclei were counted in triplicate. We assessed the migration through the uncoated filters of test cells over that in the control counterparts.

Scratch migration assay. BGC-823 and SGC-7901 cells were transfected in a 6-well plate. Forty-eight hours post-transfection, the cells were scraped with the fine end of a 10-μl pipette tip (time 0). Plates were washed twice with PBS to remove detached cells, and incubated with the complete growth medium. Cell migration into the wounded empty space was determined after 24 h and photographed.

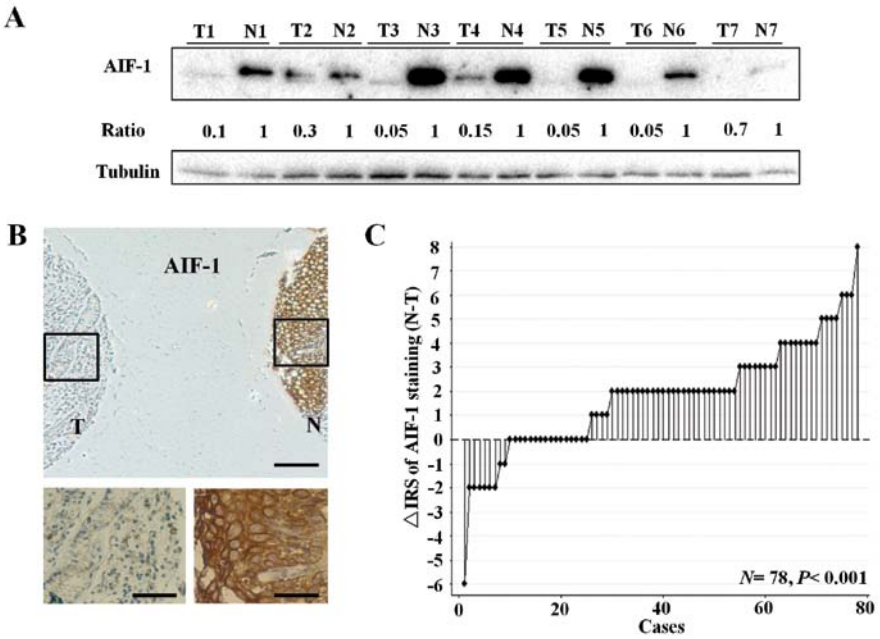

Figure 1. AIF-1 expression in primary tumors and corresponding non-tumor tissues in human gastric cancer. (A) AIF-1 protein levels in 7 cancer tissues and paired non-cancerous normal tissues of the gastric cancer patients were analyzed by western blotting. The level of protein was normalized against tubulin, and the protein levels in cancer tissues are indicated as a ratio to the paired non-cancerous normal tissues. T, tumor tissue; N, non-cancerous gastric tissue. (B) Immunohistochemical staining for AIF-1 in TMA. T, tumor tissue; N, non-cancerous gastric tissue. (Top panel, scale bar, 250 μm; bottom panel, scale bar, 50 μm). (C) The distribution of the difference in AIF-1 staining (ΔIRS = IRS N - IRS T). P-values were calculated with the Wilcoxon test. AIF-1, allograft inflammatory factor-1; TMA, tissue microarray; IRS, immunoreactivity score.

Statistical analysis. The association between AIF-1 expression and clinicopathological parameters was evaluated by Fisher's exact test. The significance of correlations between AIF-1 staining in primary tumors and their corresponding non-tumor tissues was assessed by the Wilcoxon test (grouped) and Spearman's rank-order correlation (raw scores). Probability of differences in OS as a function of time was ascertained by use of the Kaplan-Meier method, with a log-rank test probe for significance. All the statistical analyses were performed using Stata Statistical software (version 10.1; StataCorp, College Station, TX, USA). A difference with a P-value of <0.05 was deemed statistically significant.

Results

AIF-1 expression is decreased in gastric cancer when compared to that in the non-cancerous tissues. To test AIF-1 protein expression, 7 pairs of human primary gastric cancer tissues and matched normal gastric mucosa were randomly selected for western blot analysis. As a result, all of the gastric cancer tissues exhibited a significant reduction in AIF-1 when compared with that in the paired normal tissues (Fig. 1A). Furthermore, immunohistochemical staining of the gastric cancer TMA was used to further confirm AIF-1 expression in 78 gastric cancer patients. Staining of AIF-1 was mainly localized in the cytoplasm (Fig. 1B). Regarding the distribution of the differences in IRS, AIF-1 expression was significantly decreased in 53 of the 78 (67.9%) gastric cancer tissues when compared with that in the matched normal tissues (P<0.001, Wilcoxon test; Fig. 1C). The above data showed that the AIF-1 protein was reduced in gastric cancer tissues when compared with that in the gastric non-cancerous normal tissues.

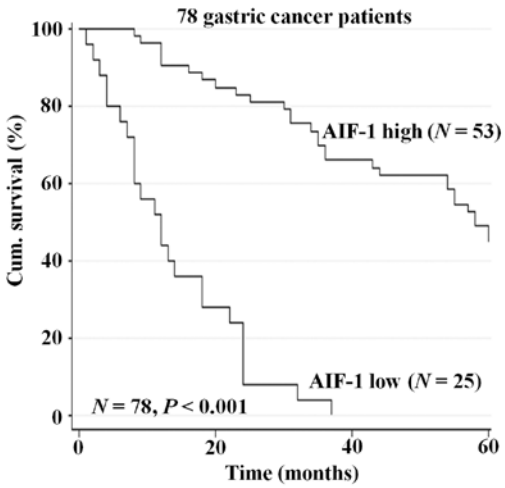

Figure 2. AIF-1 expression in gastric cancer is associated with increased overall survival (OS) in gastric cancer patients. Kaplan-Meier curves depicting OS according to expression profile of AIF-1 are shown. P-values were calculated using the log-rank test. AIF-1, allograft inflammatory factor-1.

AIF-1 expression negatively correlates with clinicopathological features of the gastric cancer patients. Immunohistochemical staining of AIF-1 levels was analyzed to determine their correlation with clinicopathological features. As shown in Table I, reduced protein expression of AIF-1 in the cancer tissues was significantly associated with malignant clinicopathological features, such as lymph node metastasis (N category), distant metastasis, TNM stage, tumor diameter and histological type (P<0.05). In addition, more

Table I. Correlation between expression levels of AIF-1 and the clinicopathological features of the gastric cancer patients (n=78).

Variables	AIF-1 expression		P-value[a]
	Low, n (%)	High, n (%)	
Total patients	25 (32.05)	53 (67.95)	
Age (years)			1.000
≤65	21 (84)	43 (81.13)	
>65	4 (16)	10 (18.87)	
Gender			0.419
Males	16 (64)	40 (75.47)	
Females	9 (36)	13 (24.53)	
Depth of invasion			0.176
T1/T2	4 (16)	17 (32.08)	
T3/T4	21 (84)	36 (67.92)	
Lymph node metastasis			<0.001
N0	1 (4)	22 (41.51)	
N1/N2/N3	24 (96)	31(58.49)	
Distant metastasis			0.004
M0	14 (56)	46 (86.79)	
M1	11 (44)	7 (13.21)	
TNM stage			<0.001
I	1 (4)	9 (16.98)	
II	1 (4)	22 (41.51)	
III	12 (48)	12 (22.64)	
IV	11 (44)	10 (18.87)	
Tumor diameter (cm)			0.041
≤5	12 (48)	36 (67.92)	
>5	13 (52)	17 (32.08)	
Histological type			0.007
Intestinal	16 (64)	16 (30.19)	
Diffuse	9 (36)	37 (69.81)	

[a]Two-sided Fisher's exact test. AIF-1, allograft inflammatory factor-1; TNM, tumor-node-metastasis.

cases with depth of T3/T4 invasion (T category) were noted in the group with low AIF-1 expression although the difference did not reach a statistically significant level. These observations suggest that deficiency in functional AIF-1 expression may contribute to clinical gastric cancer progression.

Expression of AIF-1 is an independent prognostic indicator for overall survival of gastric cancer patients. To further determine the prognostic value of AIF-1 in gastric cancer, overall survival was analyzed. Kaplan-Meier survival curves showed that reduced AIF-1 expression in gastric cancer tissues was significantly correlated with shorten overall 5-year survival in the patients in the cohort (P<0.001, Fig. 2). This suggests that expression of AIF-1 in gastric cancer tissues may be an independent biomarker of patient overall survival.

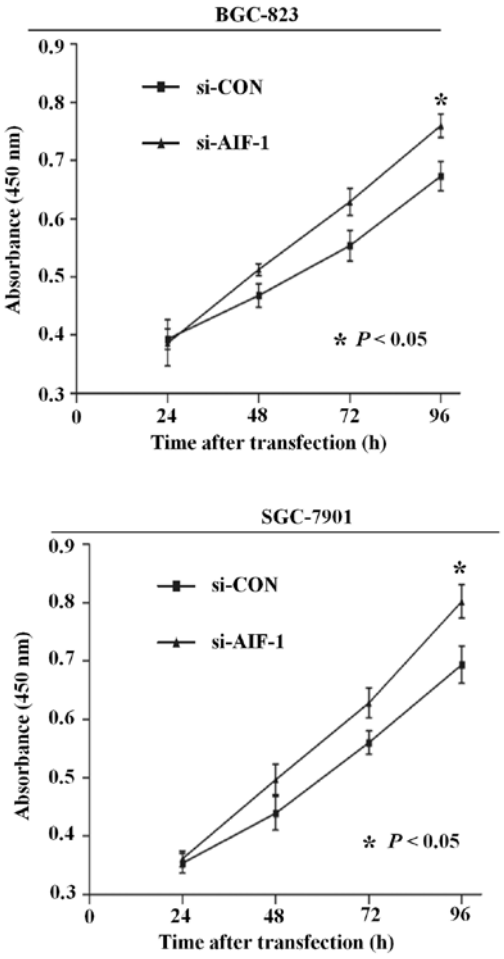

Figure 3. Knockdown of AIF-1 increases proliferation in gastric cancer cells. The number of viable BGC-823 and SGC-7901 cells at various time points after transfection of si-AIF-1 was assessed by a colorimetric water-soluble tetrazolium salt assay. AIF-1, allograft inflammatory factor-1.

Deficiency of AIF-1 expression contributes to proliferation and migration in gastric cancer cells. To determine the potential role of AIF-1 in the development of gastric cancer, we designed a series of cell culture models to investigate whether knockdown of AIF-1 affects proliferation, migration and adhesion of gastric cancer cells. As shown in Fig. 3, the ability for cell proliferation was significantly increased after knockdown of AIF-1 by gene-specific siRNA in both BGC-823 and SGC-7901 cells. Similarly, the Transwell migration assay revealed that the numbers of BGC-823 and SGC-7901 cells that migrated through the membrane into the lower chamber were significantly higher in the si-AIF-1 transfected cells when compared with these numbers in the controls (Fig. 4). In addition, AIF-1-deficient cells also demonstrated accelerated wound closure (Fig. 5). These results indicate that AIF-1 deficiency contributes to the development of the malignant phenotype in gastric cancer cells.

AIF-1 deficiency activates β-catenin expression in gastric cancer cells. Previous studies have reported that β-catenin activation plays an important role in gastric cancer (16). To test whether the role of AIF-1 is realized via β-catenin in gastric cancer cells, AIF-1 and β-catenin protein in human

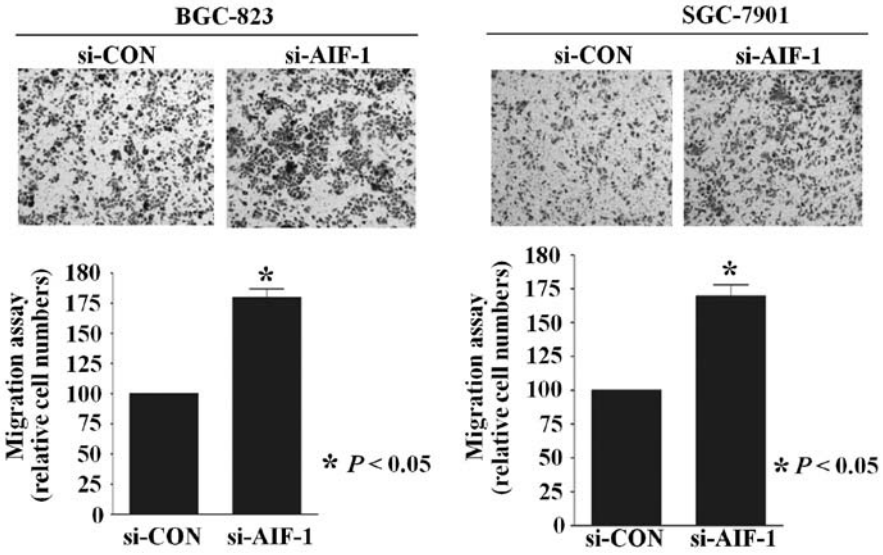

Figure 4. Knockdown of AIF-1 increases migration in gastric cancer cells as determined by the Transwell migration assay. AIF-1, allograft inflammatory factor-1.

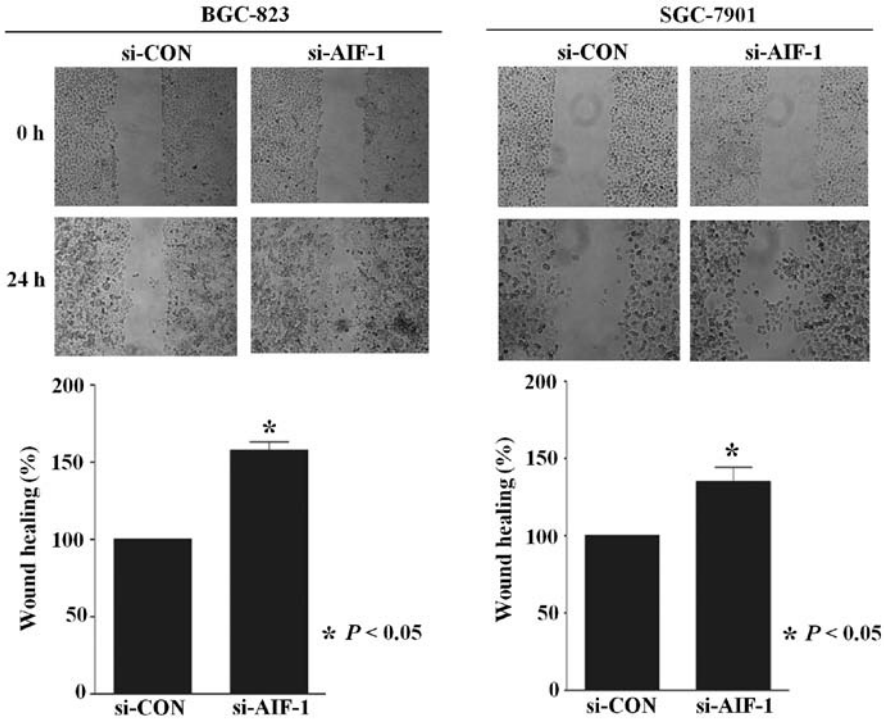

Figure 5. Knockdown of AIF-1 enhances wound healing closure in gastric cancer cells as determined by the scratch migration assay. AIF-1, allograft inflammatory factor-1.

gastric cancer cell lines was detected by western blotting. As shown in Fig. 6, the expression of β-catenin was upregulated in BGC-823 and SGC7901 cells following transfection with si-AIF-1 when compared with the control (Fig. 6). These data suggest that AIF-1 deficiency promotes β-catenin expression in gastric cancer cells.

Discussion

The assessment of biological prognostic factors is of clinical importance, particularly for diseases with poor outcome such as gastric cancer. In the present study, we investigated the role of AIF-1 expression in the prognosis of gastric cancer patients for the first time. Decreased AIF-1 expression in gastric cancer when compared to non-cancerous tissues was observed. Moreover, we showed that AIF-1 expression was associated with clinical progression and prognosis of the gastric cancer patients. In the *in vitro* models, AIF-1 deficiency promoted cell growth, migration and β-catenin expression in gastric cancer cell lines BGC-823 and SGC-7901. Thus, the functions of AIF-1 in gastric cancer cells may be realized through the suppression of β-catenin expression.

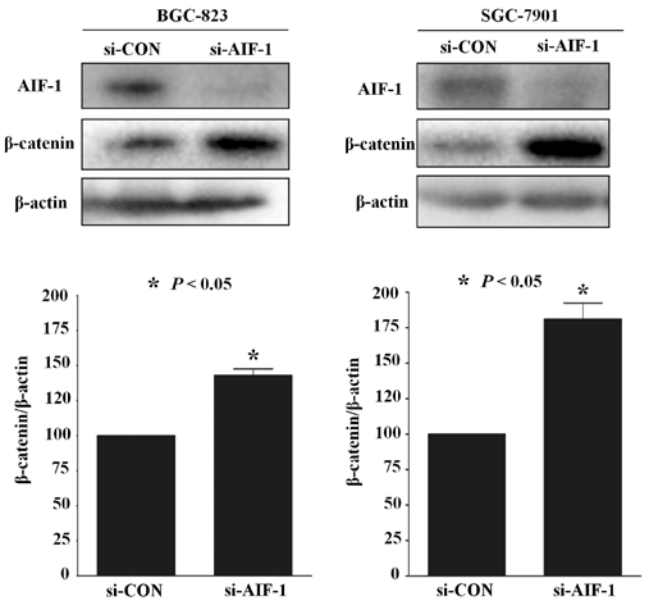

Figure 6. AIF-1 negatively regulates β-catenin expression in gastric cancer cells. The protein expression of β-catenin following transfection of si-AIF-1 in BGC-823 and SGC-7901 cells was assessed by western blotting. AIF-1, allograft inflammatory factor-1.

AIF-1 is a cytoplasmic, calcium-binding, inflammation-responsive scaffold protein that has been implicated in the regulation of inflammation. The AIF-1 gene is located on chromosome 6p21.3, which is densely clustered with genes involved in the inflammatory response, including surface glycoproteins, complement cascade, TNF-α, TNF-β and NF-κB genes (17). It has been reported that AIF-1 is closely associated with cardiac allograft vasculopathy, rheumatoid arthritis, inflammatory skin disorders and systemic sclerosis (6,7). Since AIF-1 may be involved in the cytoskeletal signaling network and may contribute to the progression of EMT (18,19), it is evident that aberrant regulation of AIF-1 may lead to tumor progression. Previous studies have revealed that AIF-1 can promote the growth of breast tumors via the activation of NF-κB signaling, which consequently upregulates the expression of cyclin D1 (9). Moreover, expression of AIF-1 was found to be upregulated in cervical cancer tissues (20). These results indicate that AIF-1 may function as an oncogene. However, in the present study, our results provided novel evidence of a strong association between AIF-1 upregulation and clinicohistopathological parameters, indicative of a more favorable outcome in gastric cancer patients. This suggests that there may be another mechanism by which AIF-1 is involved in gastric cancer progression.

We noted that the roles of β-catenin in mediating intercellular adhesion and regulation of cell growth, differentiation, invasion and metastasis have been well characterized (21,22). The β-catenin-TCF/LEF complex regulates and activates its downstream target transcription genes which are involved in the development and progression of cancer (23-25). The abnormal activation of β-catenin frequently occurs in gastric cancer and has been proven to promote tumor growth, invasion and metastasis (26,27). Furthermore, previous studies have confirmed that high β-catenin expression is an independent indicator of poor prognosis for these carcinomas and is closely correlated with enhanced tumor progression (28,29).

In the present study, we found that AIF-1 knockdown promoted β-catenin expression in gastric cancer. Our observations were consistent with the previous finding that β-catenin activity is negatively correlated with bacteria-induced inflammation (30). Further studies are required to obtain a detailed profile of the exact mechanisms involved in the regulation of β-catenin by AIF-1 in gastric tumor development.

This is the first study to report the association between AIF-1 expression and the clinicopathological features of gastric cancer. Our data demonstrate that AIF-1 functions as a tumor suppressor possibly by regulating β-catenin in gastric cancer. Moreover, loss of AIF-1 expression may represent a novel indicator for the progression and prognosis of gastric cancer. Nevertheless, despite highly significant results in our patient cohort, further studies must be carried out to evaluate whether AIF-1 may be beneficial as a future preventive and therapeutic target for gastric cancer.

Acknowledgements

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