

## ORIGINAL ARTICLE

# Polyamine synthesis enzyme AMD1 is closely associated with tumorigenesis and prognosis of human gastric cancers

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## Abstract

Adenosylmethionine decarboxylase 1 (AMD1) is a key enzyme involved in biosynthesis of polyamines including spermidine and spermine. The potential function of AMD1 in human gastric cancers is unknown. We analyzed AMD1 expression level in 319 human gastric cancer samples together with the adjacent normal tissues. The protein expression level of AMD1 was significantly increased in human gastric cancer samples compared with their corresponding para-cancerous histological normal tissues ( $P < 0.0001$ ). The expression level of AMD1 was positively associated with *Helicobacter pylori* 16sRNA ( $P < 0.0001$ ), tumor size ( $P < 0.0001$ ), tumor differentiation ( $P < 0.05$ ), tumor venous invasion ( $P < 0.0001$ ), tumor lymphatic invasion ( $P < 0.0001$ ), blood vessel invasion ( $P < 0.0001$ ), and tumor lymph node metastasis (TNM) stage ( $P < 0.0001$ ). Patients with high expression of AMD1 had a much shorter overall survival than those with normal/low expression of AMD1. Knockdown of AMD1 in human gastric cancer cells suppressed cell proliferation, colony formation and cell migration. In a tumor xenograft model, knockdown of AMD1 suppressed the tumor growth *in vivo*. Inhibition of AMD1 by an inhibitor SAM486A in human gastric cancer cells arrested cell cycle progression during G<sub>1</sub>-to-S transition. Collectively, our studies at the cellular, animal and human levels indicate that AMD1 has a tumorigenic effect on human gastric cancers and affect the prognosis of the patients.

## Introduction

Polyamines are polycationic alkylamines including putrescine, spermidine and spermine and are present in mammalian cells in millimolar concentration (1). The biosynthesis of polyamines is mainly controlled by two rate-limiting enzymes, ornithine decarboxylase (ODC) and adenosylmethionine decarboxylase 1 (AMD1). ODC catalyzes the formation of putrescine from ornithine, whereas AMD1 produces the aminopropyl donor, decarboxylated S-adenosylmethionine (dcAdoMet), required for spermidine and spermine synthesis (1). Polyamine metabolism is closely related to many fundamental cellular processes such as

differentiation, apoptosis, nucleic acid synthesis, transcription, translation, autophagy and protection from oxidative stress (1–5). The contribution of polyamines to protein translation is exemplified by the need of spermidine for formation of an uncommon amino acid hypusine that is present in eukaryotic initiation factor 5A isoform 1 (eIF5A) to prevent ribosomal stalling during the translation of messenger RNAs (mRNAs) encoding polyproline tracts (6). Such function of polyamines is linked to cancer formation as MYC oncogene drives transcription of ODC gene and increases the availability of spermidine for hypusine formation (7).

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**Abbreviations**

AMD1	adenosylmethionine decarboxylase 1
CagA	cytotoxin-associated gene A
FBS	fetal bovine serum
IHC	immunohistochemistry
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
ODC	ornithine decarboxylase
OS	overall survival
PBS	phosphate-buffered saline
PCHNT	para-cancerous histological normal tissue
RT-PCR	real-time PCR
SMOX	spermine oxidase

Polyamines plays numerous important roles for the proliferation and survival of the cells and elevated polyamine levels are required for the tumor cell transformation and tumor progression (1). A recent study indicated that the polyamine-hypusine axis functions as a new tumor suppressor network to regulate apoptosis in lymphoma (8). Heterozygous deletions encompassing AMD1 and *elF5A* often occur together in human lymphomas, and co-inhibition of both genes promote lymphomagenesis in mice (8). In prostate cancers, AMD1 and polyamine synthesis are found to be dysregulated in response to elevated mTORC1 pathway in cancer cells (9). The activation of mTORC1 leads to a reduction of AMD1 stability and an increase in AMD1 protein level, resulting in elevated polyamine synthesis (9). In a clinical trial using an mTORC1 inhibitor, the immune reactivity of AMD1 was decreased, together with a reduction in oncogenesis (9). Lately, it was found that concomitant inhibition of polyamine synthesis and polyamine uptake is able to reduce oncogenesis of neuroblastoma in mice (10). However, it is currently unknown whether AMD1 is implicated in the oncogenesis of gastric cancers. In this study, we explored the association of AMD1 expression with clinical features of gastric cancer patients and analyzed the effect of AMD1 on gastric cancer cells *in vitro* and *in vivo*.

**Materials and methods****Patients**

Three hundred and nineteen patients with gastric adenocarcinoma were randomly selected from January 2008 to December 2017 in Zhejiang Cancer Hospital, including 219 males and 100 females aged 26–84 years with an average age of 59.1 years. The gastric cancer tissue was taken from the cancer nest, and the adjacent normal tissue was obtained from the pathologically confirmed tissue from the edge of the tumor by 5 cm. The tissues were removed and stored in liquid nitrogen for a short time, then transferred to  $-80^{\circ}\text{C}$  refrigerator. According to the eighth edition of the (AJCC) guidelines of the International Anti-Cancer Union (UICC)/Joint American Committee on Cancer (11), 119 cases with stage I/II and 200 cases with stage III/IV were divided into two groups. The mean follow-up time was 120 months on 31 December 2017. The lost visit rate was 6.0% (19 cases). All patients were first diagnosed and had no preoperative radiotherapy and chemotherapy or other tumors and had complete clinical data. The study was approved by the ethics committee in Zhejiang Cancer Hospital (Ethical certification No. zjzlyy[2013]-03-79 and zjzlyy[2015]-02-125) and informed consent was signed with patients and their families.

**Antibodies and reagents**

The antibodies and inhibitor used in this study were purchased as follows: anti-phosphorylated retinoblastoma and anti-cyclin B from Cell Signaling Technology (Cat# 3590 and 12231, Boston, MA); anti-cyclin A from Santa Cruz Biotechnology (Cat# sc-136253, Santa Cruz, CA); anti-glyceraldehyde

3-phosphate dehydrogenase from Abcam (Cat# ab181602, Cambridge, UK); anti-AMD1 from Proteintech (Cat# 11052-1-AP, Chicago) and SAM486A from MedKoo (Cat# 200411, Morrisville, NC).

**Cell culture**

HEK293T cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Human gastric adenocarcinoma cells line AGS was cultured in Ham's F-12K (Kaighn's) medium containing 10% FBS, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. All the cell lines were maintained in a cell incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in humidified atmosphere. Transient transfection was performed with the polyetherimide transfection reagent (Sigma-Aldrich, St. Louis, MO) for HEK293T cells. All the cell lines were purchased from Cell bank of the SIBS, Chinese Academy of Sciences, and satisfactorily authenticated based on polymorphic short tandem repeat loci. All the cell lines were mycoplasma free and used within 6 months of receipt.

**Lentivirus packaging and infection**

For lentiviral packaging, HEK293T cells ( $6 \times 10^6$ ) were seeded in 15 cm cell culture dishes, incubated for 24 h and then transfected with 30.75  $\mu\text{g}$  of lentivirus plasmids performing with polyetherimide. At 48 h and 72 h, the virus-containing medium was collected for the first and second time, and then filtered through 0.45  $\mu\text{m}$  filter (Millipore, Danvers, MA). Next, the filtered supernatants were centrifuged at 20 000 r.p.m.,  $4^{\circ}\text{C}$  for 2 h. Afterward, the supernatants were discarded and precipitate was suspended in 100  $\mu\text{l}$  Dulbecco's modified Eagle's medium and divided into 1.5 ml tubes. For lentivirus infections, the AGS cells were cultured in six-well plates for infection by moderate virus-contained Dulbecco's modified Eagle's medium with polybrene (Sigma-Aldrich), incubated at  $37^{\circ}\text{C}$  for 24 h and replaced by Ham's F-12K (Kaighn's) medium with 10% FBS, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin.

**RNA isolation and quantitative real-time PCR**

The AGS cells were lysed in Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated under RNA-free environment according to the manufacturer's instructions. Then complementary DNA was obtained by reverse transcribing RNA with FastQuant RT Kit (Tiangen, Shanghai, China). Real-time PCR (RT-PCR) was performed with the SYBR Green PCR system (Applied Biosystems, Foster City, CA) and conducted with an ABI Prism 7900 sequence detection system (Applied Biosystems). The primers used in RT-PCR were as follows: human  $\beta$ -actin: 5'-CTGGAACGGTGAAGGTGACA-3' and 5'-AAGGGACTTCTGT AACAATGCA-3'; human AMD1: 5'-TTTCGAAGGACCG AGAAGC-3' and 5'-ACTCTCACTGGGATAGTGCG-3'.

**Cell proliferation and migration assays**

AGS cells were seeded at a density of  $3 \times 10^3$  cells/well into a 96-well culture plate and grown in 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  for various time. Cell viability was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich) as follows: 20  $\mu\text{l}$  of 5 mg/ml MTT was added to each well and incubated with cells at  $37^{\circ}\text{C}$  for 4 h. The MTT solution was then discarded, and 200  $\mu\text{l}$  dimethyl sulfoxide (Amresco, Solon, OH) was added to dissolve the formazan sediment. Finally, the optical density was detected using a microplate reader (Molecular Devices, San Jose, CA) at an absorption wavelength of 570 nm. For colony formation assay, the AGS cells were inoculated into six-well culture plate with 300 cells per well and cultured for 14 days, then stained with crystal violet and counting. For cell migration assay, the AGS cells were seeded into 24-well transwell chambers and the cells in the lower chamber were fixed in 24 h and stained with crystal violet before counting.

**Nude mice xenograft model**

All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, SIBS, Chinese Academy of Sciences. All of the experimental procedures were carried out in accordance with the Chinese Academy of Sciences ethics commission with an approval number 2010-AN-8. The cells in the logarithmic phase of growth were collected,

centrifuged and rinsed with phosphate-buffered saline (PBS) twice. The nude mice (5 weeks old, female) were injected subcutaneously with AGS cells ( $6 \times 10^6$  cells) in the outside of the forelegs. The xenograft tumor sizes were analyzed by measuring two perpendicular diameters with digital calipers every other day and calculated according to the formula:  $0.5 \times \text{length} \times \text{width}^2$ . The xenograft tumor weights were measured after the nude mice were killed and removed the tumor.

### Cell cycle analysis

The AGS cells were seeded into six-well culture plate with  $5 \times 10^5$  cells per well and induced cell cycle synchronization through serum starvation for 24 h and then exposed to 10% serum for various times. The cells were collected, centrifuged and rinsed with precooling PBS twice. Then 70% ethanol was added and the cells were fixed at 4°C overnight or -20°C for long-term storage. Finally, the cells were centrifuged and rinsed with precooling PBS once and added with 50 µg/ml propidium iodide (Sigma-Aldrich) to stain the DNA. The cell cycle phase was evaluated by flow cytometry (BD FACSCalibur, San Jose, CA) and the data was analyzed by FlowJo 7.6.

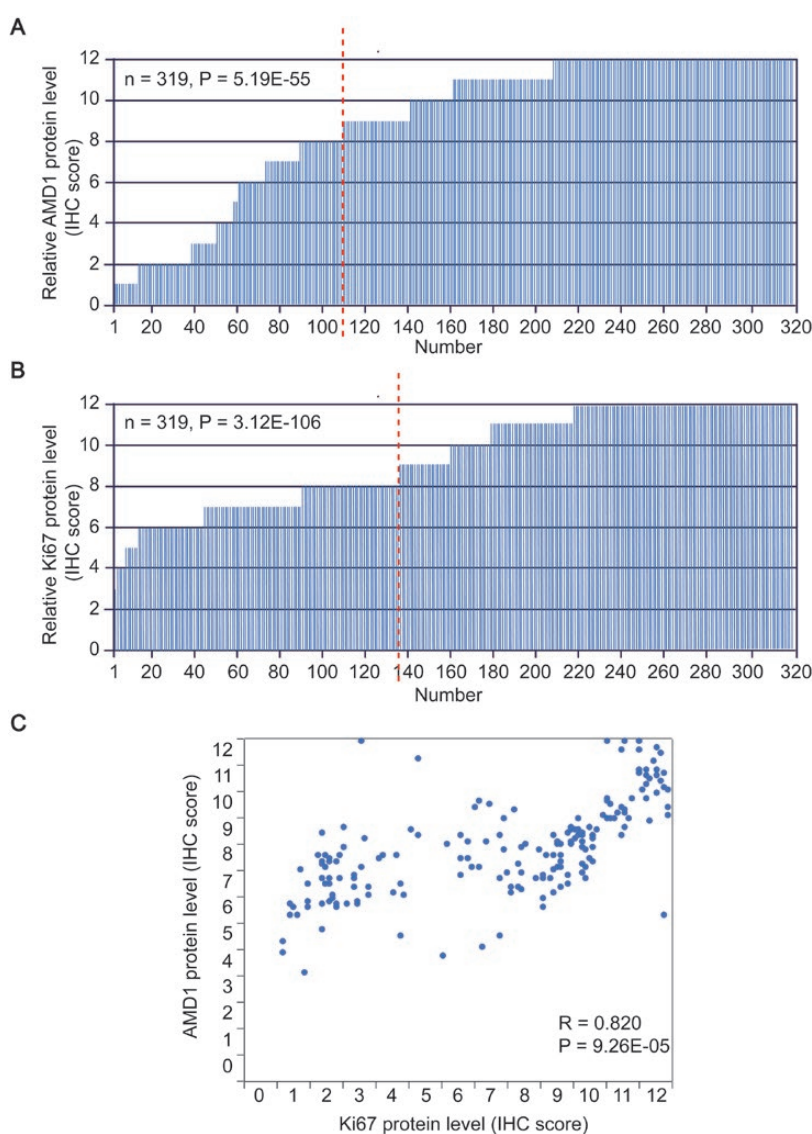
### Statistical analysis

Statistical analysis with the cellular and animal data was performed using Student's t-test. The AMD1 immunohistochemistry (IHC) score data comparisons for each clinical pathological parameter was analyzed by  $\chi^2$  test and Fisher's exact test. Patient survival analysis was evaluated by means of the Kaplan–Meier method and the significance levels were assessed by means of the log-rank test. The univariate and multivariate analysis with the Cox regression model were performed to determine prognostic factors and explore the combined effects.

## Results

### AMD1 is significantly upregulated in primary gastric cancer tissues and correlated with the clinical characteristics of the patients

A previous study revealed that the single nucleotide polymorphism of AMD1 is linked to the risk of human gastric



**Figure 1.** Expression of AMD1 is upregulated in primary gastric carcinomas and correlated with Ki67. (A) The protein level of AMD1 in 319 primary gastric cancer tissues and their adjacent normal tissues are determined by IHC. IHC score of 0–8 is considered to be low and normal expression and 9–12 is considered to be high expression. The dotted red line represents the separation between high expression group versus low/normal expression level group. (B) The protein level of Ki67 in 319 primary gastric cancer tissues are determined by IHC. IHC score of 0–8 is considered to be low and normal expression and 9–12 is considered to be high expression. (C) The correlation between AMD1 protein level and Ki67 protein level determined by IHC in 319 primary gastric cancer tissues.

cancers (12). To explore whether the expression level of AMD1 is altered in human gastric cancers, we investigated the protein expression level of AMD1 in 319 primary gastric cancer samples together with their corresponding para-cancerous histological normal tissue (PCHNT). We carried out IHC to analyze the protein level of AMD1. We also analyzed the expression level of Ki67 that is a marker of cellular proliferation and commonly used as important prognostic in many malignant diseases (13). Overall, the protein level of AMD1 was robustly increased (with IHC score = 9–12) in gastric cancer samples in comparison with PCHNT ( $P < 0.0001$ , Figure 1A). Similarly, the expression of Ki67 was significantly upregulated in gastric cancer samples compared with PCHNT ( $P < 0.0001$ , Figure 1B). At the protein level, AMD1 was increased in 66.5% (212/319) gastric cancer samples, whereas AMD1 upregulation was not significant in the

tumor-adjacent normal tissues (Table 1). Intriguingly, the protein level of AMD1 was highly correlated with the protein level of Ki67 ( $R = 0.80$ ,  $P < 0.0001$ , Figure 1C), indicating that high level of AMD1 expression is positively correlated with elevated level of cell proliferation.

We also analyzed the association of AMD1 expression level with numerous clinical characteristics of gastric cancers. The expression level of AMD1 in the gastric cancer samples were strongly correlated with *Helicobacter pylori* (*H. pylori* 16sRNA ( $P < 0.0001$ ), tumor size ( $P < 0.0001$ ), tumor differentiation ( $P < 0.05$ ), tumor venous invasion ( $P < 0.0001$ ), tumor lymphatic invasion ( $P < 0.0001$ ), blood vessel invasion ( $P < 0.0001$ ), invasive depth ( $P < 0.0001$ ), TNM stage ( $P < 0.0001$ ) and distant metastasis ( $P < 0.05$ ) (Table 1). On the other hand, AMD1 expression level was not significantly associated with the gender, age at

**Table 1.** Correlation between AMD1 expression level and clinicopathologic parameters

Clinicopathologic parameter	n	AMD1 protein level		$\chi^2$ (P-value)
		Low/normal, n (%)	High, n (%)	
Case				
Tumor	319	107 (33.5)	212 (66.5)	NA
PCHNT	319	319 (100%)	0 (0)	
Gender				
Male	219	76 (23.8)	143 (44.8)	0.273 (0.602)
Female	100	31 (9.7)	69 (21.7)	
Age at diagnosis				
<60	165	62 (19.4)	103 (32.3)	2.134 (0.144)
≥60	154	45 (14.1)	109 (34.2)	
Tumor site				
Cardia	89	28 (8.8)	61 (19.1)	0.128 (0.7206)
Body/antrum	230	79 (24.8)	151 (47.3)	
Hp 16sRNA				
Negative	62	27 (8.5)	35 (11.0)	17.917 (2.31E-05)
Positive	257	45 (14.1)	212 (66.4)	
Hp CagA				
Negative	96	39 (12.2)	57 (17.9)	2.652 (0.103)
Positive	223	68 (21.3)	155 (48.6)	
Tumor size				
< 5 cm	156	88 (27.6)	68 (21.3)	69.628 (2.20E-16)
≥5 cm	163	19 (6.0)	144 (45.1)	
Differentiation				
Low	270	83 (26.0)	187 (58.6)	5.398 (0.020)
High/medium	49	24 (7.5)	25 (7.9)	
Venous invasion				
No	126	62 (19.4)	64 (20.1)	14.584 (1.34E-04)
Yes	193	45 (14.1)	148 (46.4)	
Lymphatic invasion				
No	159	77 (24.1)	82 (25.7)	30.193 (3.91E-08)
Yes	160	30 (9.4)	130 (40.8)	
Blood vessel invasion				
Negative	146	67 (21.0)	79 (24.8)	17.407 (3.02E-05)
Positive	173	40 (12.5)	133 (41.7)	
Distant metastasis				
No	303	107 (33.5)	196 (61.5)	6.9915 (0.008)
Yes	16	0 (0.0)	16 (5.0)	
TNM stage				
I/II	119	90 (28.2)	29 (9.1)	147.830 (2.20E-16)
III/IV	200	17 (5.3)	183 (57.4)	
Invasive depth				
T1/T2	49	41 (12.9)	8 (2.5)	62.638 (2.48E-15)
T3/T4	270	66 (20.7)	204 (63.9)	

AMD1 protein upregulation is defined as IHC score = 9–12. P-value are from the Chi-square test. P-value >0.05 is considered statistically significant. Hp Cag A, *H. pylori* cytotoxin-associated gene A; Hp 16sRNA, *H. pylori* 16S ribosomal; TNM, tumor, lymph node, metastasis.

diagnosis, tumor site and *H. pylori* cytotoxin-associated gene A (CagA) (Table 1). In conclusion, these findings indicate that AMD1 expression level is significantly upregulated in gastric cancers and such increase is tightly associated with the progression and invasion of the tumors.

### The expression level of AMD1 is closely associated with the prognosis of gastric cancer patients

We next investigated the association of AMD1 expression level with the survival of the patients. We analyzed the overall survival (OS), which is defined as the time from surgery to death. The average durations of OS in patients with AMD1 upregulation (IHC score = 9–12) in the tumors were prominently shorter than those with low/normal expression (IHC score = 0–8) in the tumors ( $P < 0.0001$ , Figure 2). The average OS in patients with high expression of AMD1 was 36.0 months versus 66.6 months in patients with low/normal expression of AMD1 ( $P < 0.0001$ , Table 2). Interestingly, the association of AMD1 expression level with patient survival was strongly associated with tumor stage. The association of AMD1 expression level with the survival was more pronounced in patients at TNM stage III/IV than those at TNM stage I/II (Figure 2B). In addition, we analyzed the association of numerous clinical parameters with the patient survival (Table 2). Surprisingly, the expression level of AMD1 was the most important factor than others to correlate with the duration of patient survival (Table 2). Collectively, these data suggest that the expression level of AMD1 might be regarded as an independent marker to predict the survival and prognosis of the patients with gastric cancer.

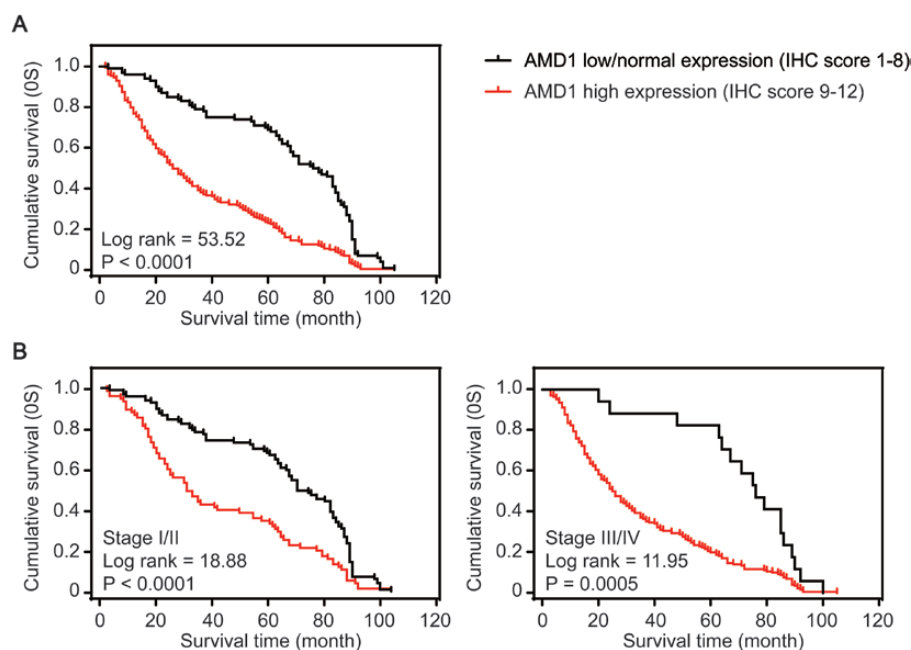
### Risk assessment of gastric cancer patients based on the expression level of AMD1

We performed detailed analysis to assess the risk of gastric cancer based on the protein expression level of AMD1. We used IHC score 0–8 for low to normal expression and 9–12 for high expression in these assays. The patients with

high expression level of AMD1 had 15.84-fold increased risk for increased invasive depth ( $P < 0.0001$ ), 4.07-fold increased risk for lymphatic invasion ( $P < 0.0001$ ), 3.17-fold increased risk for venous invasion ( $P < 0.0001$ ), 2.82-fold increased risk for blood vessel invasion ( $P < 0.0001$ ) and 33.41-fold increase risk for a late TNM stage ( $P < 0.0001$ ; Supplementary Table S1, available at *Carcinogenesis Online*). Besides, the expression level of AMD1 significantly increased the risk of distant metastasis of gastric cancer (Supplementary Table S1, available at *Carcinogenesis Online*). In summary, these data further confirmed our findings that AMD1 upregulation is closely associated with the progression in gastric cancer patients.

### AMD1 regulates proliferation and migration of human gastric cancer cells

We next investigated whether AMD1 had a direct effect on the growth and migration of AGS cell, that is, a moderately differentiated *in situ* gastric adenocarcinoma from a single biopsy specimen of an untreated human adenocarcinoma of the stomach (14). We established cell clones with endogenous AMD1 being silenced by the two different AMD1-specific short hairpin RNA (shRNA). The efficiency of AMD1 knockdown was confirmed by quantitative RT-PCR (Figure 3A) and immunoblotting (Supplementary Figure S1, available at *Carcinogenesis Online*). As analyzed by MTT assay, the cell proliferation rate of AGS cells was significantly inhibited by AMD1 knockdown (Figure 3B). Consistently, AMD1 knockdown profoundly reduced colony formation in AGS cells (Figure 3C). In addition, the migration of AGS cells as analyzed by transwell assay was significantly inhibited by AMD1 knockdown (Figure 3D). Collectively, these results suggested that AMD1 has a negative effect on cell proliferation and migration of gastric cancer cells, supporting our clinical findings that AMD1 expression level is positively correlated with the malignancy and severity of gastric cancers.



**Figure 2.** Correlation of AMD1 expression level with survival of patients with gastric cancer. The Kaplan-Meier curves of OS in patient with gastric cancer treated with primary gastrectomy according to the expression of AMD1. Overall OS is shown in (A) and its stratifications based on different TNM stages are shown in (B) according to the protein expression level of AMD1.

**Table 2.** Univariate and multivariate analysis of survival according to clinicopathologic parameters and AMD1 expression level

Clinicopathologic parameter	n	Survival (months)	OS			
			Univariate analysis		Multivariate analysis	
			HR (95% CI)	(P-value)	HR (95% CI)	(P-value)
Gender						
Male	219	46.6	1.06 (0.79–1.43)	0.683	1.06 (0.77–1.45)	0.721
Female	100	45.5				
Age at diagnosis						
<60	165	48.9	1.14 (0.87–1.51)	0.332	1.13 (0.84–1.51)	0.425
≥60	154	43.6				
Tumor site						
Cardia	89	49.0	1.15 (0.84–1.57)	0.383	1.36 (0.97–1.91)	0.075
Body/antrum	230	45.3				
Hp 16sRNA						
Negative	62	44.3	0.89 (0.66–1.20)	0.436	0.76 (0.48–1.20)	0.238
Positive	257	47.1				
Hp CagA						
Negative	96	46.5	0.98 (0.69–1.40)	0.923	1.13 (0.66–1.91)	0.665
Positive	223	46.2				
Tumor size						
<5 cm	156	51.6	1.35 (1.02–1.78)	0.035	1.35 (0.54–1.02)	0.069
≥5 cm	163	41.2				
Differentiation						
Low	270	44.6	0.61 (0.40–0.93)	0.022	0.73 (0.46–1.15)	0.17
High/medium	49	55.9				
Venous invasion						
No	126	54.3	1.72 (1.28–2.31)	2.99E-04	1.25 (0.91–1.73)	0.163
Yes	193	41.0				
Lymphatic invasion						
No	159	52.8	1.55 (1.17–2.05)	2.25E-03	1.25 (0.90–1.75)	0.187
Yes	160	39.7				
Blood vessel invasion						
Negative	146	51.2	1.46 (1.10–1.94)	8.47E-03	1.13 (0.83–1.55)	0.421
Positive	173	41.0				
Distant metastasis						
No	303	47.2	2.11 (1.26–3.53)	4.59E-03	1.47 (0.86–2.56)	0.162
Yes	16	30.9				
TNM stage						
I/II	119	59.9	2.42 (1.77–3.31)	3.20E-08	1.09 (0.59–1.42)	0.697
III/IV	200	38.1				
Invasive depth						
T1/T2	49	61.5	1.73 (1.36–2.20)	7.73E-06	1.14 (0.84–1.55)	0.386
T3/T4	270	43.3				
AMD1 expression						
1–8	107	66.6	3.45 (2.44–4.87)	2.33E-12	3.28 (2.03–5.31)	1.22E-06
9–12	212	36.0				

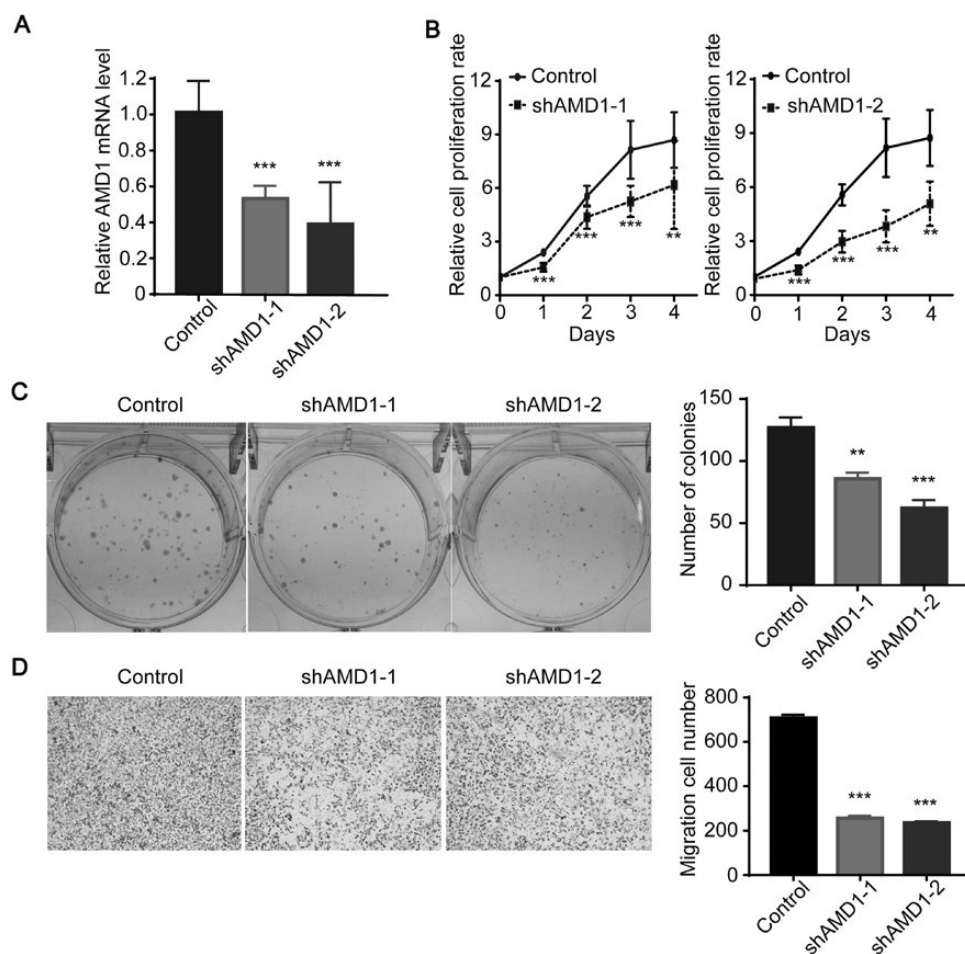
P-values are derived from univariate and multivariate Cox regression analysis. CI, confidence interval; HR, hazard ratio.

### AMD1 promotes the growth of the gastric cancer cells *in vivo*

In order to further elucidate the tumorigenic activity of AMD1 in gastric cancers, we next investigated the effect of AMD1 on tumor growth using a xenograft model. AGS cells with endogenous AMD1 being silenced by the two different AMD1-specific shRNA were inoculated into nude mice. The mice were killed in 22 days when the tumor formation became obvious. The growth of AGS cells in the mice as measured by tumor volume and tumor weight were significantly reduced by AMD1 knockdown (Figure 4). These observations, therefore, clearly indicated that AMD1 has a strong tumorigenic effect on gastric cancer cells *in vivo*.

### AMD1 inhibitor SAM486A delays the cell cycle progression of AGS cells

We next analyzed the effect of an AMD1 inhibitor on the growth and cell cycle of AGS cells. SAM486A (also known as CGP48664) is a derivative of the first-generation AMD1 inhibitor mitoguazone and has a strong and specific inhibitory effect on AMD1 (15,16). SAM486A has a growth-inhibitory effect on different types of cancers including colorectal cancer, lymphoma, neuroblastoma and melanoma with almost complete inhibition of cell growth (17–21). To analyze whether SAM486A could also affect AGS cells, we first investigated the effect of SAM486A on the cell proliferation rate. As showed in Supplementary Figure S2A, available at Carcinogenesis Online, the cell growth rate was significantly



**Figure 3.** AMD1 modulates cell proliferation, colony formation and migration of gastric cancer cells. (A) The mRNA level of AMD1 in AGS cells expressing control shRNA (control) or AMD1-specific shRNAs (shAMD1-1 and shAMD1-2) were determined by quantitative RT-PCR. (B) Effect of AMD1 knockdown on cell proliferation. The gastric cancer cells as in A were used to determine the cell proliferation rate by MTT assay at the indicated time point. (C) Effect of AMD1 knockdown on colony formation. AGS cells as in A were seeded into six-well plate with 300 cells per well and cultured for 14 days, and then used in crystal violet staining and colony counting. Statistical analysis is shown in the right panel. (D) AGS cells were seeded into 24-well transwell chambers and the cells on lower chamber were fixed in 24 h and stained with crystal violet. Statistical analysis is shown in the right panel. All the data are shown as mean  $\pm$  standard deviation, \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . The same experiments were performed at least three times with similar results.

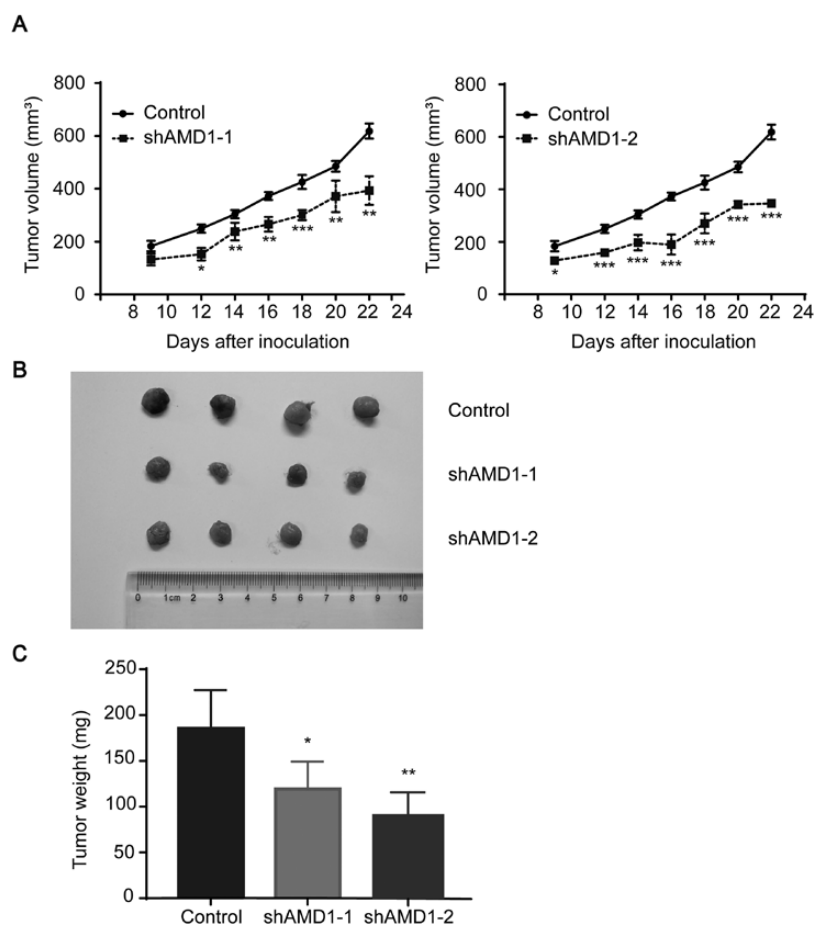
reduced by SAM486A in comparison with the control cells. We next analyzed the effect of SAM486A on cell cycle progression. AGS cells were synchronized by serum starvation and  $G_1$ -to-S transition was analyzed upon addition of 10% serum (Supplementary Figure S2B, available at *Carcinogenesis Online*). Overall, the percentage of cells at the  $G_0/G_1$  phase was significantly elevated by SAM486A treatment, whereas the percentages of the cells at S and  $G_2$  phases were significantly reduced by SAM486A treatment (Supplementary Figure S2B, available at *Carcinogenesis Online*). We also analyzed a few cell cycle-related proteins. The phosphorylation of retinoblastoma protein, a tumor suppressor that plays an important role in the negative control of cell cycle (22), was reduced by SAM486A treatment (Supplementary Figure S2C, available at *Carcinogenesis Online*). In addition, accumulation of cyclin A and cyclin B1 was delayed by SAM486A treatment (Supplementary Figure S2C, available at *Carcinogenesis Online*). These data, therefore, indicated that inhibition of AMD1 renders a negative effect on the  $G_1$ -to-S transition in gastric cancer cells.

## Discussion

In summary, our studies have provided compelling evidence that AMD1 has a tumorigenic function in human gastric cancer

cells via analyses at the cellular, animal and human levels. At the cellular level, AMD1 has a positive effect on the cell proliferation and migration of human gastric cancer cells. At the animal level, AMD1 knockdown can strongly inhibit the growth of gastric cancer cells *in vivo*. Mechanistically, AMD1 regulates cell cycle progression by affecting  $G_1$ -to-S transition. At the clinical level, AMD1 expression level was robustly upregulated in human gastric cancer samples in comparison with adjacent normal tissues. Besides, AMD1 expression level is tightly associated with *H. pylori* 16sRNA, tumor size, tumor differentiation, tumor venous invasion, tumor lymphatic invasion, blood vessel invasion, invasive depth and TNM stage. Collectively, these results demonstrate that AMD1 is a tumor-promoting gene that is closely associated with the malignancy and metastasis of human gastric cancers. Furthermore, the upregulation of AMD1 was closely associated with reduction in the survival of the gastric cancer patients. Therefore, the expression level of AMD1 might serve as an independent marker to predict the survival of gastric cancer patients.

Previous studies have linked polyamine metabolism to infection in gastric epithelial cells and *H. pylori*-mediated gastric cancer risk. It was found that *H. pylori* infection in the gastric epithelium can induce the expression of spermine oxidase (SMOX), leading to increased production of reactive



**Figure 4.** AMD1 affects the growth of gastric cancer cell xenograft in nude mice. (A) The volume of the tumors from nude mice inoculated with AGS cells expressing control vector (control) or shAMD1-1. (B) Representative images of the tumors isolated from the mice. (C) The weight of the tumors. All the data are shown as mean  $\pm$  standard deviation, \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . The same experiment was performed two times with similar results.

oxygen oxidative species and DNA damage (23). *H. pylori* could stimulate the transcription of SMOX gene and increase  $H_2O_2$  production in gastric epithelial cells, leading to DNA damage and apoptosis in these cells (24). Mechanistically, the microbial oncoprotein CagA of *H. pylori* is involved in the stimulation of SMOX by the bacteria (25). It was also found that the upregulation of SMOX by *H. pylori* infection is associated with an increased gastric cancer risk in Colombia in the Andean mountain region (26). It appears that microRNA miR-124 is involved in the regulation of SMOX transcription in response to *H. pylori* infection (27). In our study, we found that the expression level of AMD1 in the gastric cancer samples were strongly correlated with *H. pylori* 16sRNA (Table 1), indicating that *H. pylori* might also modulate the expression of AMD1 in addition to its regulation on SMOX. The combined actions of *H. pylori* on both AMD1 and SMOX might have a synergistic effect to promote progression of gastric cancers. This is an intriguing question that needs to be explored in the future.

Currently, the molecular mechanisms underlying the upregulation of AMD1 in gastric cancers are unknown. A recent study in prostate cancers indicated that AMD1 upregulation is caused by activated mTORC1 (9). Activation of the PTEN-PI3K-mTORC1 pathway occurs in many types of cancer cells. In mouse and human prostate cancer samples, the production of decarboxylated S-adenosylmethionine (dcSAM) and polyamine synthesis are upregulated. It was shown that such upregulation

is caused by mTORC1-dependent regulation of AMD1 stability. Application of an mTORC1 inhibitor everolimus could reduce the expression of AMD1 and decrease the proliferation of prostate cancer cells (9). Interestingly, we also found the mTORC1 inhibitor rapamycin significantly inhibited cell proliferation rate of AGS cells (Supplementary Figure S3A, available at *Carcinogenesis* Online). Besides, rapamycin treatments decreased the steady-state AMD1 protein levels in AGS cells and such reduction was abrogated by MG132, a proteasome inhibitor (Supplementary Figure S3B, available at *Carcinogenesis* Online). These preliminary data indicated that AMD1 protein is probably modulated by mTORC1 pathway in gastric cancer cells. This is an important issue to be addressed in the future.

According to our study, the expression level of AMD1 is closely related to the prognosis of gastric cancer, indicating that AMD1 is a promising therapeutic target for the treatment of gastric cancer in the future. Currently, a variety of chemicals have been developed to target on polyamine metabolism and polyamine uptake for cancer therapy (1). SAM486A is an AMD1 inhibitor and phase I and phase II clinical trials with this chemical have been completed for the treatment of metastatic melanoma (18). However, even though SAM486A has antitumor activity, severe toxicity occurs after its use. New chemicals to inhibit AMD1 are still under development. An ODC inhibitor, difluoromethylornithine, has been extensively investigated to treat brain tumors (28). Lately, it was found that concomitant inhibition of polyamine synthesis



and polyamine uptake is able to reduce oncogenesis of neuroblastoma in mice (10). Considering the important contribution of polyamine metabolism to gastric cancers, it will be of great potential to carry out clinical trials to target on polyamine pathway for the treatment of gastric cancers.

## Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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