Agmatinase facilitates the tumorigenesis of pancreatic adenocarcinoma through the TGFβ/Smad pathway

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Abstract. Pancreatic adenocarcinoma (PAAD) is one of the most lethal malignancies. Due to the lack of typical symptoms and difficulties in early diagnosis, PAAD has a high mortality rate. Therefore, it is essential to identify novel specific biomarkers for the application of targeted therapies. A previous study suggested that agmatinase (AGMAT) may fulfill important roles in tumor progression; however, these roles and the underlying mechanisms of AGMAT involvement in PAAD have yet to be thoroughly investigated. To address this shortcoming, in the present study the expression and prognostic significance of AGMAT were analyzed via several bioinformatics databases. Gain- and loss-of-function experiments were subsequently performed to observe the impact of AGMAT on the proliferation and metastasis of PAAD cells via Cell Counting Kit 8 (CCK-8) assay, colony formation assay, and cell migration and invasion assays in vitro. In order to probe the mechanisms involved, western blot assays were performed. AGMAT was found to be overexpressed in PAAD, and it was positively associated with a poor prognosis. Stable overexpression of AGMAT was found to lead to a marked increase in cell proliferation and metastasis through activation of the transforming growth factor-β (TGFβ)/Smad pathway, and via enhancing epithelial-mesenchymal transition (EMT). In conclusion, the results of the present study suggest that AGMAT may be an oncogene, and a pivotal mechanism has been uncovered in which AGMAT facilitates the progression of PAAD tumorigenesis through the TGFβ/Smad pathway.

Introduction

Pancreatic adenocarcinoma (PAAD) is one of the most lethal types of malignancies (1). Patients with PAAD frequently present with non-specific symptoms, such as abdominal pain and weight loss, making it difficult to diagnose PAAD at an early stage. Moreover, the vast majority of patients are found to already possess tumor metastases at the time of initial diagnosis (2,3). Despite recent advances that have been made in terms of surgical techniques, chemotherapy, and radiation therapy, the 5-year survival rate remains at a dismal 5% (4). Therefore, it is essential to identify novel specific biomarkers for the application of targeted therapy for patients with PAAD.

Agmatinase (AGMAT) belongs to the arginase family (5), and its tertiary protein structure comprises eight parallel β-sheets sandwiched in between three α-helices on either side. AGMAT is a metallohydrolase whose catalytic activity depends on Mn2+ ions (6); specifically, two Mn2+ ions bind to the high- and low-affinity sites to activate nucleophilic water molecules for catalysis (5-7). AGMAT hydrolyzes agmatine, which is an endogenous polyamine synthesized by L-arginine decarboxylase, to form urea and putrescine. It was not confirmed until 1994 that AGMAT and agmatine are synthesized in mammals (8). In mammals, previous studies have shown that agmatine is involved in a wide range of cellular biological functions; for example, regulation of insulin release from pancreatic cells (9), control of the glomerular filtration rate (10,11), and neuroprotection (12-14). Therefore, a potentially important mechanistic role for AGMAT, in terms of regulating the biological functions of agmatine in mammalian cells was revealed (15). Subsequently, numerous studies have been conducted to understand the physiological mechanisms through which the levels of agmatine are regulated; however, few reports have been published on the role of agmatine in cancer. In cancer research, AGMAT has been shown to have an important role in the development of tumors, including colorectal cancer (16) and lung adenocarcinoma (17). However, to the best of our knowledge, the potential functions and molecular mechanisms of AGMAT in PAAD have not yet been

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investigated. According to the findings of the above studies on the biological functions of AGMAT in the occurrence and development of tumors, it was possible to speculate that AGMAT may have an important role in the pathogenesis of PAAD, although its role remains to be investigated.

In the present study, it was shown that AGMAT is an oncogene that is associated with the poor prognosis of patients with PAAD through bioinformatics analysis. Moreover, it was demonstrated that AGMAT is able to promote the viability of cell proliferation and metastasis of PAAD cells in vitro. Additional mechanistic experiments demonstrated that AGMAT is able to enhance epithelial-mesenchymal transition (EMT) via the transforming growth factor-β (TGFβ)/Smad pathway. Taken together, the findings of the present study suggest that AGMAT may be a promising prognostic biomarker for PAAD, and therefore it is potentially a novel therapeutic target.

Materials and methods

Expression dataset. RNA sequencing (RNAseq) data for AGMAT was downloaded from the UCSC Xena database (http://xena.ucsc.edu/), and the expression of AGMAT was analyzed in 183 PAAD tissues and 165 adjacent normal tissues downloaded from the UCSC Xena database (http://xena.ucsc.edu/), which included PAAD tissues from The Cancer Genome Atlas (TCGA) and normal tissues from the Genotype Tissue Expression (GTEx) Project. In addition, the associations between the expression level of AGMAT and overall survival (OS) in the Kaplan–Meier plotter database (https://kmplot.com) were also downloaded. Differences in expression between tumor and normal tissues, as determined by immunohistochemical analysis in the Human Protein Atlas (https://www.proteinatlas.org/) were subsequently compared.

Plasmids, retroviral infection, and transfection. The full-length AGMAT gene was purchased from Genepl Technology Co., Ltd. and subcloned into the pCD513B vector to generate cells that stably overexpressed AGMAT. The three small hairpin RNA (shRNA) lentivirus plasmids were also purchased from Genepl Technology Co., Ltd. and cloned into a lentiviral pPLK GFP+puro vector to generate cells with stably knocked down AGMAT expression. The sequences of these three shRNAs were: shRNA#1, CGATGTGATGTCAGGTTT; shRNA#2, CAAACCACCATTATACCCGT; and shRNA#3, GGGGA GATCAGATGCTT, and the sequence of the negative control sequence used was GTTCTCCAGACCCGATT. 293T cells were transfected with DMEM, DNA and GM Easy Lentiviral Mix using MG Transgene Reagent, which was purchased from Genomeditech, according to the manufacturer's instructions. At 48 h after transfection, the lentivirus particles were collected.

Generation of stable cell lines. The human PAAD MiaPaCa-2 (cat. no. CRM-CRL-1420), SW1990 (cat. no. CRL-2172), Panc-1 (cat. no. CRL-1469), and BxPc3 (cat. no. CRL-1687) cells were obtained from American Type Culture Collection (ATCC). PaTu8988s (cat. no. CL-0303) cells were obtained from Procell Life Science & Technology Co. 293T (cat. no. GNHu17) cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained at the Central Laboratory of Shanghai Fengxian District Central Hospital. The cell lines were cultured and maintained in Gibco® DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% Gibco® fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.) in a humidified incubator supplied with 5% CO₂. The human PAAD SW1990 and BxPc3 cell lines were infected with lentivirus plus 8 µg/ml polybrene to establish the PAAD cell lines stably overexpressing or with stably knocked down AGMAT, respectively. In order to obtain stably transfected cells, the cells were cultured with puromycin (Invitrogen®; Thermo Fisher Scientific, Inc.) for 7 days. Subsequently, western blotting and reverse transcription-quantitative (RT-qPCR) analyses were used to detect both the AGMAT-knockdown efficiency and AGMAT-overexpression levels, as detailed below.

RT-qPCR. Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Subsequently, the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (Accurate Bio Technology Co., Ltd.) was used to reverse-transcribe total RNA to cDNA. A SYBR® Green Premix Pro Taq HS qPCR Kit (ROX Plus) (Accurate Bio Technology Co., Ltd.) was used to perform the qPCR experiments. The following primer sequences were all purchased from Sangon Biotech Co., Ltd. and were used for the RT-qPCR: AGMAT forward primer, 5'-CTTGTTGAAGTTCACCACCGTA-3' and reverse primer, 5'-CTTGGGAGAGACGCACTACATC-3'; GAPDH forward primer, 5'-TTGTATGCATTGGAAGGCTCA-3' and reverse primer, 5'-TGCATCATATTTGGACAGTT-3'. The 2-ΔΔCq method (18) was used to measure the relative mRNA expression levels.

Western blot analysis. Total protein was extracted from the treated cells (either stably overexpressing AGMAT or with AGMAT stably knocked down) lysed in RIPA lysis buffer. Subsequently, the proteins were separated using SDS-PAGE (10% gels) and then transferred onto a nitrocellulose membrane. The membranes were incubated with primary and secondary antibodies (see below), and the signals were visualized using a Tanon Highly-sig ECL Western Blotting Substrate Reagent Kit (#180-5001, Tanon Science and Technology Co., Ltd.). The signals were visualized using an ECL kit (Vazyme Biotech Co., Ltd.), and images were captured using a Tanon 4600 system. The following primary antibodies were used: anti-AGMAT (1:1,000; cat. no. ab231894; Abcam), anti-E-cadherin (1:5,000; cat. no. 20874-1-AP; Proteintech), anti-N-cadherin (1:1,000; cat. no. 4511; CST), and anti-β-tubulin (1:20,000; cat. no. 66240-1-Ig; Proteintech). The following secondary antibodies were used: HRP-conjugated Affinipure goat anti-mouse IgG (1:5,000; cat. no. SA00001-1; Proteintech) and HRP-conjugated AffiniPure goat anti-rabbit IgG (1:5,000; cat. no. SA00001-2; Proteintech). The relative levels of the proteins of interest were normalized against those of β-tubulin.
Cell proliferation and colony formation assays. For cell proliferation assays, the treated PAAD cells (either stably overexpressing AGMAT or with AGMAT stably knocked down) were seeded at a density of 1,000 cells/well in 96-well plates and cultured for 24, 48, 72, and 96 h. Aliquots of 10 µl Cell Counting Kit-8 (CCK-8) reagents (Dojindo, Inc.) were added to each well, and the mixture was incubated at 37˚C for 1 h. Subsequently, the absorbance at 450 nm was measured. For the colony formation assays, the treated PAAD cells (either stably overexpressing AGMAT or with AGMAT stably knocked down) were seeded onto a 6-well plate at a density of 1,000 cells/well and cultured for 10 or 14 days. After the incubation period, whole colonies were treated with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min at room temperature, prior to capturing images with a camera (Canon, Inc.).

Cell migration and invasion assays. Migration assays of PAAD cells overexpressing AGMAT or with AGMAT knocked down were performed using Transwell chambers (Corning, Inc.). Invasion assays were performed using Corning BioCoat Matrigel Invasion Chamber (Corning, Inc.). For the invasion assay, 5x10^5 cells were inoculated into each chamber in triplicate. For the migration assay, the bottom chamber was filled with culture medium containing 10% FBS, and 5x10^5 cells were suspended in serum-free medium and plated in the upper chamber. After incubation for 24 h at 37˚C, the cells on the lower surface of the membrane were fixed with 4% formaldehyde in PBS and stained with 0.1% crystal violet for 30 min at room temperature. Subsequently, the cells were counted under a fluorescence microscope (Olympus Corporation; magnification, x200).

Statistical analysis. GraphPad Prism 8 (GraphPad Software, Inc.) was used for the statistical analysis. Data are presented as the means ± standard error of the mean (SEM) or the means ± standard deviation (SD) of three independent experiments. The Student's t-test or one-way ANOVA followed by post hoc Dunnett's test was used to estimate the significant differences between different groups. P<0.05 was considered to indicate a statistically significant difference.

Figure 1. AGMAT is upregulated in PAAD and is positively associated with a poor prognosis. (A) Analysis of the expression of AGMAT in PAAD tumor tissues and adjacent normal pancreatic tissues. (B) Survival analysis revealed that PAAD patients with AGMAT overexpression had shorter overall survival (P=0.01567). (C) Immunohistochemical analysis indicated that there was a high expression of AGMAT in PAAD (https://www.proteinatlas.org/ENSG00000116771-AGMAT/pathology/pancreatic+cancer#img), whereas no expression was observed in normal tissue (https://www.proteinatlas.org/ENSG00000116771-AGMAT/tissue/pancreas#img). ***P<0.001. AGMAT, agmatinase; PAAD, pancreatic adenocarcinoma.
Results

AGMAT is upregulated in PAAD and positively associated with a poor prognosis. In order to explore the mechanism through which AGMAT is dysregulated in PAAD, the expression levels of AGMAT in PAAD tumor tissues and adjacent normal pancreatic tissues were first investigated. The AGMAT mRNA expression data of 183 PAAD tumors and 165 adjacent pancreatic tissues were analyzed using the online bioinformatics tool UCSC Xena. The results from this analysis showed that AGMAT was significantly upregulated in the PAAD tumor tissues (Fig. 1A). Accordingly, the expression levels of AGMAT were found to be inversely correlated with the OS rate. The survival analysis revealed that the PAAD patients with AGMAT overexpression had higher mortality rates and shorter OS rates compared with patients with lower expression levels of AGMAT (Fig. 1B). Furthermore, the immunohistochemical results showed that there were no appreciable levels of AGMAT expression in normal pancreatic tissues, although there was high expression of AGMAT in PAAD (Fig. 1C). Taken together, these results showed that the expression levels of the AGMAT gene was markedly upregulated in PAAD, suggesting that AGMAT may exert a positive effect in PAAD and could be of use as a prognostic biomarker.

AGMAT promotes PAAD cell proliferation and colony formation in PAAD. The protein and mRNA expression levels of AGMAT in various PAAD cell lines, including PaTu8988s, MiaPaCa-2, SW1990, Panc-1 and BxPc3 cells, were first examined by western blot and RT-qPCR analyses (Fig. 2A). As a result of these experiments, SW1990 cells were selected to construct the stably overexpressing AGMAT cells, and BxPc3 cells were used to construct cells that had stably knocked down AGMAT using a lentiviral vector. The efficiency of stable overexpression, and knockdown efficiency, of AGMAT was analyzed by both western blot and RT-qPCR analyses (Fig. 2B and C).

To explore the biological function of AGMAT in PAAD, the proliferation of AGMAT-overexpressing SW1990 cells and AGMAT-knockdown BxPc3 cells were examined via CCK-8 assays and colony formation assays, respectively. The results of the CCK-8 assays showed that cell proliferation was significantly enhanced after stably overexpressing AGMAT (Fig. 3A). The same results were obtained in the colony formation assays, AGMAT could promote the formation of pancreatic cancer cell colonies (Fig. 3C and D). Conversely, the CCK-8 assays revealed that cell proliferation was significantly inhibited after the stable knockdown of AGMAT (Fig. 3B), and according to the cell formation assays, AGMAT knockdown resulted in a significant suppression of the numbers of cell colonies formed (Fig. 3E and F). Therefore, these results suggest that AGMAT exerts an important role in the growth of cells in vitro.

Effects of AGMAT on migration and invasion of pancreatic cancer cells. Subsequently, the function of AGMAT in PAAD metastasis was examined. As shown in Fig. 4A, the results revealed that both cell migration and invasion were enhanced...
after stably overexpressing AGMAT in SW1990 cells. In contrast, cell migration and invasion were inhibited in the AGMAT-knockdown BxPc3 cells, as shown in Fig. 4D. Then, the numbers of cells that had migrated and invaded were counted and analyzed, as shown in Fig. 4B, C, E and F. These results demonstrated that AGMAT could promote the migration and invasion capabilities of the PAAD cells.

AGMAT induces EMT in PAAD cells and accelerates the process induced by the TGFβ/Smad signaling pathway. Epithelial-mesenchymal transition (EMT) refers to the transition of epithelial to mesenchymal cells. During this process, the cells lose their epithelial characteristics, including their polarity, which confers on them a migratory behavior (19,20). At the same time, the protein expression levels of three key epithelial
marker proteins (E-cadherin, vimentin and N-cadherin) are characteristically altered, which leads to a decrease in the adhesion of cells, and a loss of polarity and tight junctions (19). The matrix metalloproteinases (MMPs) are both a large family and an important class of proteolytic enzymes, which are able to degrade various protein components in the extracellular matrix, destroy the histological barrier of tumor cell invasion, and serve a key role in tumor invasion and metastasis (21). A previous study revealed that the overexpression of MMP2 and MMP9 is associated with tumors (22). These findings motivated us to examine the effect of AGMAT on the EMT-associated proteins, MMP2 and MMP9, in PAAD cells. To meet this end, western blot analysis was performed. The results obtained showed that the SW1990-constructed cell line that stably overexpressed AGMAT exhibited a significantly suppressed expression levels of E-cadherin and significantly upregulated levels of N-cadherin, vimentin, MMP2 and MMP9 (Fig. 5A). Conversely, the opposite trends were showed in BxPc3 cells with AGMAT-knockdown (Fig. 5B). Taken together, all the above results demonstrated that overexpression of AGMAT could promote cell migration and invasion in PAAD cells through promoting EMT.

Subsequently, the mechanism of AGMAT in PAAD cells was explored in more precise detail. A previous study demonstrated that TGFβ is an important factor in the tumor microenvironment; in particular, it was shown that TGFβ is able to promote tumor metastasis via inducing the so-called EMT (23). Therefore, we focused on whether AGMAT could accelerate the process of EMT by inducing the TGFβ/Smad signaling pathway in PAAD. To meet this aim, western blotting was used to detect the protein expression levels of TGFβ1, Smad4 and p-Smad2/3, which serve important roles in the pathway. As anticipated, AGMAT positively regulated the TGFβ/Smad signaling pathway. The protein expression levels of p-Smad2/3, Smad4 and TGFβ1 were significantly upregulated in SW1990 cells with AGMAT.

Figure 4. Effects of AGMAT on migration and invasion of PAAD cells. (A and D) Analysis of the properties of cell migration and invasion in SW1990 cells with AGMAT-overexpression (OE) and BxPc3 cells with AGMAT-knockdown (sh-AGMAT) by Transwell assay. (B and E) The number of migrated cells were subsequently counted and analyzed. (C and F) The number of invaded cells were subsequently counted and analyzed. Data are represented as means ± SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. the Vector or sh-NC group. AGMAT, agmatinase; PAAD, pancreatic adenocarcinoma.
overexpression, whereas the expression levels of these proteins were significantly decreased in BxPc3 cells with AGMAT expression knocked down (Fig. 6A and B). Collectively, these results indicated that AGMAT could induce EMT in PAAD cells, and that this process of induction was accelerated via the TGFβ/Smad signaling pathway.

Discussion

In the present study, a novel function of AGMAT in PAAD was uncovered. First, it was shown that the AGMAT mRNA levels were overexpressed in PAAD tissues and cells. Functionally, AGMAT was found to promote the growth and aggressiveness.
of PAAD cells in vitro. During tumor development, epithelial-mesenchymal transition (EMT) has been demonstrated to fulfill a role in the progression of PAAD (24), and the present study revealed that AGMAT could promote cell migration and invasion in PAAD cells through promoting EMT. In addition, several studies have shown that the TGFβ/Smad signaling pathway is a critical regulator of EMT (24,25). Therefore, it was crucial to investigate whether AGMAT could induce EMT via the TGFβ/Smad signaling pathway. The mechanistic analysis suggested that AGMAT could indeed induce EMT via the TGFβ/Smad signaling pathway. However, the associations between AGMAT, EMT and the TGFβ/Smad signaling pathway need to be further explored utilizing TGFβ receptor inhibitors or agonists, among other approaches. In pursuing these avenues, the mechanisms of AGMAT in PAAD will be delineated more precisely.
AGMAT significantly affects the polyamine biosynthetic pathway, functioning as the key enzyme of the polyamine metabolism alternative pathway (26). Previous studies have demonstrated that abnormalities in enzymes may be linked with a number of diseases, especially cancer (17). In a previous study, colon cancer samples were found to have low levels of agmatine (27). Furthermore, agmatine was found to suppress the proliferation of tumor cells, which were derived from colon, liver, neuronal, leukemia, among other types of cancer (28,29). Based on the aforementioned studies, we hypothesized that AGMAT may hydrolyze agmatine, thereby suppressing the antitumor function of agmatine and facilitating the progression of PAAD. For almost a decade, studies that have targeted polyamine metabolism have intensely focused on cancer treatment and prevention, as regulating polyamine levels is clearly one of the most potentially effective strategies for treating cancer. Therefore, examining more closely the molecular regulatory mechanisms of polyamine metabolism will be of great significance for the treatment of cancer. In the future, our studies will focus on whether and how AGMAT may hydrolyze agmatine to inhibit the antitumor properties in PAAD.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
XZ and YZ conceived and designed the experiments and wrote the paper. YZ and XZ participated in all experiments. LC, YZ and YX contributed to the plasmid construction and cell transfection and interpretation of the experimental results. CW and XL performed and analyzed the WB experiments. YX and YZ performed and analyzed the IF experiments. JC and XZ revised the manuscript and analyzed all data. XZ, YZ and JC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript for publication.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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