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AK5, a novel prognosis marker, inhibits apoptosis and promotes autophagy as well as proliferation in human gastric cancer

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Abstract. – OBJECTIVE: To explore the relationship between AK5 and gastric cancer.

MATERIALS AND METHODS: The *in situ* levels of AK5 in the GC tissues from 255 patients were detected by immunohistochemistry (IHC). The correlation between AK5 expression and the clinicopathological parameters was analyzed by Pearson correlation, and the prognostic factors were identified by Cox regression analysis. The transcriptome data of 14 human GC cell lines deposited in the CCLE database were analyzed, and two lines were selected for functional studies. AK5 was knocked down in the AZ521 and MKN74 cells using siRNA, and their proliferation and apoptosis were evaluated by Cell Counting Kit-8 (CCK-8) assay and Annexin-V staining, respectively. In addition, the apoptosis and autophagy of the markers were detected by Western blotting.

RESULTS: Patients expressing high AK5 levels in the tumor tissues had significantly shorter survival compared to low-expressing group. In addition, AK5 expression was associated with T stage and N stage and was an independent prognostic factor. AK5 knockdown in the AZ521 and MKN74 cells significantly inhibited proliferation and autophagy, and increased apoptosis.

CONCLUSIONS: AK5 is a potential prognostic marker and therapeutic target for GC.

Key Words:
Adenylate kinase 5, AK5, Gastric cancer, Biomarker, Potential drug target.

Introduction

Gastric cancer (GC) ranks third and fifth in terms of cancer-related mortality and incidence worldwide[1]. The current therapeutic modalities for GC include surgery, radiotherapy, chemotherapy, and molecular targeted therapy[2]. Nevertheless, the overall 5-year survival rate among GC patients is still less than 50% due to ineffective early diagnosis[3]. Therefore, it is essential to identify novel markers for detecting tumors at early stage with high specificity and sensitivity. Although PIK3CA, CDH1 and HER2 have been identified as potential GC markers, they have performed poorly in clinical studies[4]. To this end, studies have increasingly focused on elucidating the molecular basis of GC initiation and progression to screen for more effective early diagnostic markers[5-8].

Adenylate kinase 5 (AK5) is an intermediate of the AMPK signaling pathway[9,10], and is dysregulated in various pathophysiological conditions like anterograde amnesia[11], prosopagnosia[12], and cancer[13]. High level of methylated AK5 is associated with breast cancer prognosis, and studies also correlate it with GC, although the precise mechanistic role is unknown. We analyzed the *in situ* expression level of AK5 in GC tissues, and its correlation with clinico-pathological parameters such as age, gender, clinical staging, etc. In addition, the effect of AK5 silencing on the proliferation, apoptosis, and autophagy of human GC cells was assessed. Our results show that

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AK5 is an independent prognostic risk factor and a potential oncogene, in addition to being a novel drug target for GC.

Materials and Methods

Sample Collection

Tissue specimens were obtained from 255 GC patients who underwent tumor resection at the Affiliated Hospital of Jiangnan University from 2011 to 2013. None of the patients received radiotherapy or chemotherapy prior to surgery. GC was confirmed by histopathological examination, and the patients were graded according to the latest UICC criteria[14]. The patients were followed up till November 2018. The investigation was approved by the Hospital Ethics Committee and all patients provided signed informed consent.

Bioinformatics Analysis

Survival analysis was performed using data from GEPIA (http://gepia.cancer-pku.cn/)[15], ESCA, STAD, and COAD databases, and the cutoff value was the median (50%) survival. The data for the expression profile of AK5 in GC cells was obtained from CCLE (https://portals.broadinstitute.org/).

Immunohistochemistry (IHC)

IHC was performed using a specific kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol[16]. Briefly, the 5 μm-thick paraffin tissue sections were deparaffinized, rehydrated, boiled in citrate buffer for antigen retrieval, and blocked with 5% bovine serum albumin (BSA; Beyotime Biotechnology, Songjiang, Shanghai, China) at room temperature for 30 minutes. The sections were then incubated overnight with anti-AK5 antibody (1:300, Abcam, Cambridge, MA, USA) at 4°C, followed by incubation with the horseradish peroxidase (HRP)-labeled secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. After color development with liquid diaminobenzidine (DAB) substrate (Invitrogen, Carlsbad, CA, USA), the sections were rinsed with tap water and mounted for microscopic examination. Two pathologists blinded to the sample identity determined the staining intensity and positivity rate of AK5. The percentage of positively stained cells was graded as 0, 1 (1%-29%), 2 (30%-69%) and 3 (70-100%), and the staining intensity as 0 (negative), 1 (weakly stained), 2 (moderately stained), and 3 (strongly stained). The total immunological response score (IRS) was calculated by multiplying the staining intensity score with the positive cell score, and graded as 0-1: "-", 2-3: "+", 4-6: "++" and 6-9: "+++". Based on the IRS, the samples were stratified into positive AK5 expressing (4-9) and negative AK5 expressing (0-3) groups[17].

Cell Culture

The human GC cell lines AZ521 and MKN74 from American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS: Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 UI/ml penicillin (Mediatech, Herndon, VA, USA) and 100 μg/ml streptomycin (Mediatech, Herndon, VA, USA) at 37°C with 5% CO_2 in a humidified atmosphere.

Western Blotting

Cells grown in 6-well plates were washed thrice with cold phosphate-buffered saline (PBS; Beyotime, Songjiang, Shanghai, China) and scraped, and homogenized with 200 μl radio immunoprecipitation (RIPA) buffer (Invitrogen, Carlsbad, CA, USA) containing protease inhibitors. The homogenates were transferred to 5 ml tubes and kept on ice for 30 min. The lysates were centrifuged at 13500 rpm for 15 min at 4°C, and the supernatants were denatured with 1X sodium dodecyl sulphate (SDS) loading buffer (Solarbio, Tongzhou, Beijing, China) at 100°C for 10 min. Equal amount of proteins per sample were resolved by 12% or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Solarbio, Tongzhou, Beijing, China), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The latter were blocked with 5% BSA in Tris-Buffered Saline and Tween-20 (TBST) at room temperature for 2 h, and incubated overnight with primary antibodies against AK5 (1:1000, Cell Signaling Technology, Danvers, MA, USA), P21 (1:3000, Abcam, Cambridge, MA, USA), PARP (1:1000, Abcam, Cambridge, MA, USA), Caspase-3 (1:2000, Abcam, Cambridge, MA, USA), Bcl-2 (1:5000, Abcam, Cambridge, MA, USA), LC3B (1:1000, Abcam, Cambridge, MA, USA), P62 (1:2000, Abcam, Cambridge, MA, USA), and β-actin (1:5000, Abcam, Cambridge, MA, USA) at 4°C. The positive bands were visualized by enhanced

chemiluminescence (ECL; Shanghai Shenggong Biotechnology, Songjiang, Shanghai, China), and analyzed using Image J software (National Institutes of Health; NIH, Bethesda, MD, USA).

Cell Transfection

Chemically synthesized AK5-siRNA1 (NM_174858.3: CGGAGATCCTTTCTA-AGAAATGTAA), AK5-siRNA2 (NM_174858.3: CAGAACGATATGGATTCCAATACAT), AK5-siRNA3 (NM_174858.3: GAAATG-GAGTCTTATTGCCAAGATA), and the matching scramble control siRNAs were purchased from Ribo Company (Guangzhou RiboBio, Guangzhou, Guangdong, China). The siRNAs were transiently transfected into MGC803 cells by using Lipofectamine 4000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h of transfection, the cells were harvested and AK5 protein levels were determined by Western blotting.

Cell Viability Assay

The cells were seeded into 96-well plates at the density of 1 x 10^4 cells/well in 100 µl media. Six replicates were tested for each sample. The cells were cultured as described, and incubated with 10 µl CCK-8 reagent (Beyotime, Songjiang, Shanghai, China) per well. After incubating for 1 hour, the absorbance at 450 nm was measured[18].

Flow Cytometry

Cells were seeded in 60-mm tissue culture plates at the density of 1×10^6 cells/dish, and cultured for 48 h. Both the adherent and non-adherent cells were harvested and washed twice with ice-cold PBS (Solarbio, Tongzhou, Beijing, China). After staining with Annexin V-FITC (Beyotime, Songjiang, Shanghai, China) according to the manufacturer's instructions, the apoptotic cells were detected by flow cytometry (BD Biosciences, San Jose, CA, USA) and analyzed using FlowMax software (TreeStar Inc., Ashland, OR, USA).

Statistical Analysis

The Chi-square test, correlation analysis, survival analysis, and COX univariate and multivariate analyses were conducted using the R 3.5.2 program (www.r-project.org). GraphPad Prism 6 (GraphPad, San Diego, CA, USA) was used for *t*-test analysis, and $p < 0.05$ was considered statistically significant.

Results

High AK5 Expression is Predictive of Poor Prognosis in GC

Analysis of TCGA transcriptome data showed that AK5 expression levels were not associated with the overall survival (OS) of esophageal cancer and colon cancer patients (log rank p = 0.21 and 0.29 respectively, Figure 1A and 1C, respectively), but were significantly associated with gastric cancer OS [HR of the AK5 high expression group = 1.8, log rank p = 0.00051, p (HR) = 0.00058, Figure 1B]. Therefore, high levels of AK5 indicate significantly worse prognosis in GC patients, making it a potential risk factor.

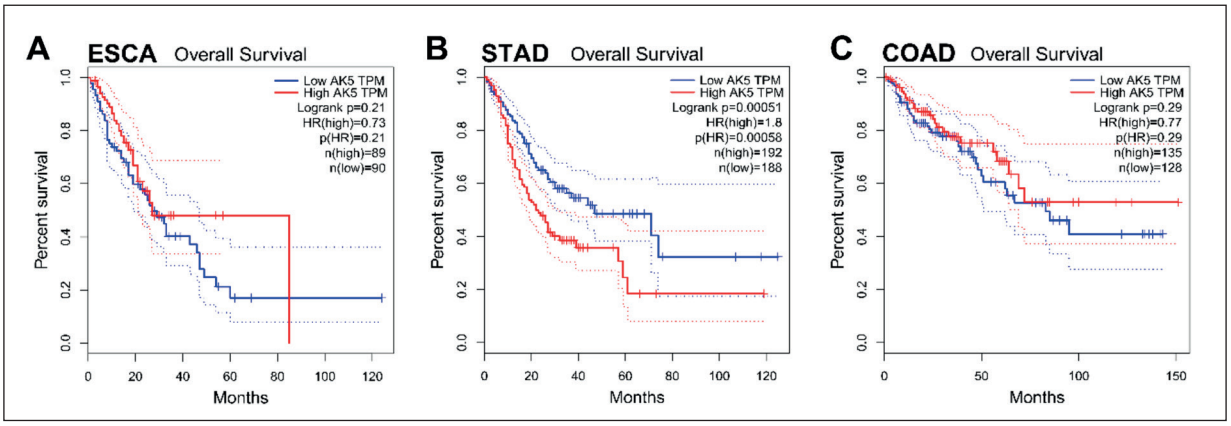

Figure 1. The relationship between overall survival and AK5 expression levels in the gastrointestinal tissues. **A,** Relationship between AK5 expression and prognosis in esophageal cancer: HR (high) = 0.73, p (HR) = 0.21. **B,** Relationship between AK5 expression and prognosis in GC: HR (high) = 1.8, p (HR) = 0.00058. **C,** Relationship between AK5 expression and prognosis in colon cancer: HR (high) = 0.77, p (HR) = 0.29.

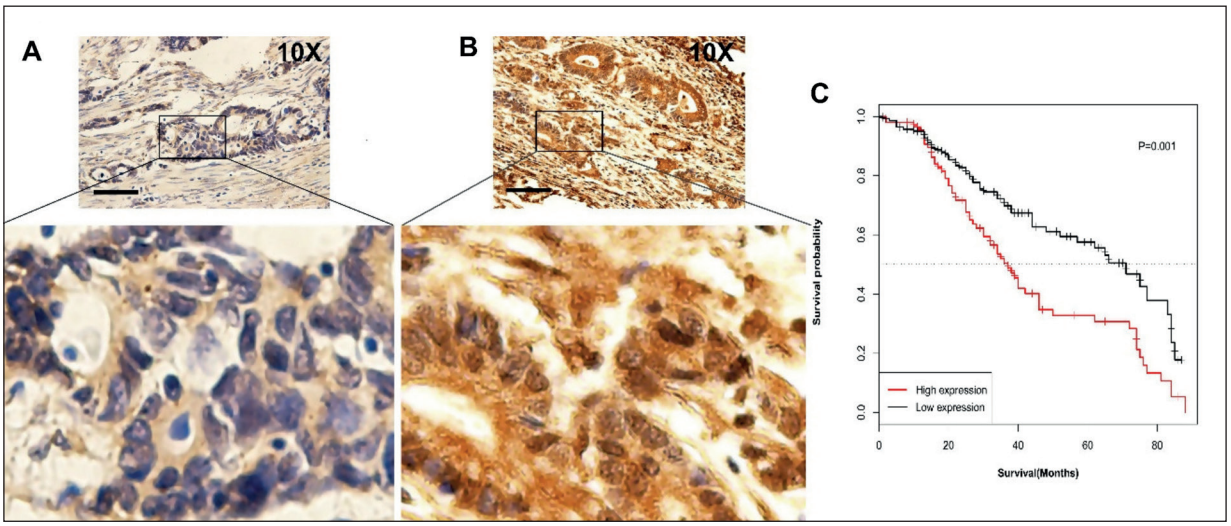

Figure 2. Relationship between *in situ* AK5 levels and GC prognosis. **A,** Representative picture of AK5 negative tissue. **B,** Representative picture of AK5 positive tissue. **C,** Kaplan-Meier survival curves of AK+ and AK- GC patients. Magnification = 40×, Scale bar = 50 μm.

To further validate the *in silico* data, we analyzed AK5 protein expression in the tumor tissues resected from GC patients. Of the 255 cases, 151 were negative (Figure 2A) and 104 were positive (Figure 2B) for AK5. Consistent with the bioinformatics results, the median survival was only 37 months in AK5-positive group compared to the 71 months in AK5-negative group (p = 0.001; Figure 2C). Taken together, high levels of AK5 promote GC progression.

AK5 Expression Level is Associated with T Stage and N Stage, and is an Independent Prognostic Factor for GC

As shown in Table I, the positive expression of AK5 was significantly correlated with T stage and N stage ($p < 0.05$), but not with age, gender, TNM stage, and differentiation ($p > 0.05$). In addition, the COX univariate analysis showed a significant association of both T-stage and AK5 expression levels with prognosis ($p < 0.05$), while

Table I. Relationship between AK5 expression and clinico-pathological features of GC patients.

Characteristic	No. Cases	Positive AK5	Negative AK5	X^2	p-value
Age					
<=60	130	57	73	0.787	0.375
> 60	125	47	78		
Gender					
Female	139	60	79	0.517	0.472
Male	116	44	72		
T stage					
T1-T2	137	36	101	24.517	**7.37E-07**
T3-T4	118	68	50		
N stage					
N0	61	32	29	3.912	**0.048**
N1-N3	194	72	122		
TNM stage					
I-II	195	74	121	2.283	0.131
III	60	30	30		
Differentiation					
Well/moderately	120	45	75	0.772	0.380
Poorly	135	59	76		

the multivariate analysis showed that only AK5 expression was an independent prognostic factor (p=0.014) as shown in Table II.

AK5 Knockdown in Human GC Cell Lines Inhibits Proliferation and Autophagy and Promotes Apoptosis

The relative AK5 mRNA levels of 14 GC cell lines were analyzed from the CCLE cell transcriptome sequencing data, and ranged between 4.43-4.70 (Figure 3A). Accordingly, we selected AZ521 and MKN74 as the *in vitro* models for subsequent experiments. We also designed and tested three pairs of AK5 siRNA, and based on the degree of AK5 protein inhibition, selected siRNA1 for the functional assays (Figure 3B).

AK5 silencing significantly reduced cell viability on the third day post-transfection (Figure 3C), and increased the percentage of apoptotic cells (Figure 3D). Consistent with this, the cell cycle inhibitor protein P62 and the pro-apoptotic proteins PARP and Caspase-3 were upregulated, while the anti-apoptotic Bcl-2 was downregulated in the AK5-knockdown cells (Figure 3E). In addition, the ratio of LCII/I decreased significantly in the absence of AK5, indicating autophagy inhibition (Figure 3E). Taken together, AK5 promotes GC development at the cellular level by increasing proliferation and autophagy, and inhibiting apoptosis.

Discussion

Gastric cancer is one of the most commonly diagnosed tumors of the digestive tract, and is associated with significantly high morbidity and mortality due to late diagnosis. Therefore, it is essential to identify novel early diagnostic markers of GC to improve treatment outcome and prognosis. Previous bioinformatics studies have identified AK5 as a potential prognostic marker for GC, but not of other gastrointestinal malignancies like esophageal and colon cancers, indicating a specific biological function in GC. In addition, the AK5 gene is associated with anterograde amnesia and prosopagnosia disease, and one study reported high levels of methylated AK5 in breast cancer[19]. Nevertheless, no study so far has investigated the clinical significance of AK5 in GC.

We analyzed the *in situ* levels of AK5 in the tumor tissues resected from GC patients, and found that the positive expression of AK5 was significantly associated with the T stage, but not with age, gender, M stage, TNM stage, and differentiation. This indicates that AK5 is likely involved in the initiation of GC, rather than metastasis and differentiation. Furthermore, patients expressing high AK5 levels in the tumors had significantly shorter median survival and worse prognosis compared to those lacking AK5, indicating that the latter may function as an oncogene in GC. While both AK5 expression levels and T stage were associated with prognosis, the multivariate analysis showed that only AK5 expression was an independent prognostic factor for GC, and therefore has clinical significance.

To further study the biological function of AK5 in GC, we knocked down the gene in two human GC cell lines, and found that it inhibited proliferation and increased apoptosis rates. In addition, the autophagy flux indicator LCII/I was significantly decreased following AK5 silencing, whereas the autophagy substrate P62 was increased. Therefore, AK5 likely promotes GC progression by increasing proliferation and inhibiting apoptosis. Since autophagy is increasingly being implicated as a pro-proliferative and anti-apoptotic mechanism[20], we hypothesize that AK5 exerts its tumorigenic effects at the molecular level by inhibiting autophagy. These findings are consistent with the fact that AK5 is involved

Table II. Univariate and multivariate COX regression analysis of the prognostic factors of GC.

Characteristics	Hazard Ration	CI 95	*p*-value	Hazard Ration	CI 95	*p*-value
Age (≥ 60 *vs*. < 60)	1.11	0.77-1.62	0.57			
Gender (Female *vs*. Male)	0.95	0.65-1.39	0.802			
Differentiation (Well/moderately *vs*. Poorly)	0.74	0.51-1.07	0.113			
T stage (T3-T4 *vs*. T1-T2)	1.58	1.08-2.3	**0.018**	1.31	0.87-1.96	0.196
N stage (N1-N3 *vs*. N0)	1.4	0.92-2.1	0.112			
TNM stage (III-IV *vs*. I-II)	1.07	0.7-1.64	0.751			
AK5 Expression (High *vs*. Low)	4.54	2.37-6.78	**0.001**	2.61	1.41-3.9	**0.014**

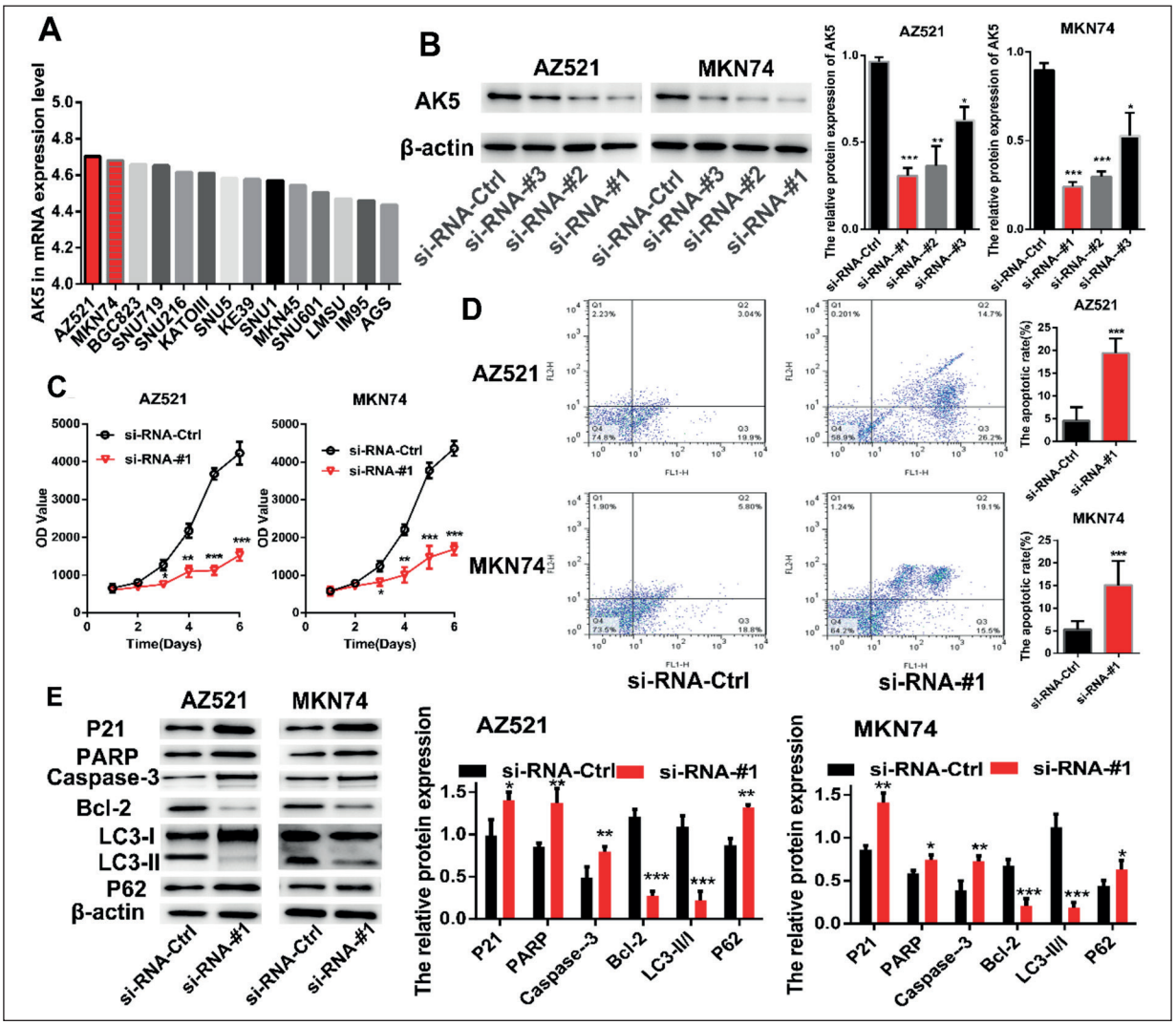

Figure 3. Mechanistic role of AK5 in GC cells. **A,** AK5 transcript levels in 14 GC cell lines as per the CCLE database **B,** Immunoblots showing AK5 protein levels in AZ521 and MKN74 lines transfected with the different siRNA constructs **C,** Proliferation of AZ521 and MKN74 cell lines after AK5 knockdown. **D,** Effect of silencing AK5 on the apoptosis rate of AZ521 and MKN74 cells. **E,** Immunoblots showing expression levels of apoptosis and autophagy markers in AK5-knockdown GC cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

in the AMPK signaling pathway[21], which is closely related to cell proliferation, apoptosis, and autophagy[22]. Therefore, it is possible that inhibiting AK5 alters tumor cell metabolism and induces autophagy through the AMPK signaling pathway.

Although targeted therapy against GC hinging on specific molecular markers is increasingly gaining attention, only a few genes have been clinically validated, and several potential target genes have not yet been identified. We have shown for the first time that AK5 is an independent prognostic factor and potential therapeutic target for GC. However, this is still a proof-of-concept study, which needs to be validated by multi-center clinical studies on larger patient cohorts. In addition, the molecular mechanism underlying the role of AK5 in the development and progression of GC needs further elucidation.

Conclusions

Overall, our data strongly demonstrated that AK5 is associated with advanced cancer and is an unfavorable prognostic factor. Inhibiting AK5 reduced cell viability by blocking autophagy and promoting apoptosis, making it a potential prognostic marker and therapeutic target for GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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References

1) Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.
2) Fan GF, Pan JJ, Fan PS, Zhang TY, Liu YB, Huang J, Weng CT, Liu M, Duan QH, Wu Y, Tang LL, Yang GH, Dai HB, Zhu ZQ. The clinical observation of verapamil in combination with interventional chemotherapy in advanced gastric cancer. Eur Rev Med Pharmacol Sci 2018; 22: 5508-5518.
3) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.
4) Shi HZ, Wang YN, Huang XH, Zhang KC, Xi HQ, Cui JX, Liu GX, Liang WT, Wei B, Chen L. Serum HER2 as a predictive biomarker for tissue HER2 status and prognosis in patients with gastric cancer. World J Gastroenterol 2017; 23: 1836-1842.
5) Wen J, Zheng T, Hu K, Zhu C, Guo L, Ye G. Promoter methylation of tumor-related genes as a potential biomarker using blood samples for gastric cancer detection. Oncotarget 2017; 8: 77783-77793.
6) Wang H, Li B, Liu Z, Gong J, Shao L, Ren J, Niu Y, Bo S, Li Z, Lai Y, Lu S, Gao J, Shen L. HER2 copy number of circulating tumour DNA functions as a biomarker to predict and monitor trastuzumab efficacy in advanced gastric cancer. Eur J Cancer 2018; 88: 92-100.
7) Pan HX, Bai HS, Guo Y, Cheng ZY. Bioinformatic analysis of the prognostic value of ZNF860 in recurrence-free survival and its potential regulative network in gastric cancer. Eur Rev Med Pharmacol Sci 2019; 23: 162-170.
8) Zhang HQ, Zhao G, Ke B, Ma G, Liu GL, Liang H, Liu LR, Hao XS. Overexpression of UBE2C correlates with poor prognosis in gastric cancer patients. Eur Rev Med Pharmacol Sci 2018; 22: 1665-1671.
9) Doliba NM, Babsky AM, Doliba NM, Wehrli SL, Osbakken MD. AMP promotes oxygen consumption and ATP synthesis in heart mitochondria through the adenylate kinase reaction: an NMR spectroscopy and polarography study. Cell Biochem Funct 2015; 33: 67-72.
10) Lai Y, Hu X, Chen G, Wang X, Zhu B. Down-regulation of adenylate kinase 5 in temporal lobe epilepsy patients and rat model. J Neurol Sci 2016; 366: 20-26.
11) Ng AS, Kramer J, Centurion A, Dalmau J, Huang E, Cotter JA, Geschwind MD. Clinico-pathological correlation in adenylate kinase 5 autoimmune limbic encephalitis. J Neuroimmunol 2015; 287: 31-35.
12) Tuzun E, Rossi JE, Karner SF, Centurion AF, Dalmau J. Adenylate kinase 5 autoimmunity in treatment refractory limbic encephalitis. J Neuroimmunol 2007; 186: 177-180.
13) Jan YH, Lai TC, Yang CJ, Lin YF, Huang MS, Hsiao M. Adenylate kinase 4 modulates oxidative stress and stabilizes HIF-1alpha to drive lung adenocarcinoma metastasis. J Hematol Oncol 2019; 12: 12.
14) Tanaka S, Komatsu S, Ohta A, Furuke H, Kumano T, Imura K, Shimomura K, Ikeda J, Taniguchi F, Shioaki Y. [Validation of the 8th Edition of the UICC TNM Classification for Stage Gastric Cancer]. Gan To Kagaku Ryoho 2019; 46: 502-504.
15) Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 2017; 45: W98-W102.
16) Zhang LH, Wang Z, Li LH, Liu YK, Jin LF, Qi XW, Zhang C, Wang T, Hua D. Vestigial like family member 3 is a novel prognostic biomarker for gastric cancer. World J Clin Cases 2019; 7: 1954-1963.
17) Boutrup RJ. Analysis of stem cells and their activity in human skeletal muscles by immunohistochemistry. Methods Mol Biol 2019; 2045: 235-244.
18) Zhang LH, Li LH, Cai YF, Zhang PF, Hua D. Association of LINC00957 expression with poor survival and chemoresistance in human colorectal cancer. Frontiers Oncol 2019; 9: 776-785.
19) Miyamoto K, Fukutomi T, Akashi-Tanaka S, Hasegawa T, Asahara T, Sugimura T, Ushijima T. Identification of 20 genes aberrantly methylated in human breast cancers. Int J Cancer 2005; 116: 407-414.
20) Zhang W, Li Z, Zhang Q. Leptin inhibits apoptosis of nucleus pulposus cells via promoting autophagy. Eur Rev Med Pharmacol Sci 2019; 23: 4065.
21) Ionescu MI. Adenylate kinase: a ubiquitous enzyme correlated with medical conditions. Protein J 2019; 38: 120-133.
22) Dong HW, Zhang LF, Bao SL. AMPK regulates energy metabolism through the SIRT1 signaling pathway to improve myocardial hypertrophy. Eur Rev Med Pharmacol Sci 2018; 22: 2757-2766.