Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:1219-1229
DOI: 10.1159/000487453
Published online: February 15, 2018
Accepted: January 09, 2018

Original Paper

Overexpression of Lymphocyte Antigen 6 Complex, Locus E in Gastric Cancer Promotes Cancer Cell Growth and Metastasis

Yan Lv[a] Yu Song[b] Chen Ni[b] Shaokai Wang[b] Zhipeng Chen[b]
Xiaojing Shi[b] Qin Jiang[d] Cong Cao[a,c,e] Yun Zuo[b]

[a]Center of Translational Medicine, The First People Hospital of Zhangjiagang City, Soochow University, Suzhou, [b]Department of Oncology, The First People Hospital of Zhangjiagang City, Soochow University, Suzhou, [c]Jiangsu Key Laboratory of Neuropsychiatric Diseases Researchs and Institute of Neuroscience, Soochow University, Suzhou, [d]The Affiliated Eye Hospital of Nanjing Medical University, Nanjing, [e]North District, The Municipal Hospital of Suzhou, Suzhou, China

Key Words
Ly6e • Gastric cancer • Proliferation • Apoptosis • Invasion

Abstract
Background/Aims: Lymphocyte antigen 6 complex, locus E (LY6E) is a member of the lymphostromal cell membrane Ly6 superfamily protein. The present study investigated the clinical significance and potential biological function of LY6E in gastric cancer (GC). ***Methods:*** LY6E mRNA and protein expressions in human GC tissues and GC cells were tested. Relationship between LY6E expression and the GC patients' clinicopathologic characteristics was analyzed. LY6E was silenced by siRNA in the cultured GC cells. ***Results:*** The RNA expression microarray profiling assay results demonstrated that *LY6E mRNA* was significantly increased in multiple human GC tumor tissues. Immunohistochemistry (IHC) staining analysis revealed that 59 of 75 (78.7%) GC specimens were LY6E positive. LY6E over-expression in human GC was correlated with the histology grade, AJCC stage, N classification, lymphatic invasion, and tumor location. Notably, functional LY6E expression was also detected in AGS and other established GC cell lines. LY6E knockdown by targeted-siRNA inhibited AGS cell survival and proliferation. Meanwhile, the LY6E siRNA induced G1-S cell cycle arrest and apoptosis in AGC cells. Additionally, AGC cell migration was also inhibited by LY6E knockdown. Expressions of tumor-suppressing proteins, including PTEN (phosphatase and tensin homolog) and E-Cadherin, were increased in LY6E-silenced AGS cells. ***Conclusion:*** LY6E over-expression in GC is potentially required for cancer cell survival, proliferation and migration.

Y. Lv, Y. Zuo and Y. Song contributed equally to this work.

Qin Jiang, Yun Zuo
and Cong Cao

The Affiliated Eye Hospital, Nanjing Medical University, Department of Oncology,
The First People Hospital of Zhangjiagang City, (China)
E-Mail Jqin710@vip.sina.com, zuoyunlab@163.com, caocong@suda.edu.cn

Introduction

Gastric cancer (GC) is one of the leading causes of morbidity and mortality worldwide [1]. Due to the improvement of sanitation [2], *Helicobacter pylori* infection control [3] and the availability of GC screening [4], the global GC-ASMR (age-standardized cancer mortality rates) declined (-2.7% in men and -2.8% in women) [5]. Yet, GC is still the second most common cause of cancer death [6].

The current clinical treatment options for GC include surgical resection and chemo-/targeted-therapies [7]. However, the early symptoms of GC are not typical. Most patients are therefore diagnosed at late-stages with lymph node/distant metastases, or with relapse [7]. It has been reported that early GC patients with timely treatment could have the five-year overall survival rate over 94% [8]. Therefore, the identification of novel and more efficient biomarkers for GC is important [9-12]. These novel markers shall offer important practical clinical significance [9, 13-15]. Additionally, understanding the molecular events underlying the progression of GC is necessary to the development of novel therapeutic strategies [9, 13-15].

Molecular profiling of GC can be performed using microarray-based gene expression analysis [16-18]. We performed differential expression analysis using this technology to measure genomic or transcriptional expression of GC tissues (along with the adjacent normal gastric tissues). The data show that LY6E (lymphocyte antigen 6 complex, locus E) was one predominant upregulated-gene in GC tissues.

LY6E is a member of the lymphostromal cell membrane Ly6 superfamily protein [19]. It is also known as stem cell antigen 2 (SCA2) or thymic shared antigen-1 (TSA-1). Ly6 family members locate on human chromosome 8, the 8q24.3 locus. They are evolutionary conserved [20]. At least twenty (20) different human Ly6 proteins have been identified thus far, which are categorized as either transmembrane or secretory proteins [21]. The majority of the studies on Ly6 superfamily proteins focused on their physiological importance in T cell development [22] and cell adhesion [23]. However, the biological function and clinical significance of LY6E in human GC are largely unknown. Here, we show that overexpression of LY6E in GC promotes cancer cell growth and metastasis.

Materials and Methods

GC tissue specimens

Human GC samples were collected from 75 patients who underwent GC surgical resection at The First People Hospital of Zhangjiagang City, Soochow University (Zhangjiagang, China) between 2014 and 2016. Informed consent was obtained from each patient, and this study was approved by the Ethical Committee of The First People Hospital of Zhangjiagang City and Soochow University. For RNA isolation and protein extraction, GC tissues and matched surrounding normal tissues were immediately snap-frozen in liquid nitrogen, and were stored at -80°C until further usage. For histological examination and immunohistochemistry staining, tissues were fixed using 4% buffered formalin and paraffin-embedded.

Cell lines

Human gastric cell lines, including AGS, BGC-823, MGC-803 and SGC-7901, were acquired from the Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution in a 37°C incubator containing 5% CO_2. All cell culture reagents were purchased from the GIBCO (Nanjing, China)

Gene expression microarray profiling

A total of three pairs fresh GC tumor tissues and surrounding normal gastric tissues were prepared. The total RNA was extracted using TRIzol reagents (Invitrogen, Carlsbad, CA). After quantification, 100 ng of total RNA was labeled and hybridized to the Human GeneExpression Microarray (RiboBio Co.

Guangzhou, China). Sequencing was performed on the Illumina HiSeq 2500 platform in RiboBio (RiboBio Co.). The differential expression of the genes between the tumor and paired-normal tissues was defined as: Average fold change ⩾ 2.0 and $P < 0.05$. Hierarchical clustering and heat map generation were performed.

Table 1. Primer sequences for quantitative PCR

Primer	Primer sequence	Size (bp)
LY6E (NM_001127213.1)	F: 5'- GGCATTGGGAATCTCGTGACAT-3' R: 5'- GCCGCACTGAAATTGCACAGAA -3'	156
PTEN (NM_000314.6)	F: 5'- ATTAGTGCTGTTGCTAGTTC-3' R: 5'- GCAACAATCATTAGGCTTTC-3'	194
E-Cadherin (NM_004360.3)	F: 5'- GAGAACGCATTGCCACATACAC-3' R: 5'- AAGAGCACCTTCCATGACAGAC-3'	164
GADPH (NM_001256799.2)	F: 5'- CACCCACTCCTCCACCTTTG-3' R: 5'- CCACCACCCTGTTGCTGTAG-3'	110

Real-time quantitative polymerase chain reaction (qRT-PCR)

The detailed protocol of qRT-PCR was described in detail in our previous studies [24-26]. Total RNA was extracted, quantified and reversely-transcribed into complementary DNA via the First-Strand cDNA Synthesis Kit (Thermo Fisher, Shanghai, China). The qRT-PCR reactions were then performed on an ABI-7600 system (Applied Biosystems, Shanghai, China) with SYBR Green PCR Master Mixes (Thermo Fisher). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA* was always tested as the reference gene and internal control. Response: For each assay, melt curve analysis was performed to calculate product melting temperature. the $2^{-\Delta\Delta Ct}$ method was applied to quantify targeted gene expression change. The *mRNA primers* for qRT-PCR were listed in Table 1. All mRNA primers were designed, synthesized and verified by Sangon Biotech (Shanghai China).

Western blotting assay

Western blotting analysis was carried out using the described method [27-30]. Same set of lysate samples were run in sister gels to examine different proteins. Following antibodies were utilized: LY6E (BS61207, Bioworld technology, 1:1000 dilution), PTEN (9552, Cell Signaling; Danvers, MA, 1:1000 dilution), E-Cadherin (3195, Cell Signaling, 1:1000 dilution) and GAPDH (2188, Cell Signaling, 1:1000 dilution). Secondary antibodies (A0208, HRP-labeled Goat Anti-Rabbit IgG, Beyotime, 1:10000 dilution, A0216, HRP-labeled Goat Anti-Mouse IgG, Beyotime, 1:10000 dilution) were employed.

Immunohistochemistry (IHC) staining

The detailed protocol of IHC staining was described early [24]. In brief, the GC tumor tissues and adjacent normal tissues were fixed in 4% paraform, embedded in paraffin, and cut into 4-μm sections. The paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol, boiled in antigen retrieval solution and incubated with fresh 3% hydrogen peroxide in methanol to quench endogenous peroxidase. Then, the sections were blocked, incubated with anti-LY6E polyclonal antibody (NBP1-68553, Novus Biologicals, 1 mg/mL). Patient samples were scored by two pathologists independently who were blind to clinicopathological characteristics. For each sample, 500 cells from five randomly chosen fields were counted. The expression level of LY6E in GC and adjacent normal tissues was scored as the proportion of immunopositive staining area ("0" = 0%, "1" = 1–25%, "2" = 26–50%, "3" = 51–75% and "4" = 76–100%) multiplied by the intensity of staining ("0" = negative, "1" = weak, "2" = moderate, "3" = strong). Expression level of LY6E was categorized as weak or strong according to the median value of total score.

RNA small interference

Three LY6E siRNAs (siRNA1: 5'- GCUUCUCCUGCUUGAACCATT-3', siRNA2: 5'- GCAUUGGGAAUCUCGUGACTT-3' and siRNA3: 5'- GCCAGAGCUUUCUGUGCAATT-3') and a non-specific scramble control siRNA (siNC) were synthesized by GenePharm (Shanghai, China). The siRNA was transfected to the AGS cells by Lipofectamine 2000 regent (Invitrogen, Carlsbad, CA) per the manufacture's instruction. After 24 hours, knockdown of LY6E was determined by qRT-PCR and Western blotting assay.

Cell viability assay

The Cell Counting Kit-8 (CCK-8) assay was employed to assess the cell viability. Cells were seeded at a density of 2×10^3 cells/well. After the applied treatment, 10 μL CCK-8 solution (CCK-8, Dojindo, Japan) was added and was incubated for another 2 hours. Optical density (OD) of each well at 450 nm was measured with a microplate reader (Bio-Rad Laboratories).

EdU assay

The proliferation of AGS cells was evaluated using EdU Apollo®567 *In vitro* Imaging Kit (Ribo Bio, China) according to the manufacturer's protocol. The fluorescence dye 5-ethynyl-20-deoxyuridine (EdU) was added, and cells were incubated for additional 2 hours. Cell nuclei were then visualized under a fluorescent microscope (Leica, DM 4000, Germany)

Cell cycle distribution analysis

Cells were harvested and fixed with ice-cold ethanol, which were then stained with 20 μg/mL propidium iodide (PI, Sigma, St. Louis, MO) and 100 μg/mL RNase (Sigma). DNA content was analyzed by using a Navios Flow Cytometer (Beckman Coulter, Brea, CA). Cell cycle analysis was carried out by the MultiCycle software.

Cell apoptosis analysis

Following the applied treatment, cells were harvested and washed. Cells were then resuspended in 1X Binding Buffer, and were stained with Annexin V (10 μg/mL, BD Pharmingen) and PI (10 μg/mL), which were immediately detected by a Navios Flow Cytometer (Beckman Coulter, Brea, CA). The cells undergoing apoptosis were quantified by calculating the percentage of Annexin V [31].

Wound healing assay

Briefly, fibronectin (10 μg/mL, Sigma) was plated onto 6-well tissue culture plates, and the wells were washed with PBS and plated with AGC cells. The cell layer were carefully scratched with a 10 μl plastic tip to produce a straight line and washed twice with fresh medium. After 12 hours, cells were imaged under a light microscope to monitor the wound healing process. Mitomycin (1.0 μg/mL Sigma) was always added to exclude the influence of cell proliferation.

In vitro invasion assays

Invasion assays were tested by the Corning chambers with 8 μm pore filters (Corning, New York, NY), which were pre-coated with 1 mg/mL Matrigel (BD Biosciences, Shanghai, China). The detailed protocols were descried early [24]. AGS cells (5×10^4 cells of each chamber) starved overnight were added to the upper chamber in serum-free medium, and the lower chamber was filled with completed medium (containing 10% FBS). After 24h of incubation, cells that invaded to the lower surface of the chamber were fixed, stained and counted [24]. Mitomycin (1.0 μg/mL Sigma) was always added to exclude the influence of cell proliferation.

Statistical analysis

All data were analyzed via the SPSS 20.0 analysis. Chi-square test was used to examine possible correlations between LY6E expression and patients' clinical pathological features. The comparison between two groups was performed by the Student's t-test. Comparison of more than two groups was performed by one-way ANOVA. Statistical significance was defined as *P < 0.05, **P < 0.01, ***P < 0.001.

Results

LY6E is overexpressed in GC tissues

A total of three fresh GC tumor tissue specimens and the adjacent normal gastric tissue specimens were subjected to RNA expression microarray profiling assay. The differential expression of the genes between the tumor and paired-normal tissues were defined. Among which, ten (10) different genes were significantly overexpressed in the cancer tissues (fold change≥ 2 *vs.* normal tissues, P < 0.05, Fig. 1A). *LY6E* was one of the most predominant upregulated genes (Fig. 1A). To verify the results of the microarray analysis, real-time quantitative-PCR ("qRT-PCR") was employed to measure *LY6E mRNA* expression in thirty (30) different human GC tumor tissues. We compared it with that in normal gastric tissues. Results in Fig. 1B showed that *LY6E mRNA* expression in GC tissues was significantly higher than that in the non-cancerous normal tissues (P < 0.001). Western blotting assay results demonstrated that LY6E protein expression was also higher in GC tissues (Fig. 1C and D). These results suggest that LY6E is overexpressed in human GC tissues.

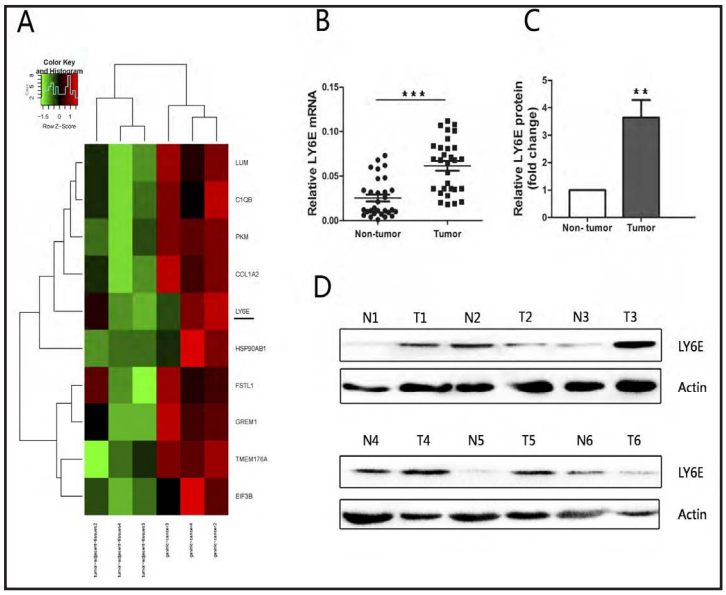

Fig. 1. LY6E is overexpressed in GC tissues. (A) Heat map representation of mRNA microarray showed that ten (10) genes were overexpressed in GC tumor tissues (as compared with the paired non-tumor tissues, fold change ⩾ 2, P<0.05). Notably, LY6E is one predominant gene upregulated in GC tumor tissues. Each column represents an individual sample and each row represents one protein-coding mRNA. Expression values were normalized. Red, upregulated genes; Green, downregulated genes; Black, unchanged genes. (B) The LY6E mRNA expression in GC ("Tumor") and paired surrounding normal gastric tissues ("Non-tumor", n=30) was examined by qRT-PCR, *** P<0.001. (C) Quantified results of LY6E protein expression in GC tissues and paired normal tissues were shown (n=30). GAPDH protein was tested as the loading control. (D) Western blotting assay of LY6E protein expression in GC (T1-T6) and normal tissues (N1-N6) was shown.

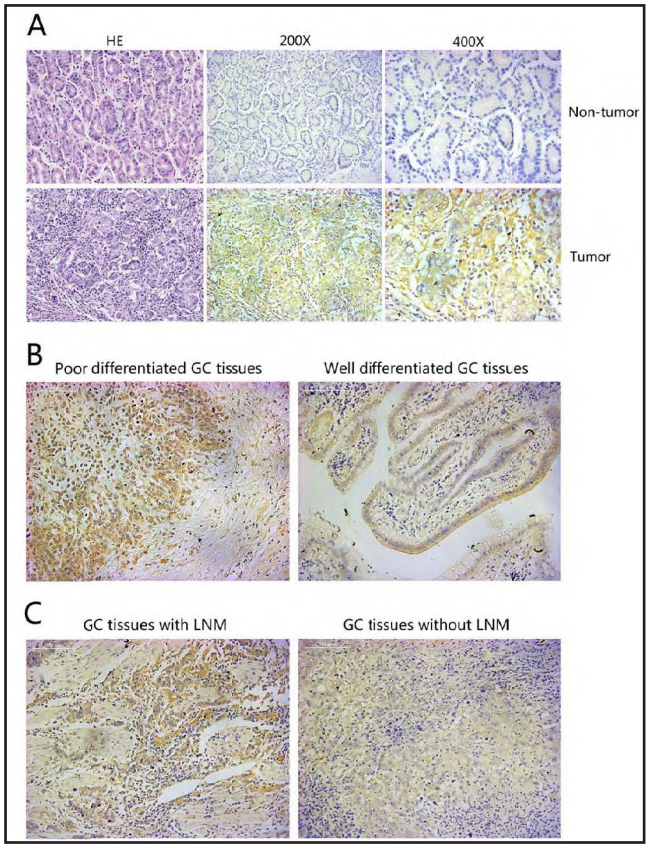

Fig. 2. The clinical significance of LY6E expression in GC. (A) Tumor and non-tumor samples are identified via the Hematoxylin-Eosin (HE) staining; LY6E protein expression was tested by immunohistochemistry (IHC) staining in seventy-five (75) tumor tissues and paired surrounding non-tumor tissues. LY6E-positive staining was predominantly enriched in the membrane and cytoplasm of GC cells. (B) GC with poor differentiation showed higher expression of LY6E. (C) GC with lymph node metastasis (LNM) showed higher expression of LY6E. Bar=100 μm (B and C).

The clinical significance of LY6E expression in GC

Next, we analyzed the correlation between LY6E expression and several key GC clinical characteristics. The expression of LY6E was examined by immunohistochemistry (IHC)

staining in GC specimens. A total of seventy-five (75) informed-consent patients with GC surgical resection were enrolled. The GS clinical features, including the patients' age, gender, tumor size, histology grade, AJCC stage, T/N classification, tumor location, lymphatic invasion and vascular invasion, were included. The IHC staining assay results demonstrated that LY6E protein was enriched in GC cell membrane and cytoplasm (Fig. 2A). LY6E protein was over-expressed in 78.7% (59 of 75) of the tested GC tissue samples (Fig. 2A, Table 2). As shown in Table 2, LY6E overexpression was correlated with histology grade (Fig. 2B, Table 2), AJCC stage, N classification and lymphatic invasion (Fig. 2C, Table 2), as well as the tumor location ($P < 0.05$, Table 2). LY6E expression was yet not correlated with patient's age and gender, nor with tumor size, T classification and vascular invasion (Table 2).

Table 2. Relationship between LY6E expression and clinicopathologic characteristics. Abbreviation: AJCC, American Joint Committee on Cancer.Bold fonts, $P < 0.05$

Characteristics	n	LY6E expression			P value
		Negative	Weak	Strong	
Age					
≥60	42	8	20	14	
<60	33	8	18	7	0.502
Gender					
Male	55	9	29	17	
Female	20	7	9	4	0.204
Histologic grade					
Poor	22	3	8	11	
Moderate/ Well	49	13	30	10	0.023
Tumor size (cm)					
≥5	44	10	22	12	
<5	31	6	16	9	0.830
AJCC stage					
I	7	3	2	2	
II	31	10	16	5	
III	37	3	20	14	0.042
T classification					
T_1+T_2	14	4	7	3	
T_3+T_4	61	12	31	18	0.708
N classification					
N_0	24	10	8	6	
N_{1-3}	51	6	30	15	0.011
Tumor location					
Cardiac part	15	2	4	9	
Fundus of stomach	2	0	0	2	
Body of stomach	14	3	10	1	
Pyloric part	34	9	20	5	
Multiple parts	10	2	4	4	0.011
Lymphatic invasion					
Absent	62	15	33	14	
Present	14	1	5	8	0.030
Vascular invasion					
Absent	71	15	38	18	
Present	4	1	0	3	0.064

LY6E expression in human GC cell lines

We next tested expression of LY6E in a panel of established GC cell lines, including AGS, BGC-823, MGC-803, and SGC-7901 [32]. Among them, AGS is a poorly differentiated GC cell line [32], and it had the highest expression level of *LY6E mRNA* (Fig. 3A) and protein (Fig. 3B). In order to study the possible function of LY6E in GC cell behaviors, a panel of three distinct LY6E siRNAs (siRNA1, siRNA2 and siRNA3, with non-overlapping sequence) were applied and transfected to AGS cells. siRNA efficiency was tested qRT-PCR assay (Fig. 3C) and Western blotting assay (Fig. 3D). Results clearly demonstrated that among all the tested siRNAs, the LY6E siRNA3 had the highest efficiency

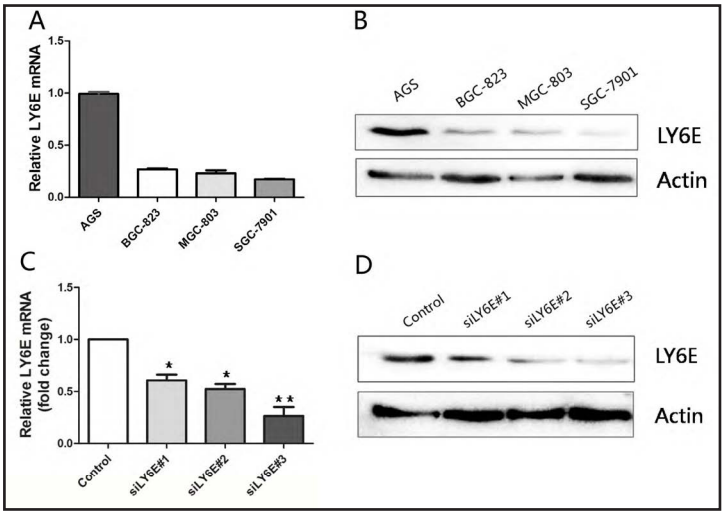

Fig. 3. LY6E expression in human GC cell lines. (A) qRT-PCR analysis of LY6E mRNA expression in listed human GC cell lines (AGS, BGC-823, MGC-803 and SGC-7901). (B) Quantification of LY6E protein expression in the four human GC cell lines. (C) AGS cells were transfected with 100 nM of listed LY6E siRNA ("1/2/3") or scramble control siRNA ("Ctrl") for 24 hours, mRNA (C) and protein (D) expressions of LY6E were tested by qRT-PCR assay and Western blotting assay, respectively. *P<0.05, **P<0.01 vs. "Ctrl".

in downregulating LY6E (Fig. 3C and D). Thus, LY6E siRNA3 was selected for further experiments.

LY6E knockdown inhibits AGS cell survival, proliferation and cell cycle progression

AGS cells with/without LY6E siRNA were subjected to various functional assays. The Cell Counting Kit-8 (CCK-8) viability assay results showed that knockdown of LY6E by the siRNA3 inhibited AGS cell survival (Fig. 4A). The CCK-8 optic density (OD) was significantly decreased in the LY6E-siRNA3 cells, as compared to that in "Mock" (un-treated) and control-siRNA ("siNC") cells (Fig. 4A). EdU incorporation is a well-established marker of cell proliferation. Results demonstrated that LY6E knockdown significantly reduced EdU staining in AGC cells (Fig. 4B, data were quantified in Fig. 4C). In addition, cell cycle distribution analysis showed that LY6E knockdown resulted in reduced percentages of cells in S-phase and G2-M phase, but increased percentage of G0/G1 phase cells (Fig. 4D and E). These results indicat-

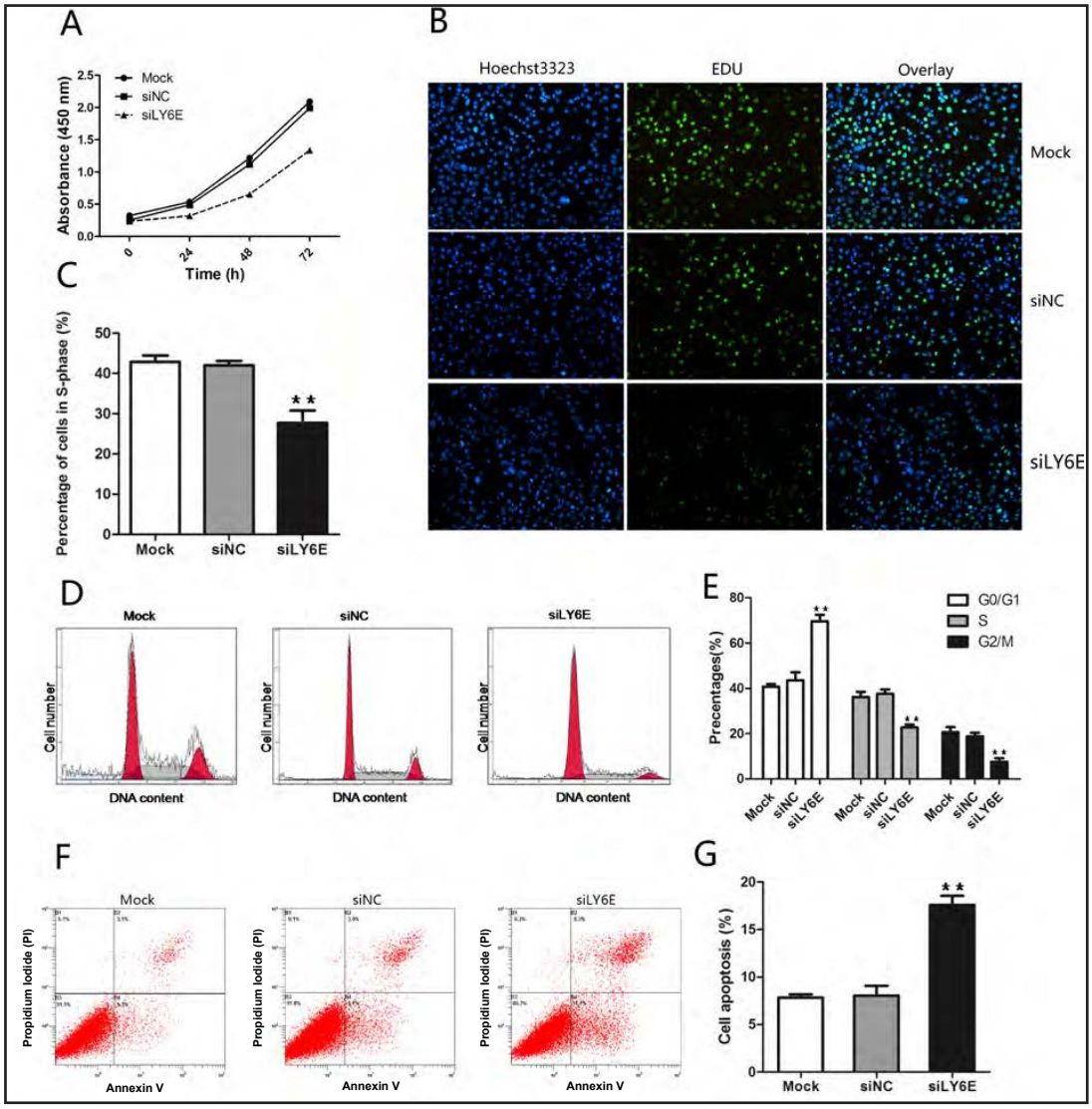

Fig. 4. LY6E knockdown inhibits AGS cell survival, proliferation and cell cycle progression. AGS cells were transfected with 100 nM of LY6E siRNA3 ("siLY6E") or scramble control siRNA ("siNC"); (A) Cell viability was determined by CCK-8 assay; (B and C) Cell proliferation was measured using EdU assay at 48 hours; (D and E) The cell cycle distribution was analyzed by PI-FACS assay at 48 hours; (F and G) Cell apoptosis was performed with double staining of Annexin V FITC-PI (at 48 hours). All data were representative results of three independent experiments. "Mock": non-treated cells; **P<0.01 vs. "siNC".

ed G1-S arrest trigged by LY6E knockdown in AGS cells. Additionally, Annexin V FACS assay results revealed that LY6E siRNA3 induced significant apoptosis (Annexin V ratio increase) in AGS cells (Fig. 4F and G). Together, these results show that LY6E knockdown inhibits AGS cell survival, proliferation and cell cycle progression, but provoking cell apoptosis.

LY6E promotes GC cells migration and invasion in vitro

The potential effect of LY6E on GC cell migration was also analyzed. The wound healing assay results demonstrated that AGS cells with LY6E siRNA3 displayed decreased migration ability, as compared with the "Mock" and "siNC" cells (Fig. 5A). Further, transwell assay results in Fig. 5B demonstrated that LY6E knockdown significantly inhibited the invasion of the AGS cells (Fig. 5B). It should be noted that, to exclude the possible influence of cell proliferation, mitomycin (1.0 μg/mL Sigma) was always added to AGC cells.

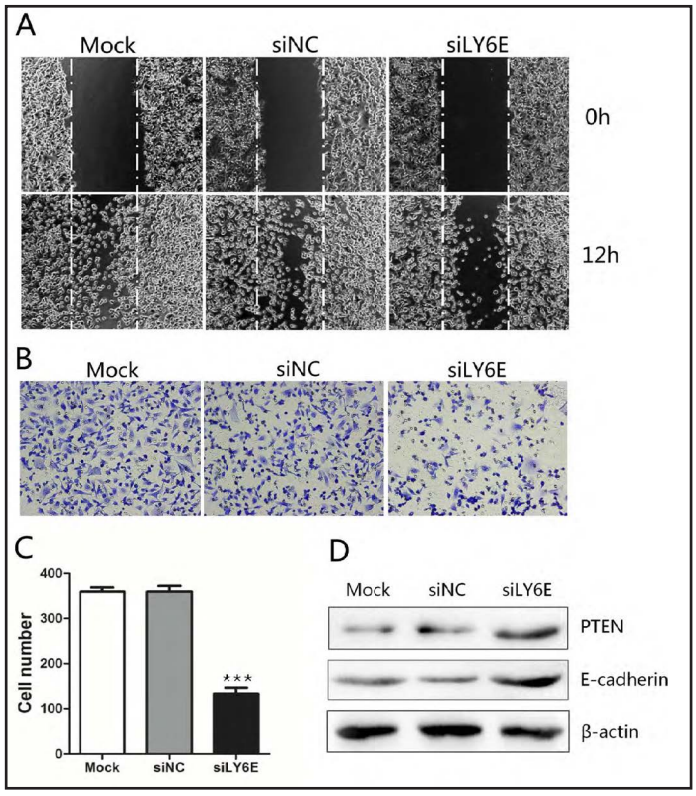

Fig. 5. LY6E knockdown inhibits AGS cell migration and invasion in vitro. AGS cells were transfected with 100 nM of LY6E siRNA3 ("siLY6E") or scramble control siRNA ("siNC"); (A) In vitro cell migration was tested by the wound healing assay (at 12 hours); (B and C) Invasion assay was carried out in Matrigel-coated Transwell chambers (at 24 hours). Cells that migrated into the lower well were stained, photographed and counted; (D) Expressions of listed proteins were tested by the Western blotting assay (at 24 hours). All assays were performed in triplicate. ***P<0.001 vs. "siNC".

Thus far, our results indicate that LY6E is important for AGC cell survival, proliferation and migration. The potential mechanism was then analyzed. It has been previously shown that LY6E could downregulate tumor-suppressor proteins, including phosphatase with tensin homology (PTEN) [33], and E-Cadherin [34], thus promoting cell proliferation and invasion. In this study, we show that LY6E knockdown by LY6E siRNA3 increased expressions of PTEN (increase by 183.4%) and E-Cadherin (increase by 87.1%) (Fig. 5D and E) in AGC cells. Based on these results, we propose that LY6E knockdown boosts expressions of tumor-suppressor proteins, which might be responsible for inhibition of cell proliferation, migration and invasion.

Discussion

GC is one common cancer [35]. In recent years, with the improvement of the awareness and treatment of GC, the morbidity and mortality have been declined [8, 9, 15]. Yet, it is still the second leading cause death in the world [36-38]. Nearly one million of new cases of GC occurred each year alone, and half of them were in China [39, 40].

GC develops as a biologically heterogeneous disease involving multiple genetic and epigenetic alterations [9, 13, 15, 41]. Aberrant gene expression profiles have been associated

with GC progression, prognosis and pathogenesis [9, 13, 15, 41, 42]. In the present study, gene expression microarray was applied to screen differentially expressed genes in GC tissues. Our data identified LY6E as a potential novel oncogenic protein for human GC.

LY6E is involved in cell signalling transduction, cell adhesion, immune regulation and drug resistance [21, 33, 34, 43]. It is over-expressed in human malignancies, including head and neck squamous cell carcinomas and lung, and oesophageal cancers [21, 33, 34, 43]. Dysregulated *LY6E mRNA* was also detected in neuroblastoma [44], bladder cancer [45], and breast cancer [46]. In the present study, the IHC staining analysis revealed that 59 of 75 (78.7%) human GC specimens were LY6E positive. The multivariate analysis results demonstrated that LY6E over-expression in GC was correlated with the histology grade, AJCC stage, N classification, lymphatic invasion, and tumor location. LY6E expression was also detected in multiple established human GC cell lines. These results suggest that abnormal expression of LY6E could possibly be a novel predictive biomarker and therapeutic target protein of human GC.

The precise function of LY6E in carcinogenesis is still largely unknown. Uncontrolled cellular proliferation is one key feature of cancer cells. Besides, cell invasion/migration is essential to the metastasis of cancer cells. In the current study, we show that expression of LY6E is detected in four GC cell lines, AGS, BGC-823, MGC-803, and SGC-7901. Among them, poorly-differentiated AGS cells showed highest expression of LY6E. Further *in vitro* studies showed that LY6E knockdown by targeted-siRNA inhibited AGC cell proliferation, survival, migration and invasion. LY6E siRNA also induced AGS cell cycle arrest and apoptosis. These studies suggested that LY6E is involved in GC cell progression and invasion.

The study by Yeom et al*.,* has shown that LY6E downregulates PTEN at mRNA level, thereby activating PI3K-AKT signaling [33]. Knockdown of LY6E has been shown to inhibit expression of ZEB1, an epithelial to mesenchymal transition (EMT) inducer and an inhibitor of E-Cadherin [35]. In line with these findings, our present study showed that siRNA-mediated knockdown of LY6E increased PTEN and E-Cadherin expression in AGS cells. We propose that LY6E-mediatd downregulation of tumor suppressor proteins (PTEN and E-Cadherin) could be linked to tumor cell progression. Over-activation of PI3K-AKT signaling is one of the most defined pro-cancerous signaling in GC [9]. We propose that over-expressed LY6E might also contribute to PI3K-AKT hyper-activation by downregulating PTEN in GC.

Conclusion

In conclusion, LY6E over-expression in GC cells is potentially important for cell survival, proliferation and migration. LY6E could be a novel oncogenic protein for efficient diagnosis and treatment of GC.

Acknowledgements

This work was generously supported by the grants from the National Natural Science Foundation of China (81302195, 31371139, 81571282, and 81771457) and by the Natural Science Foundation of Jiangsu province (BK20130301, BK20170060), and by grants from the Affiliated Zhangjiagang Hospital of Soochow University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

All authors listed in the current study carried out the experiments, participated in the design of the study and performed the statistical analysis, conceived of the study, and helped to draft the manuscript.

Disclosure Statement

No potential conflicts of interest were disclosed.

References

1 Brenner H, Rothenbacher D, Arndt V: Epidemiology of stomach cancer. Methods Mol Biol 2009;472:467-477.
2 Parkin DM: The global health burden of infection-associated cancers in the year 2002. Int J Cancer 2006;118:3030-3044.
3 Peleteiro B, La Vecchia C, Lunet N: The role of Helicobacter pylori infection in the web of gastric cancer causation. Eur J Cancer Prev 2012;21:118-125.
4 Choi KS, Suh M: Screening for gastric cancer: the usefulness of endoscopy. Clin Endosc 2014;47:490-496.
5 Hashim D, Boffetta P, La Vecchia C, Rota M, Bertuccio P, Malvezzi M, Negri E: The global decrease in cancer mortality: trends and disparities. Ann Oncol 2016;27:926-933.
6 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
7 Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H: Gastric cancer. Lancet 2016;388:2654-2664.
8 Shah MA: Gastrointestinal cancer: targeted therapies in gastric cancer-the dawn of a new era. Nat Rev Clin Oncol 2014;11:10-11.
9 Wadhwa R, Song S, Lee JS, Yao Y, Wei Q, Ajani JA: Gastric cancer-molecular and clinical dimensions. Nat Rev Clin Oncol 2013;10:643-655.
10 Li S, Zhang H, Ning T, Wang X, Liu R, Yang H, Han Y, Deng T, Zhou L, Zhang L, Bai M, Ge S, Ying G, Ba Y: MiR-520b/e Regulates Proliferation and Migration by Simultaneously Targeting EGFR in Gastric Cancer. Cell Physiol Biochem 2016;40:1303-1315.
11 Liu JF, Nie XC, Shao YC, Su WH, Ma HY, Xu XY: Bleomycin Suppresses the Proliferation and the Mobility of Human Gastric Cancer Cells Through the Smad Signaling Pathway. Cell Physiol Biochem 2016;40:1401-1409.
12 Li W, Fan M, Chen Y, Zhao Q, Song C, Yan Y, Jin Y, Huang Z, Lin C, Wu J: Melatonin Induces Cell Apoptosis in AGS Cells Through the Activation of JNK and P38 MAPK and the Suppression of Nuclear Factor-Kappa B: a Novel Therapeutic Implication for Gastric Cancer. Cell Physiol Biochem 2015;37:2323-2338.
13 Hudler P: Challenges of deciphering gastric cancer heterogeneity. World J Gastroenterol 2015;21:10510-10527.
14 Smyth EC, Cunningham D: Gastric cancer in 2012: Defining treatment standards and novel insights into disease biology. Nat Rev Clin Oncol 2013;10:73-74.
15 Smith K: Gastric cancer: new biologic therapy effective as second-line treatment in gastric cancer. Nat Rev Gastroenterol Hepatol 2013;10:694.
16 Tan IB, Ivanova T, Lim KH, Ong CW, Deng N, Lee J, Tan SH, Wu J, Lee MH, Ooi CH, Rha SY, Wong WK, Boussioutas A, Yeoh KG, So J, Yong WP, Tsuburaya A, Grabsch H, Toh HC, Rozen S, Cheong JH, Noh SH, Wan WK, Ajani JA, Lee JS, Tellez MS, Tan P: Intrinsic subtypes of gastric cancer, based on gene expression pattern, predict survival and respond differently to chemotherapy. Gastroenterology 2011;141:476-485, 485 e471-411.
17 Lei Z, Tan IB, Das K, Deng N, Zouridis H, Pattison S, Chua C, Feng Z, Guan YK, Ooi CH, Ivanova T, Zhang S, Lee M, Wu J, Ngo A, Manesh S, Tan E, Teh BT, So JB, Goh LK, Boussioutas A, Lim TK, Flotow H, Tan P, Rozen SG: Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. Gastroenterology 2013;145:554-565.
18 Waller T, Gubala T, Sarapata K, Piwowar M, Jurkowski W: DNA microarray integromics analysis platform. BioData Min 2015;8:18.
19 Gumley TP, McKenzie IF, Sandrin MS: Tissue expression, structure and function of the murine Ly-6 family of molecules. Immunol Cell Biol 1995;73:277-296.
20 Kong HK, Park JH: Characterization and function of human Ly-6/uPAR molecules. BMB Rep 2012;45:595-603.
21 Luo L, McGarvey P, Madhavan S, Kumar R, Gusev Y, Upadhyay G: Distinct lymphocyte antigens 6 (Ly6) family members Ly6D, Ly6E, Ly6K and Ly6H drive tumorigenesis and clinical outcome. Oncotarget 2016;7:11165-11193.
22 Randle ES, Waanders GA, Masciantonio M, Godfrey DI, Boyd RL: A lymphostromal molecule, thymic shared Ag-1, regulates early thymocyte development in fetal thymus organ culture. J Immunol 1993;151:6027-6035.
23 Classon BJ, Boyd RL: Thymic-shared antigen-1 (TSA-1). A lymphostromal cell membrane Ly-6 superfamily molecule with a putative role in cellular adhesion. Dev Immunol 1998;6:149-156.

24 Lv Y, Si M, Chen N, Li Y, Ma X, Yang H, Zhang L, Zhu H, Xu GY, Wu GP, Cao C: TBX2 over-expression promotes nasopharyngeal cancer cell proliferation and invasion. Oncotarget 2017;10.18632/oncotarget.17084
25 Li ZW, Cai S, Liu Y, Yang CL, Tian Y, Chen G, Cao C: Over-expression of Galphai3 in human glioma is required for Akt-mTOR activation and cell growth. Oncotarget 2016;10.18632/oncotarget.10995
26 Gong YQ, Huang W, Li KR, Liu YY, Cao GF, Cao C, Jiang Q: SC79 protects retinal pigment epithelium cells from UV radiation via activating Akt-Nrf2 signaling. Oncotarget 2016;7:60123-60132.
27 Yang L, Zheng LY, Tian Y, Zhang ZQ, Dong WL, Wang XF, Zhang XY, Cao C: C6 ceramide dramatically enhances docetaxel-induced growth inhibition and apoptosis in cultured breast cancer cells: a mechanism study. Exp Cell Res 2015;332:47-59.
28 Zhang YM, Zhang ZQ, Liu YY, Zhou X, Shi XH, Jiang Q, Fan DL, Cao C: Requirement of Galphai1/3-Gab1 signaling complex for keratinocyte growth factor-induced PI3K-AKT-mTORC1 activation. J Invest Dermatol 2015;135:181-191.
29 Li KR, Yang SQ, Gong YQ, Yang H, Li XM, Zhao YX, Yao J, Jiang Q, Cao C: 3H-1, 2-dithiole-3-thione protects retinal pigment epithelium cells against Ultra-violet radiation via activation of Akt-mTORC1-dependent Nrf2-HO-1 signaling. Sci Rep 2016;6:25525.
30 Jia X, Wang F, Han Y, Geng X, Li M, Shi Y, Lu L, Chen Y: miR-137 and miR-491 Negatively Regulate Dopamine Transporter Expression and Function in Neural Cells. Neurosci Bull 2016;32:512-522.
31 Zhao Q, Yang X, Cai D, Ye L, Hou Y, Zhang L, Cheng J, Shen Y, Wang K, Bai Y: Echinacoside Protects Against MPP(+)-Induced Neuronal Apoptosis via ROS/ATF3/CHOP Pathway Regulation. Neurosci Bull 2016;32:349-362.
32 Ji D, Zhang Z, Cheng L, Chang J, Wang S, Zheng B, Zheng R, Sun Z, Wang C, Zhang Z, Liu R, Zhang X, Liu X, Wang X, Li J: The combination of RAD001 and MK-2206 exerts synergistic cytotoxic effects against PTEN mutant gastric cancer cells: involvement of MAPK-dependent autophagic, but not apoptotic cell death pathway. PLoS One 2014;9:e85116.
33 Yeom CJ, Zeng L, Goto Y, Morinibu A, Zhu Y, Shinomiya K, Kobayashi M, Itasaka S, Yoshimura M, Hur CG, Kakeya H, Hammond EM, Hiraoka M, Harada H: LY6E: a conductor of malignant tumor growth through modulation of the PTEN/PI3K/Akt/HIF-1 axis. Oncotarget 2016;7:65837-65848.
34 AlHossiny M, Luo L, Frazier WR, Steiner N, Gusev Y, Kallakury B, Glasgow E, Creswell K, Madhavan S, Kumar R, Upadhyay G: Ly6E/K Signaling to TGFbeta Promotes Breast Cancer Progression, Immune Escape, and Drug Resistance. Cancer Res 2016;76:3376-3386.
35 Gupta GP, Massague J: Cancer metastasis: building a framework. Cell 2006;127:679-695.
36 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87-108.
37 Siegel RL, Miller KD, Jemal A: Cancer Statistics, 2017. CA Cancer J Clin 2017;67:7-30.
38 Siegel RL, Miller KD, Jemal A: Cancer statistics, 2016. CA Cancer J Clin 2016;66:7-30.
39 Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J: Cancer statistics in China, 2015 CA Cancer J Clin 2016;66:115-132.
40 Chen W: Cancer statistics: updated cancer burden in China. Chin J Cancer Res 2015;27:1.
41 Qu Y, Dang S, Hou P: Gene methylation in gastric cancer. Clin Chim Acta 2013;424:53-65.
42 Garcia-Gonzalez MA, Lanas A: Genetic susceptibility and gastric cancer risk: the importance of meta-analyses as a statistical tool. Gastroenterol Hepatol 2014;37:421-426.
43 Asundi J, Crocker L, Tremayne J, Chang P, Sakanaka C, Tanguay J, Spencer S, Chalasani S, Luis E, Gascoigne K, Desai R, Raja R, Friedman BA, Haverty PM, Polakis P, Firestein R: An Antibody-Drug Conjugate Directed against Lymphocyte Antigen 6 Complex, Locus E (LY6E) Provides Robust Tumor Killing in a Wide Range of Solid Tumor Malignancies. Clin Cancer Res 2015;21:3252-3262.
44 Asgharzadeh S, Pique-Regi R, Sposto R, Wang H, Yang Y, Shimada H, Matthay K, Buckley J, Ortega A, Seeger RC: Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification. J Natl Cancer Inst 2006;98:1193-1203.
45 Lee JS, Leem SH, Lee SY, Kim SC, Park ES, Kim SB, Kim SK, Kim YJ, Kim WJ, Chu IS: Expression signature of E2F1 and its associated genes predict superficial to invasive progression of bladder tumors. J Clin Oncol 2010;28:2660-2667.
46 Kao KJ, Chang KM, Hsu HC, Huang AT: Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: implications for treatment optimization. BMC Cancer 2011;11:143.