XAGE-1b Promotes the Proliferation, Invasion and Metastasis of Gastric Cancer

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Research

Keywords: XAGE-1b; Gastric cancer; CLDN6; CHGA

Posted Date: July 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-47552/v1

Abstract

Background X antigen family member 1B (XAGE-1b), a member of XAGE subfamily and GAGE family, is upregulated in some malignant tumors and has been associated with the proliferation, invasion and metastasis of tumors. However, the biological roles of XAGE-1b in gastric cancer (GC) still remain unclear.

Methods We detected the expression of XAGE-1b in 60 paired fresh tissues of GC patients by real-time RT-PCR. Kaplan-Meier survival curve was explored to analyze 5-year survival time of GC patients. Function experiments were performed to estimate the role of XAGE-1b on the proliferation, invasion and metastasis of GC cells. Informatic analysis was applied to investigate the potential mechanisms.

Results XAGE-1b was obviously upregulated in GC tissues. XAGE-1b was correlated significantly with poor prognosis of GC patients. XAGE-1b markedly promoted the proliferation and invasion in GC cell lines in vitro. Knockdown of XAGE-1b promoted the pulmonary metastatic ability in nude mice. Moreover, XAGE-1b was positively or negatively correlated with the expression of CLDN6 and CHGA, which regulated the progression of GC.

Conclusions XAGE-1b could act as an oncogene in GC, which provides a potential biological marker or treatment target for GC.

Background

Gastric cancer is one of the most common malignant tumors. In many countries, the mortality of GC ranks the first in digestive system malignant tumors[1, 2]. Moreover, early gastric cancer patients have better prognosis than advanced gastric cancer, which has a higher risk of recurrence and metastasis[3]. Nowadays, GC gradually tends to affect the young, and the most of early gastric cancer patients have no obvious symptoms, the diagnostic rate of GC is still low[4]. Therefore, it is of great interest to explore the molecular mechanisms and effective therapeutic approaches for GC.

XAGE-1b belongs to XAGE subfamily and GAGE family. XAGE-1b protein contains a nuclear localization region, which is similar to other GAGE/PAGE proteins. With similar expression patterns and sequence, it also belongs to the CT (cancer-testis) antigen family. There are 4 transcript variants of XAGE-1, include XAGE-1a, XAGE-1b, XAGE-1c and XAGE-1d. XAGE-1b is characterized as wide expression spectrum, high expression rate and strong immunogenicity, which also leads to a variety of transcription variations. It has been reported that XAGE-1b was highly expressed in melanoma[5, 6], lung cancer[7–13], prostate cancer[14, 15] and hepatocellular carcinoma[16, 17], and was correlated with the tumor poor prognosis. Moreover, XAGE-1b could promote invasion and metastasis of adenoid cystic carcinoma[18, 19].

However, the biological role of XAGE-1b in GC has not been reported. In present study, we investigated the role and potential mechanisms of XAGE-1b in GC, which maybe deepen our understanding and provide novel treatment target or biological marker for clinical GC patients.

Methods

Cell culture

The human GC lines (SGC7901, BGC823, MGC803, MNK45 and AGS) were taken from Central South University Xiangya School of Medicine Affiliated Haikou Hospital. GC cells were maintained in RPMI-1640 medium (BI, China) with 10% fetal bovine serum (FBS, BI). GC cells were cultured in a cell incubator with 5% CO_2, at 37 ℃.

Transfection

For knocking down XAGE-1b, lentivirus with a shRNA vector (5'-GCTGCATCAGTCAAACACC -3') was designed in Jikai (Shanghai, China). The shRNA vector targeting XAGE-1b was transfected into HEK293T cells. Then, 1 ml viral supernatant with 4ul of polybrene was added into GC cells for stable transduction. After that, the medium was replaced by the complete culture medium with 10% FBS.

GC tissues and Tissue RNA extraction

The total of 60 pairs of fresh GC tissues and normal mucosa samples were collected from Central South University Xiangya School of Medicine Affiliated Haikou Hospital. The Biomedical Ethics Committee of Haikou People's Hospital and the Research Ethics Committee of Zunyi Medical University approved the study. Written informed consent was acquired from the GC patients.

The cleaned mortars were placed in 180 ℃ oven and baked for 5 hours. The mung bean sized fresh tissues were put into a mortar with appropriate liquid nitrogen, grinded into powder. Then, 1 ml Trizol was added into the mortar for homogenate cracking.

Real time RT-PCR

Trizol reagent (Invitrogen, USA) was used to extract RNA and cDNA was synthesized according to the instructions for Takara reverse kit (TaKaRa, China). Primer sequences of XAGE-1b were obtained from PCR Primer 5.0 software. The specific sequences of primers were as follows: XAGE-1b (upstream-primer: 5'AAACCAGCTTGCGTTGTTTCAG3'; downstream-primers:5'CGCA

TGTTCACTGGGCGTCTT3'). The reaction system of reverse transcription was prepared following the instructions of Takara RT-PCR kit. Relative expression quantity between the experimental group and the control group was represented by folders = $2^{-\Delta\Delta CT}$. The experiment was repeated three times.

Cell proliferation assay

CCK8 assay kit (C0038, Beyotime, China) was used to detect cell proliferation. 1×10^3 GC cells with 100 μl culture medium were seeded into 96-well plates. The proliferation assay was performed by adding 10 μl CCK8 reagent into wells for incubation 2 h and measured continuously for 7 days respectively. The absorbance (OD) value of each pore at 450 nm was measured. The experiment was repeated three times.

In vitro invasion assay

Invasion Boyden Chamber (BD Biosciences, Foster city, USA) plated by matrigel was used to detect cell invasion. The complete culture medium was added into the lower chamber as the chemotactic factor. In each chamber, 2×10^5 cells with serum-free RPMI 1640 medium were added into the upper compartment. After incubation for 48 h, the chambers were fixed with alcohol and stained with hematoxylin. The number of cells passed through the membrane with matrigel was examined by the microscope in 5 random visual fields and counted the average number of cells. The experiment was repeated three times.

Metastasis in the mouse models

For tail vein metastasis assay, a total of 1×10^6 cells were injected into the tail veins of nude mice. After 8 weeks, the nude mice were killed, and lungs were removed for further analysis. The lung tissues were fixed, dehydrated, embedded, sectioned and subjected to pathological observation by the microscope. Metastatic tumors were observed by H&E staining and the number of metastasis was assessed by counting metastatic lesions in each tissue section.

Bioinformatic analyses based on online databases

The samples diagnosed with GC were from the TCGA database (http://cancergenome.nih.gov/) and were used to analyze the differential expression of XAGE-1b, CLDN6 and CHGA. Survival analysis was conducted based on the expression value of the genes and follow-up time of the GC patients. The expression correlation of XAGE-1b, CLDN6 and CHGA in GC was analyzed by GEPIA (http://gepia.cancer-pku.cn/detail.php?clicktag=matrix).

Statistical analysis

The Graphpad Prism 6.0 was used to analyze data. Mean ± SD was used to express the experimental data. $P < 0.05$ was regarded as statistical difference. Statistical analysis methods included independent samples *t*-test, one-way ANOVA, two-way ANOVA and Mantel-Cox test.

Results

XAGE-1b is upregulated in human GC tissues and correlated with the prognosis of GC patients

To investigate the potential role of XAGE-1b in GC, the expression of XAGE-1b in 60 pairs of fresh GC tissues was performed by qRT-PCR. Compared with normal specimens, the expression of XAGE-1b was significantly upregulated in GC tissues (Fig. 1A).

Kaplan-Meier survival curve was performed to analyze the prognostic relationship between XAGE-1b expression level and overall survival time of GC patients. The results showed that patients with high expression of XAGE-1b had a significantly poor 5-year survival rate compared with the low expression of XAGE-1b ($p = 0.04$, Fig. 1B).

Construction of interference stable cell lines

To investigate the potential role of XAGE-1b in GC progression, the endogenous expression of XAGE-1b in GC cell lines was explored. In five GC cell lines, the mRNA expression level of XAGE-1b was highest in BGC823 cells, and gradually decreased in AGS, MNK45, MGC803 and SGC7901 (Fig. 2A). BGC823 and AGS cells were infected by lentivirus particles. QRT-PCR was used to detect effective transfection of lentivirus in BGC823 (Fig. 2B) and AGS cells (Fig. 2C). Compared with NC group, the mRNA expression of XAGE-1b was significantly downregulated in shXAGE-1b group (Fig. 2B and C).

Effects of XAGE-1b on the proliferation and invasion of GC in vitro

Next, to demonstrate the role of XAGE-1b in the proliferation and invasion of GC, we performed loss-function assays to explore changes in malignant biological function of GC cells in vitro. CCK8 assay revealed that knockdown of XAGE-1b significantly reduced the proliferation rate of BGC823 (Fig. 3A) and AGS cells (Fig. 3B). Boyden chamber assay showed that cells with knockdown of XAGE-1b passing through the number of membrane significantly reduced in BGC823 (Fig. 3C) and AGS cells (Fig. 3D). The above results indicated that XAGE-1b promoted proliferation and invasion of GC cells in vitro.

Effect of XAGE-1b on GC metastasis in vivo

To confirm the effect of XAGE-1b on metastasis in vivo, we respectively injected BGC823/shXAGE-1b cells and BGC823/shCtrl cells into the tail vein of nude mice (n = 5), then we observed lung colonization capacity. After 8 weeks, we removed the lungs, then performed general views of the lung tissues (Figure 4A) and pathology changes in H□E sections (Figure 4B). We found that the number of metastatic nodules in shCtrl group was more than that in XAGE-1b-knockdown group (Figure 4C). Knockdown of XAGE-1b obviously inhibited lung metastatic capacity. These results demonstrate that XAGE-1b profoundly promotes metastasis of GC cells in vivo.

Potential mechanism analyses using bioinformatic methods

Above results revealed a novel oncogene, XAGE-1b, in GC. To make clear the mechanisms of XAGE-1b in the progression of GC, we analyzed the differential expressed genes in GC. Over-expressed and Under-expressed genes were displayed based on the chromosomes (Fig. 5A), and selected twenty genes with the highest scores. Further correlation analysis was applied to analyze the expression of XAGE-1b and the selected genes. XAGE-1b was positively and negatively correlated with CLDN6 (Fig. 5B) and CHGA (Fig. 5C), respectively. Next, to explore the potential clinical implication of CLDN6 and CHGA, the results analyzed based on the online database suggested that CLDN6 was upregulated in GC tissues (Fig. 5D). Further overall survival analysis of GC patients showed that GC patients with higher expression of CLDN6 led to a decreased survival time than GC patients with lower expression of CLDN6 (Fig. 5E). Further analyses were conducted for another potential gene, CHGA. CHGA was downregulated in GC tissues (Fig. 5F). However, CHGA wasn't significantly correlated with the prognosis of GC (Fig. 5G). These results

suggest that XAGE-1b could be regarded as an oncogene for GC progression through the potential downstream molecules, CLDN6 and CHGA.

Taken together, these results indicated that increased XAGE-1b expression is frequent event in human GC tissues. XAGE-1b was correlated with the prognosis of GC, and regulated the proliferation, invasion and metastasis in vitro and in vivo. Correlated expression patterns with CLDN6 and CHGA implied that XAGE-1b maybe function as an oncogene through these molecules.

Discussion

GC is a common malignant tumor with high morbidity and mortality. Even though the advancement in the gastrectomy and chemotherapy of GC patients, treatment failure occurs frequently due to the drug resistance[20]. Thus, it is urgent to explore novel diagnostic markers or treatment therapies for GC.

XAGE-1b is considered as a tumor serological marker which assisted clinical diagnosis[7, 14]. Moreover, significant dysregulated levels of XAGE-1b were observed in several tumors[5–19, 21]. However, XAGE-1b expression in GC and its role in the prognosis of GC patients remain unclear. In this study, we found that XAGE-1b was obviously up-regulated in GC tissues and correlated significantly with poor overall survival. The result of XAGE-1b is compatible with other tumor, which reported that XAGE-1b$^+$ group with melanoma showed poorer prognosis[5]. These findings demonstrate that XAGE-1b may be related to biological events of GC.

In previous studies, XAGE-1b increased the growth and metastasis of tumor cells[19, 22]. To address the effects of XAGE-1b on the biological events of GC in vitro and in vivo, the endogenous expression of XAGE-1b was investigated, and the lentivirus was applied to knockdown XAGE-1b expression in GC cell lines. Knockdown XAGE-1b obviously prohibited GC cell proliferation, invasion and pulmonary metastatic capability. It is suggested that XAGE-1b expression is closely related to the proliferation, invasion and metastasis of GC.

Next, to explore the potential mechanisms of XAGE-1b in the progression of GC, we analyzed the correlation between XAGE-1b and other differential expressed genes in GC. Correlation analysis showed that XAGE-1b was positively and negatively correlated with CLDN6 and CHGA, respectively. As a tight junction protein, claudin-6 (CLDN6) belongs to a family of transmembrane proteins[23]. Our results and previous studies[24, 25] suggested that CLDN6 was upregulated in GC tissues and correlated with a decreased survival time in GC patients. Human chromogranin-A (CHGA), a member of the chromogranin/secretogranin family, has been reported to be associated with several tumors[26–28]. Even though no significant association between CHGA expression and overall survival time of GC patients was observed (Fig. 5G), CHGA has been reported to be an early diagnosis biomarker for GC[26, 29]. Here, our preliminary results suggest that XAGE-1b could be regarded as an oncogene for GC progression through the potential downstream molecules, CLDN6 and CHGA. However, further experiments need to be applied to investigate the direct regulatory patterns among XAGE-1b, CLDN6 and CHGA.

Conclusions

In general, our study reported a novel oncogene, XAGE-1b, in the progression of gastric cancer. Mechanistically, we demonstrated that the upregulated expression of XAGE-1b was significantly correlated with the prognosis of GC. Knockdown of XAGE-1b prohibited the proliferation and invasion of GC cells in vitro, and inhibited the metastasis in vivo. Bioinformatic analyses revealed that XAGE-1b was correlated with CLDN6 and CHGA. These findings proved the role of XAGE-1b in GC, and provided the potential mechanisms underlying the promotive effects of XAGE-1b on gastric cancer. Moreover in vitro and in vivo experiments are needed to validate the findings.

Abbreviations

GC: gastric cancer; XAGE-1b: X antigen family member 1B; CT: cancer-testis; FBS: fetal bovine serum; CLDN6: claudin-6; CHGA: chromogranin-A.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

All the authors contributed to this work. Fei Geng designed the experiments and revised the manuscript. Hui Men, Zhi-wei Zhang, Gui-feng Lu, Tan Shun, Wen-Hui Hu, Xin Yang carried out the experiments. Hui Men wrote the paper. Hui Men collected and analyzed data. All authors had final approval of the submitted and published versions.

Funding

This work was supported by the National Natural Science Foundation of China (No. 31860271), Opening fund of the State Key Laboratory of Basic Pharmacology ([2017]382), Joint Fund for Science and Technology of Zunyi Science and Technology Bureau ([2017]07) and Youth Science and Technology Personnel Training Project ([2017]197).

Availability of data and materials

On reasonable request, the corresponding author and the first author will provide the original data and materials' information involved in the study.

Ethics approval and consent to participate

The study was approved by the Biomedical Ethics Committee of Haikou People's Hospital and the Research Ethics Committee of Zunyi Medical University. Written informed consent was acquired from the

GC patients.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures

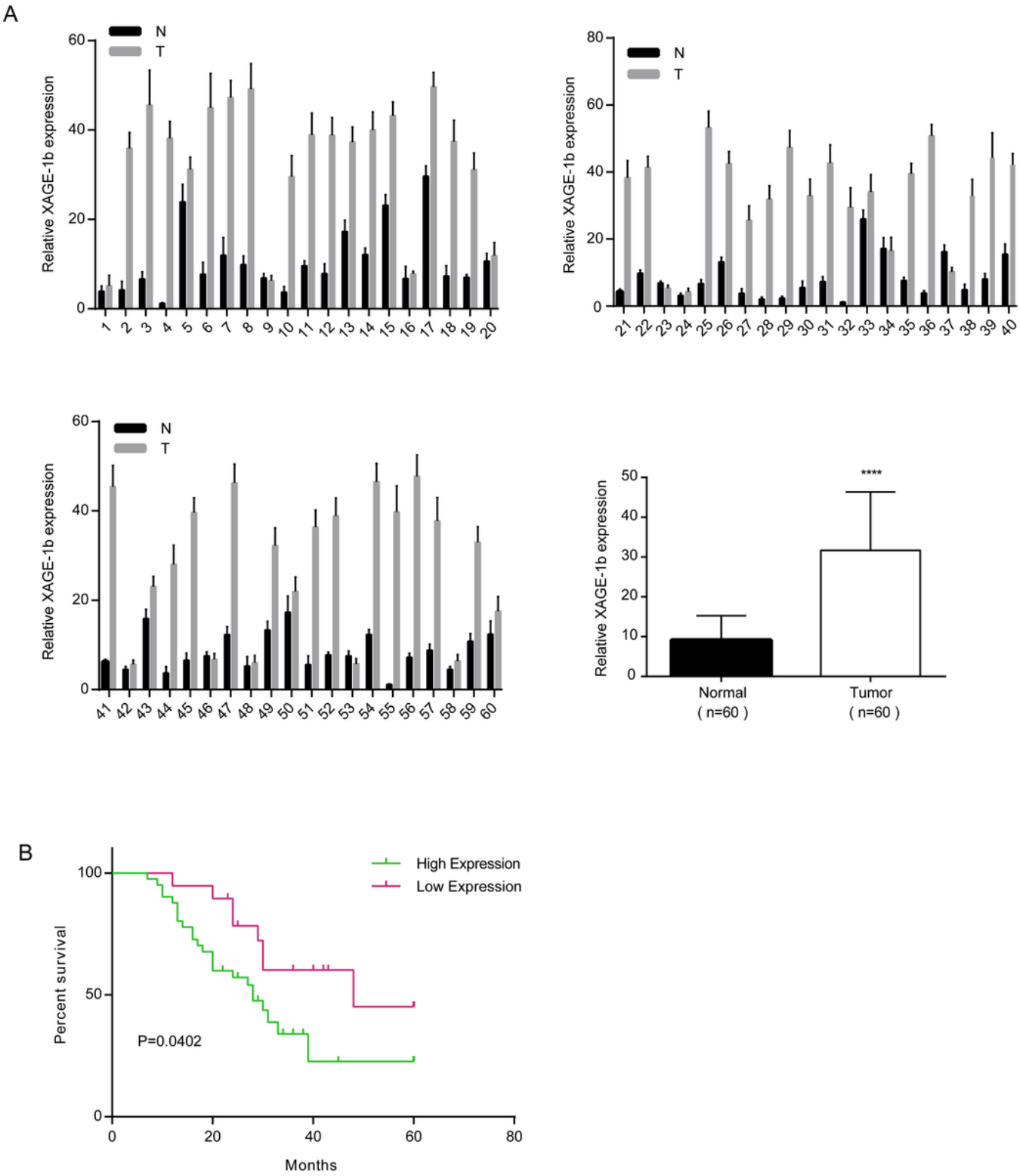

Figure 1

XAGE-1b is upregulated in human GC tissues and correlated with the prognosis of GC patients. (A)The mRNA expression level of XAGE-1b was upregulated in GC tissues. (B) XAGE-1b expression was negatively correlated with the prognosis of GC patients. N, normal tissues; T, gastric tissues. *** p □ 0.001.

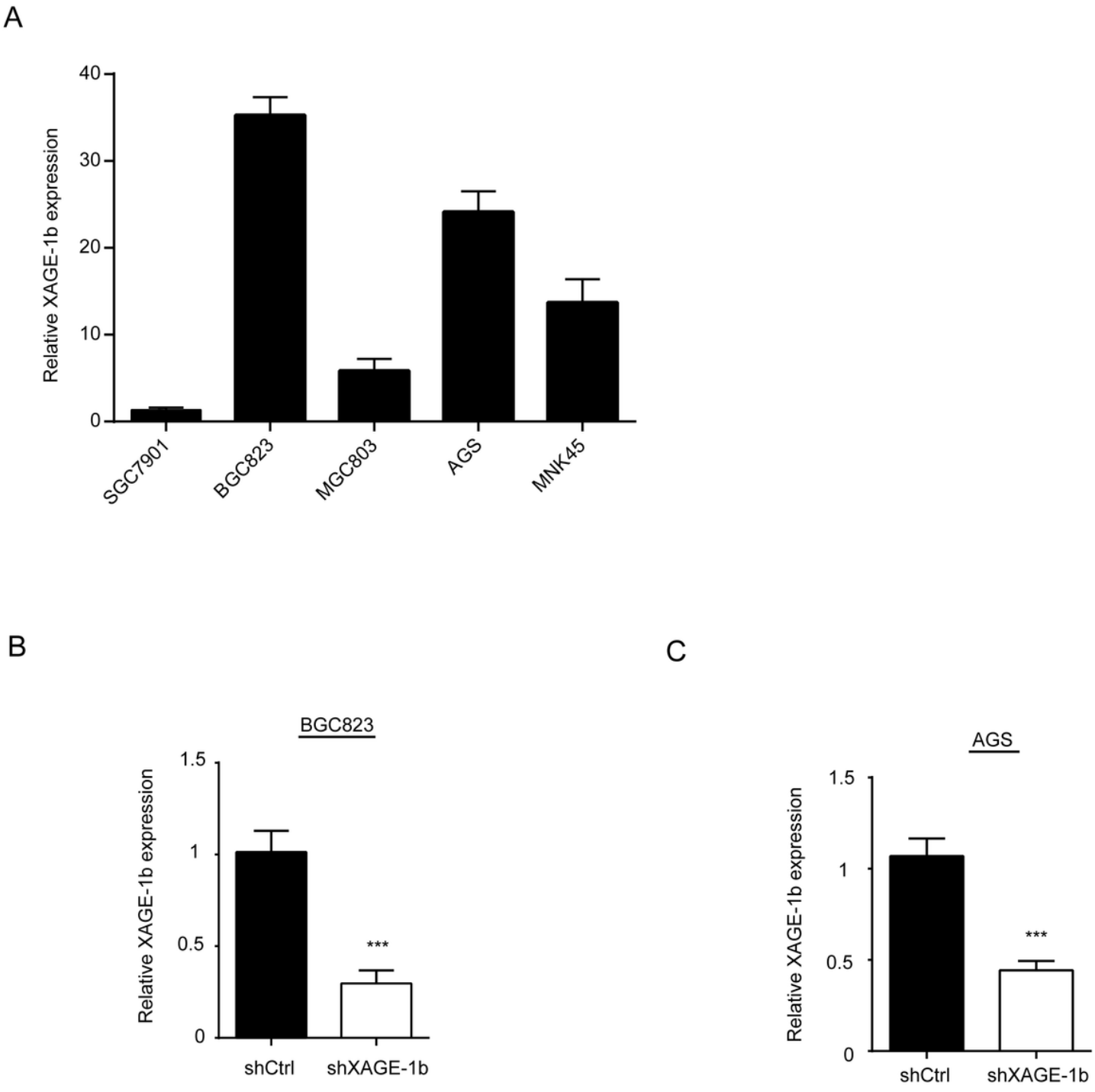

Figure 2

Construction of interference stable cell lines. (A) Relative expression of XAGE-1b in 5 GC cell lines. (B) Relative expression of XAGE-1b in BGC823 cells transfected with shRNA of XAGE-1b. (C) Relative expression of XAGE-1b in AGS cells by qRT-PCR. shCtrol, empty lentivirus control; shXAGE-1b, shRNA of XAGE-1b. *** p □ 0.001.

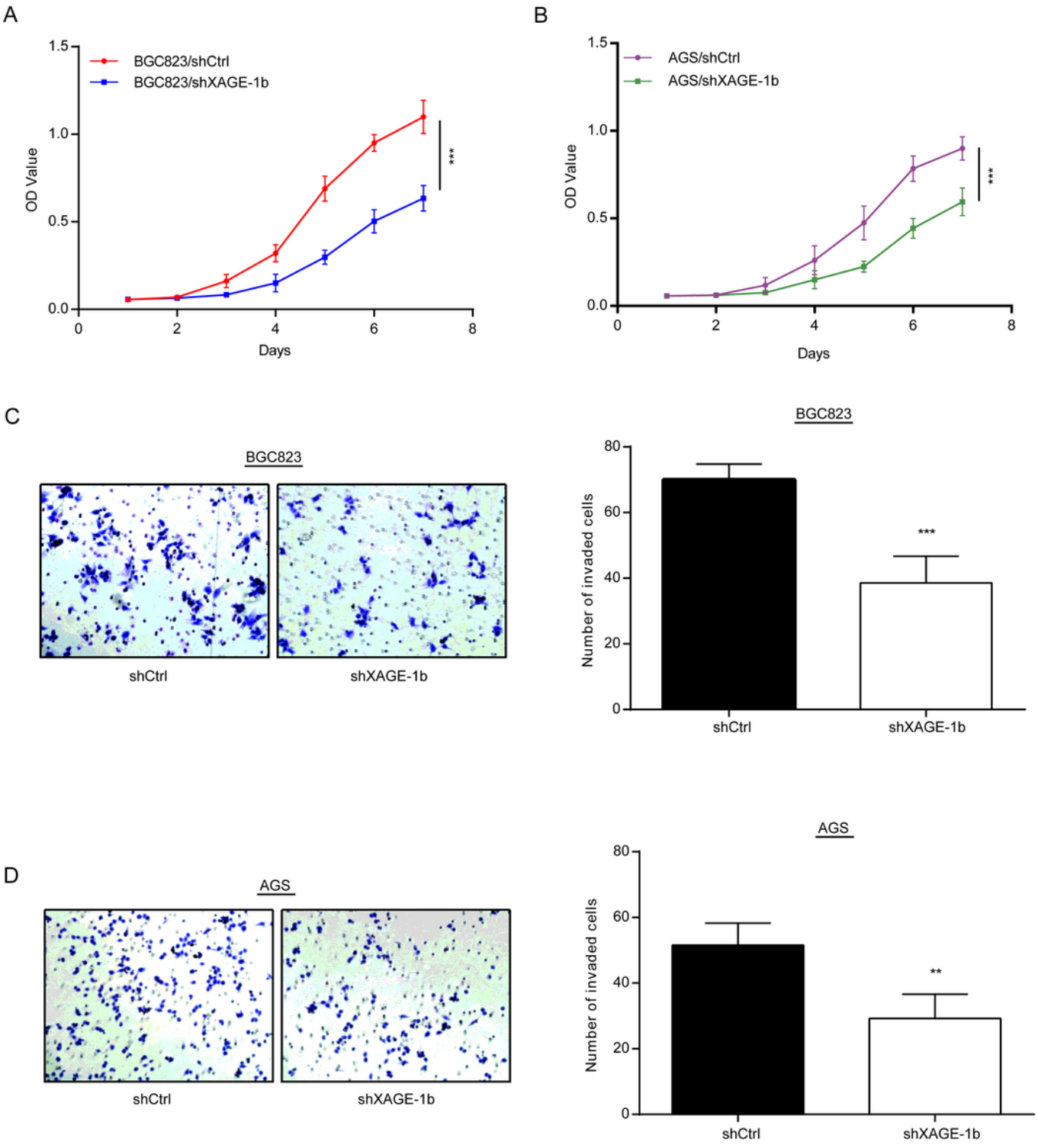

Figure 3

Effects of XAGE-1b on the proliferation and invasion of GC in vitro. (A) Effect of XAGE-1b knockdown on BGC823 cell proliferation by CCK-8 assay. (B) Knockdown of XAGE-1b prohibited AGS cell proliferation. (C) Effect of XAGE-1b knockdown on BGC823 cell invasion by Boyden chamber assay. (D) Knockdown of XAGE-1b suppressed the invasion of AGS cells. ** p □ 0.01, *** p □ 0.001.

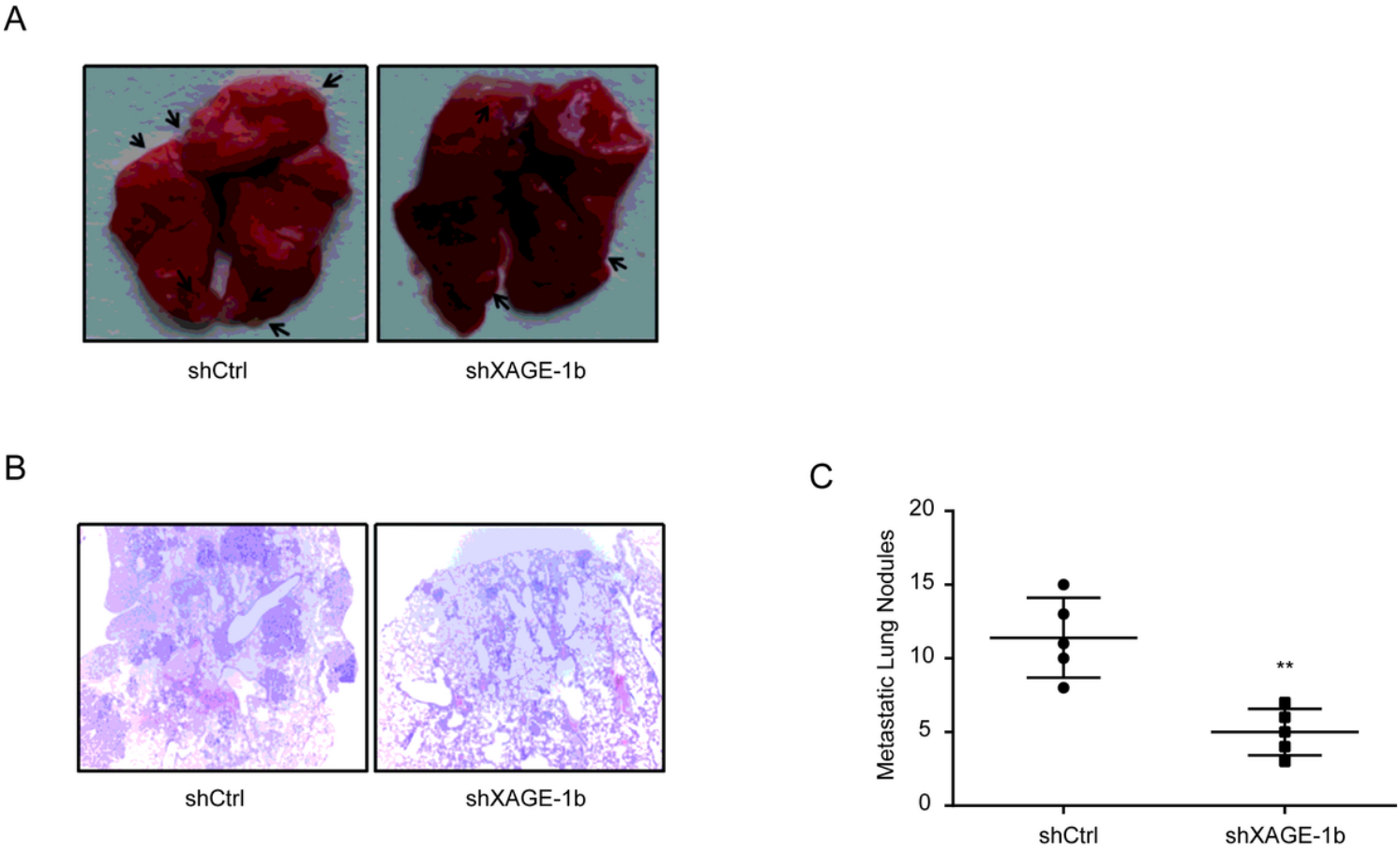

Figure 4

Effect of XAGE-1b on GC metastasis in vivo. (A) Pulmonary metastasis of nude mice injected with BGC823/shXAGE-1b and BGC823/shCtrol cells by general views of the lung tissues. (B) Pulmonary metastasis of nude mice injected with BGC823/shXAGE-1b and BGC823/shCtrol cells by pathology changes in H□E sections(×20). (C)The number of metastatic nodules per mice was counted under the microscope. ** p □ 0.01.

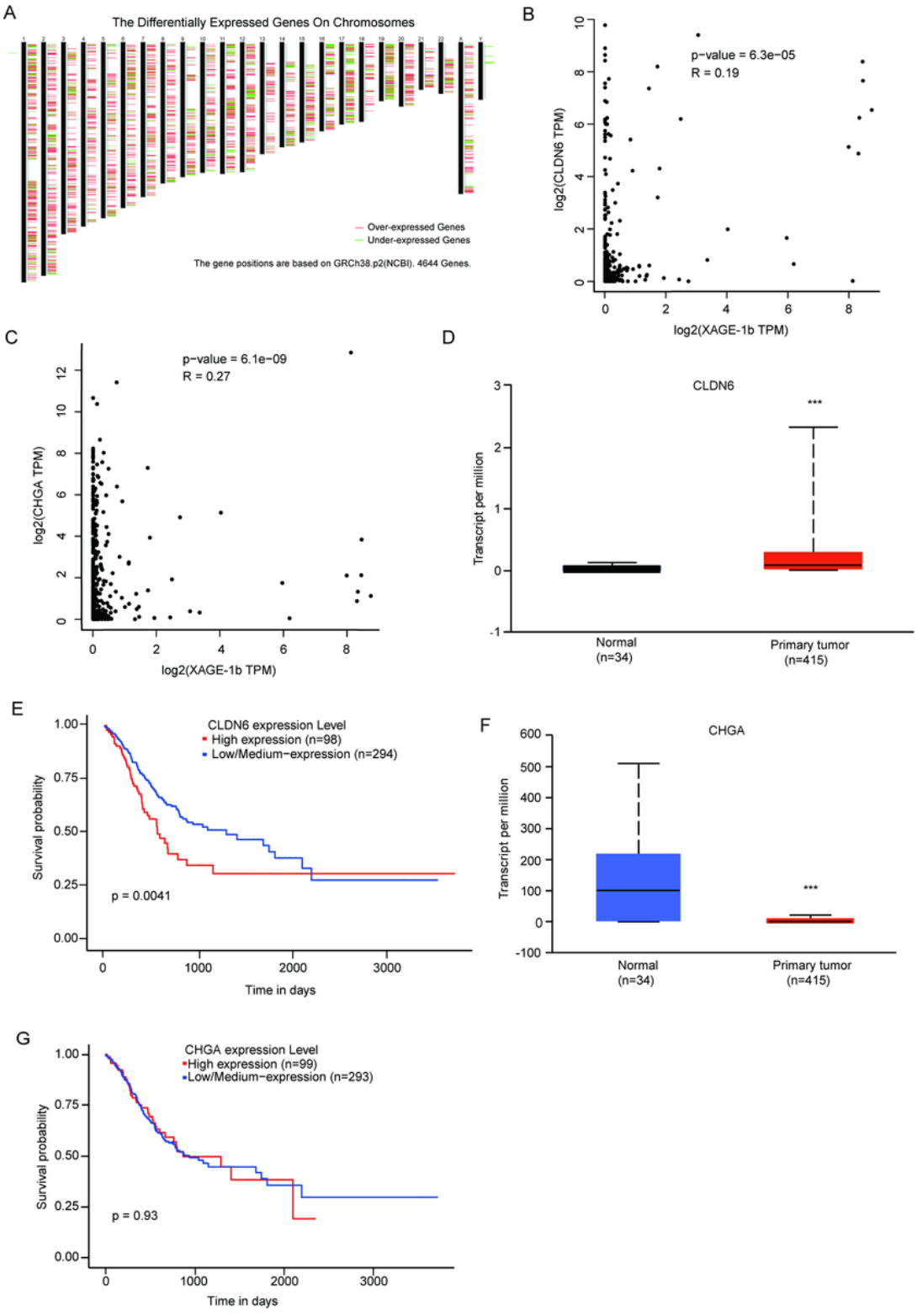

Figure 5

Potential mechanism analyses using bioinformatic methods. (A) The differentially expressed genes in GC. (B) XAGE-1b was positively correlated with CLDN6. (C) XAGE-1b was negatively correlated with CHGA. (D) CLDN6 was increased in clinical GC tissues. (E) CLDN6 was significantly correlated with the prognosis of GC patients. (F) CHGA was downregulated in GC specimens. (G) The expression level of CHGA was not correlated with the prognosis of gastric cancer. *** p □ 0.001.