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Significance of Anoctamin 6 in progression and prognostic prediction of gastric adenocarcinoma

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Summary. Background. Gastric cancer is one of the most lethal malignancies worldwide with surgery as the only curative therapy. However, postoperative overall survival of gastric cancer is far from satisfactory although significant improvement has been made in adjuvant therapies. Gastric cancer is characterized as highly heterogeneous and illustrating the molecular mechanisms is invaluable for both identification of novel prognostic biomarkers and development of therapeutic drugs. Here we aimed to investigate the participation of Anoctamin 6 (ANO6) in gastric adenocarcinoma.

Methods. Immunohistochemical (IHC) staining was used to explore the expression pattern of ANO6 in tumor tissues from gastric adenocarcinoma patients (n=108). Clinicopathological data was subjected to Kaplan-Meier survival and Cox multivariate analyses to evaluate prognostic predictors. Overexpression and silencing procedures were performed on gastric cancer cell lines to investigate the functional mechanisms of ANO6 in regulating tumor development.

Results. Higher ANO6 expression showed a positive correlation with advanced tumor stage of gastric cancer. Univariate and multivariate analyses revealed that ANO6 was an independent prognostic factor for overall survival of gastric cancer. An *in vitro* study demonstrated that ANO6 can promote cell proliferation while silencing ANO6 significantly downregulated cell viability.

Conclusion. High ANO6 expression in gastric cancer indicates poor clinical outcomes, and ANO6 may act as a potential target for novel therapy development targeting gastric cancer.

Key words: Gastric adenocarcinoma, ANO6, Prognosis, Proliferation

Introduction

Gastric cancer is the fifth most common cancer and the third most common cause of cancer-related death worldwide (Smyth et al., 2020). Although the incidence of gastric cancer is decreasing, treatment and prognostic prediction are far from satisfactory (Siegel et al., 2020). Major risk factors of gastric cancer include genetic heredity and *Helicobacter pylori* (*H. Pylori*) infection; however, gastric cancer is a highly heterogeneous disease in both molecular alterations and phenotypes (Wang and Wang, 2021). More and more histological prognostic biomarkers have been identified in gastric cancer. For example, HtrA serine peptidase 3 (HTRA3) has been reported to participate in gastric cancer progression via the NF-κB pathway, YAP1/WWTR1/ TAZ pathway, and TGFβ pathway (Ji et al., 2020a). Similarly, Na^+/K^+-ATPase alpha1 subunit (ATP1A1), a critical component for maintaining cellular osmolality, was also involved in gastric cancer development and prognosis (Nakamura and Shiozaki, 2021). Besides proteins, several microRNAs and circRNAs (Alessandrini et al., 2018; Ji et al., 2020b; Chen et al., 2021a,b; Xu et al., 2021) were also indicated to play vital roles in gastric cancer prognosis. Identifying more prognostic predictive biomarkers is essential for personalized treatment as well as for novel therapy development.

The anoctamin (ANO) protein family is a kind of voltage-gated calcium-activated anion channel on the cell membrane, which is critical for embryo development (Rock and Harfe, 2008; Yang et al., 2008; Tian et al., 2012). Although the atomic resolution structure of the anoctamin members has been obtained, their involvement in malignancies has not been fully illustrated. One of the major mechanisms of anoctamins on cancer pathobiology is their role in regulating intracellular Ca^{2+} levels (Kunzelmann et al., 2019). Previous studies suggested that anoctamins can control membrane exocytosis by setting Ca^{2+} levels near the plasma membrane, and/or by controlling the intracellular Cl- concentration, subsequently affecting cell survival and cell death (Tian et al., 2012; Kunzelmann et al., 2019). ANO6 physiologically acts as a regulator of

phospholipid scrambling in platelets and its mutations can result in Scott syndrome, a rare human bleeding disorder (Millington-Burgess and Harper, 2020). Interestingly, using Ehrlich-Lettre ascites carcinoma cells as the experimental model, Jacobsen and his colleagues showed that ANO6-knockdown affected the speed of cell migration although they did not dig into the detailed mechanisms (Jacobsen et al., 2013). Till now, the tumor related role of ANO6 was only reported in glioma (Xuan et al., 2019). According to Xuan et al, ANO6 expression level was positively associated with tumor stage. In addition, ANO6 inhibition significantly suppressed the viability and invasion of glioma cells, while ANO6 overexpression led to the opposite effects. Meanwhile, ANO6 knockdown inhibited the phosphorylation level and nuclear translocation of extracellular signal-regulated kinase (ERK) protein to inhibit ERK signaling.

The expression patterns of ANO members in gastric cancer have not been well-characterized, and here we aimed to initially investigate the expression and function of ANO6 in gastric cancer. In the current study, we firstly tested the protein expression of ANO6 in gastric adenocarcinoma tissues and assessed its clinical significance in predicting patients' survival after surgical resection. The oncogenic role of ANO6 was also validated by cellular assays, which demonstrated its involvement in gastric cancer proliferation.

Materials and methods

Online database

Gene expression data of ANO6 in gastric cancer tissues and normal stomach tissues were retrieved from Gene expression profiling interaction analysis (GEPIA) (https://gepia.cancer-pku.cn/) online tools.

Kaplan–Meier curves for ANO6 were generated with the online Kaplan–Meier Plotter (https://www.kmplot.com/). Briefly, gastric adenocarcinoma patients were grouped into two groups according to the median mRNA levels of ANO6 from RNAseq data. The overall survival and relapse-free survival were analyzed and compared between the two groups using log-rank test.

Patients and samples

We retrospectively collected a cohort of gastric adenocarcinoma patients (n=176) that underwent R0 resection and D2 gastrectomy in our hospital during 2013-2016. Among them, 17 cases were recorded with unclear tumor location, 3 cases with unknown tumor size, 16 cases underwent preoperative chemotherapy, and 32 cases absent of follow-up. After excluding the above patients (n=68), a total of 108 cases were enrolled in this study. All these patients were followed-up by phone and medical records. None of the patients received any preoperative chemotherapy or radiotherapy. None of the patients exhibited distant metastasis at the time of surgical treatment. All diagnoses were based on pathological tests and staged according to the American Joint Committee on Cancer (AJCC) tumor/node/metastasis (TNM) classification 7th edition. The clinical characteristics of all gastric adenocarcinoma patients are presented in Table 1. This study was approved by the Ethic Committee of Third Affiliated Hospital of Nanchang University. Written informed consent was obtained from each participant.

Immunohistochemistry (IHC) staining and scoring

IHC was conducted to evaluate the protein expression level of ANO6 in all 108 gastric adenocarcinoma tissue specimens using the streptomycin-horseradish peroxidase (HRP) method according to the conventional procedures (Liu et al., 2017). After pretreatment, sample slides were incubated with ANO6 primary antibody (ab243446, Abcam) at 1:500 dilution and then labeled with an HRP conjugated secondary antibody. The slides were finally stained with 3,3'-diaminobenzidine (Han et al., 2020).

The IHC results were assessed by two independent pathologists based on both staining intensity (ranging 0-3) and percentage of positively stained cells (ranging 0-4). Then the IHC score was obtained by multiplying the two scores above (ranging 0-12). The average values from independent pathologists were used as the final IHC score. All the 108 patients were divided into two groups, namely a high-ANO6 expression group (IHC

Table 1. Correlations between ANO6 expression with patients' characteristics.

Characteristics	Cases (n=108)	ANO6 protein expression		P value
		Low (n=52)	High (n=56)	
Age				
≤53 yrs	53	23	30	0.332
>53 yrs	55	29	26	
Sex				
Female	39	23	16	0.090
Male	69	29	40	
Localization				
Cardia/fundus	26	10	16	0.524
Body	53	27	26	
Antrum/pylorus	29	15	14	
Tumor diameter				
≤5.0 cm	64	35	29	0.101
>5.0 cm	44	17	27	
T stage				
T1/T2	43	27	16	0.013*
T3/T4	65	25	40	
Differentiation				
Well/moderate	53	28	25	0.339
Poor	55	24	31	
TNM stage				
Stage I-II	45	32	13	<0.001*
Stage III	63	20	43	

score≥6.0, n=56) and a low-ANO6 expression group (IHC score<6.0, n=52) according to the median IHC score (6.0).

Cell culture and transfection

Human gastric epithelial cell line (GES-1) and three gastric adenocarcinoma cell lines (MKN28, MKN45, and AGS-1) were all purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (GIBCO) at 37 degrees with 5% CO_2 atmosphere (Liu et al., 2021b).

Knockdown assay was conducted by transient transfection of siRNAs (sc-96071 and sc-37007; Santa Cruz, CA, USA) with Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions (Liu et al., 2021a). Overexpression assay was realized by transient transfection of pcDNA3.1-vector or pcDNA3.1-ANO6 plasmids into cultured cells with Lipofectamine 3000. The pcDNA3.1 plasmids were also purchased from GenePharma and validated by DNA sequencing.

Protein extraction and immunoblotting

Total protein was extracted with RIPA buffer (Beyotime, Shanghai, China) from cultured cells and protein concentration was determined using the BCA method (Thermo Fisher Scientific). Approximately 30 μg of total proteins were subjected for western blot analyses as we described before (Yao et al., 2021) using 1:1000 dilution of ANO6 primary antibody (PA5-35240, Thermo Fisher Scientific). The immunoreactivity was routinely detected with a chemiluminescent HRP substrate (Beyotime) and electrogenerated chemiluminescence (ECL) imaging system.

Cell counting kit-8 (CCK-8) assay

The CCK-8 assay was performed to evaluate cell proliferation ability (Chen et al., 2021b). Briefly, transfected cells were seeded into 96-well plates at the density of 5,000 cells/well. The CCK-8 solution was added at 8, 24, 48, 72, and 96 h after cell seeding according to the standard procedure. Absorbance at 570 nm was recorded using a microplate spectrophotometer. Each group was seeded in triplicate and repeated three independent times.

Statistics

All statistical analyses were conducted using the SPSS Software Version 22.0 (SPSS Inc., Chicago, IL, USA). Pearson chi-square test was used to calculate the correlations between ANO6 expression and clinicopathological features of patients. Overall survival was calculated by the Kaplan–Meier method and compared with the log-rank test. Multivariate Cox proportional hazards regressions model was used to identify independent prognostic factors. $P<0.05$ was considered to be of statistical significance.

Results

Patients' information

We retrospectively enrolled a cohort of gastric adenocarcinoma patients (n=108) that underwent D2 gastrectomy in our hospital during 2013-2016. All tumor samples were formalin-fixed and paraffin-embedded (FFPE) for IHC test. The median age of our cohort was 53 years old, ranging 34-75 years old. Among all the patients, 39 were female and 69 were male. Twenty-six patients had lesions located in gastric cardia or fundus, 53 cases with gastric body location, while the other 29 cases with antrum or pylorus location. The median tumor diameter was 4.3 cm, ranging 0.2-9.4 cm. As for the invasion depth, 9 cases were diagnosed with T1 stage, 34 cases with T2 stage, 46 cases with T3 stage, and the other 19 cases with T4 stage. Only 9 cases showed good differentiation, 44 cases with moderate-poor differentiation and the other 55 cases with poor-signet differentiation. Finally, 14 cases were classified as TNM stage I, 31 cases as TNM stage II, and the other 63 cases with TNM stage III.

Expression level and clinicopathological correlation of ANO6

Tissue specimens from all 108 gastric cancer patients were subjected to IHC staining to evaluate the expression of ANO6 in tumor tissues. ANO6 predominantly showed cytoplasmic localization in both primary lesions and

Table 2. Correlations between ANO6 expression with gastric cancer pathogenesis.

| Factors	Cases (n=108)	ANO6 protein expression		P value
		Low (n=52)	High (n=56)	
H. pylori infection				
Negative	26	12	14	0.086
Positive	47	18	29	
Unknown	35	22	13	
TP53 mutation				
Wild type	62	26	36	0.134
Mutation	46	26	20	
HER2 status				
Positive	19	7	12	0.204
Negative	67	31	36	
Unknown	22	14	8	
Ki67 level				
≤25%	17	11	6	0.327
26-50%	26	12	14	
>50%	65	29	36	

Data was calculated by Chi-square test.

ANO6 promotes gastric cancer proliferation

concomitant metastatic lymph nodes (Fig. 1A), however it exhibited low expression or negative staining in certain tumor samples (Fig. 1B). By comparing the IHC scores, we found that patients with TNM stage III showed higher protein expression of ANO6 than those with TNM stage I-II (Fig. 1C, P=0.005).

To better investigate clinicopathological involvement of ANO6 in gastric cancer, patients were separated into two groups according to the median IHC score as described in the Method. Chi-square test demonstrated that ANO6 was positively correlated with lesion invasion depth (Table 1, P=0.013). Similarly, patients within the high-ANO6 group were more prevalent with TNM stage III (43/63, 68.3%), while
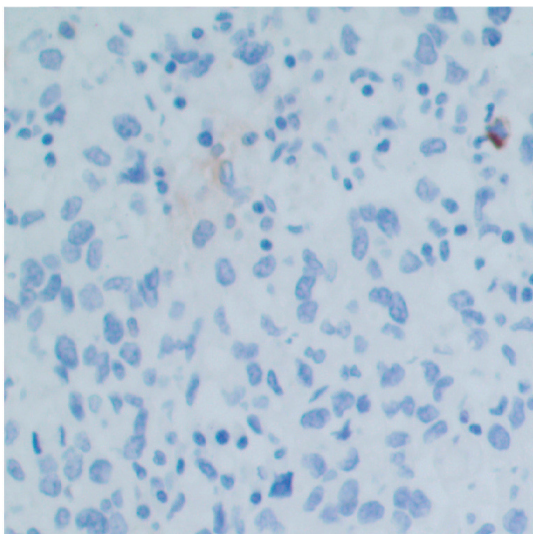
A High ANO6 in gastric cancer tissue
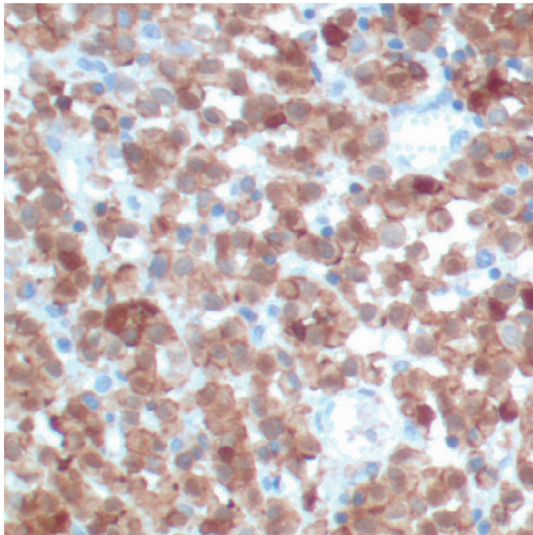
High ANO6 in metastatic lymph node
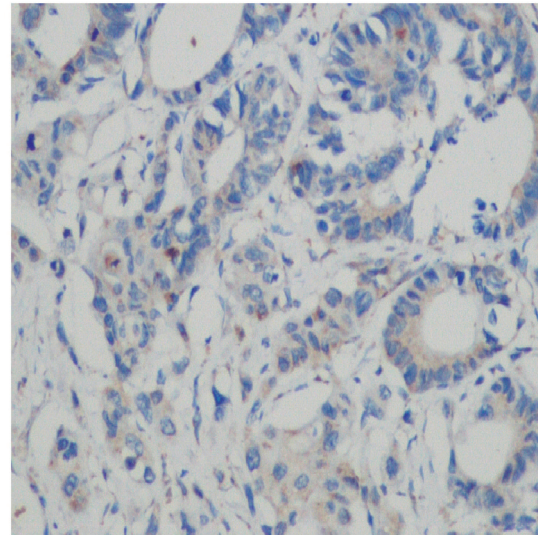
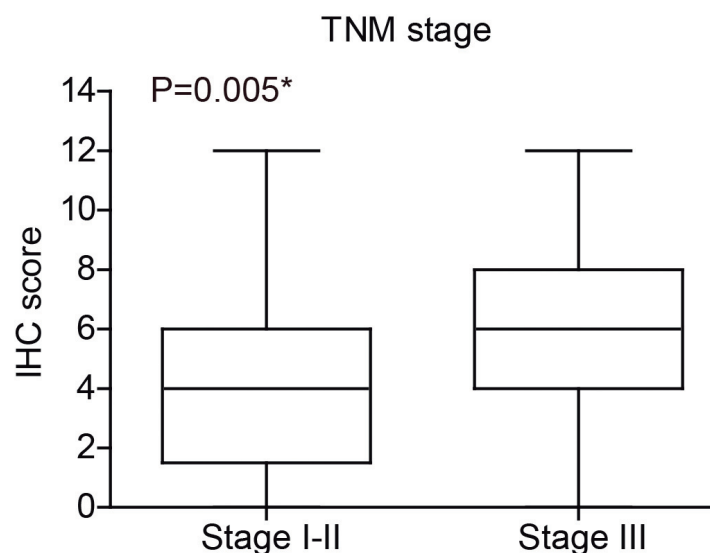

B Low ANO6 in gastric cancer tissue

C

Fig. 1. Protein expression of ANO6 in gastric cancer tissues. **A.** IHC images showed representative high ANO6 protein expression in gastric cancer samples and positive expression in metastatic lymph nodes from the same patient. **B.** Representative low ANO6 protein expression in gastric cancer samples by IHC staining. **C.** ANO6 showed higher immunoreactivity in tumor tissues with TNM stage III than that in tumor tissues with TNM stage I-II. Data were compared by unpaired Student's t-test. A, B, x 400.

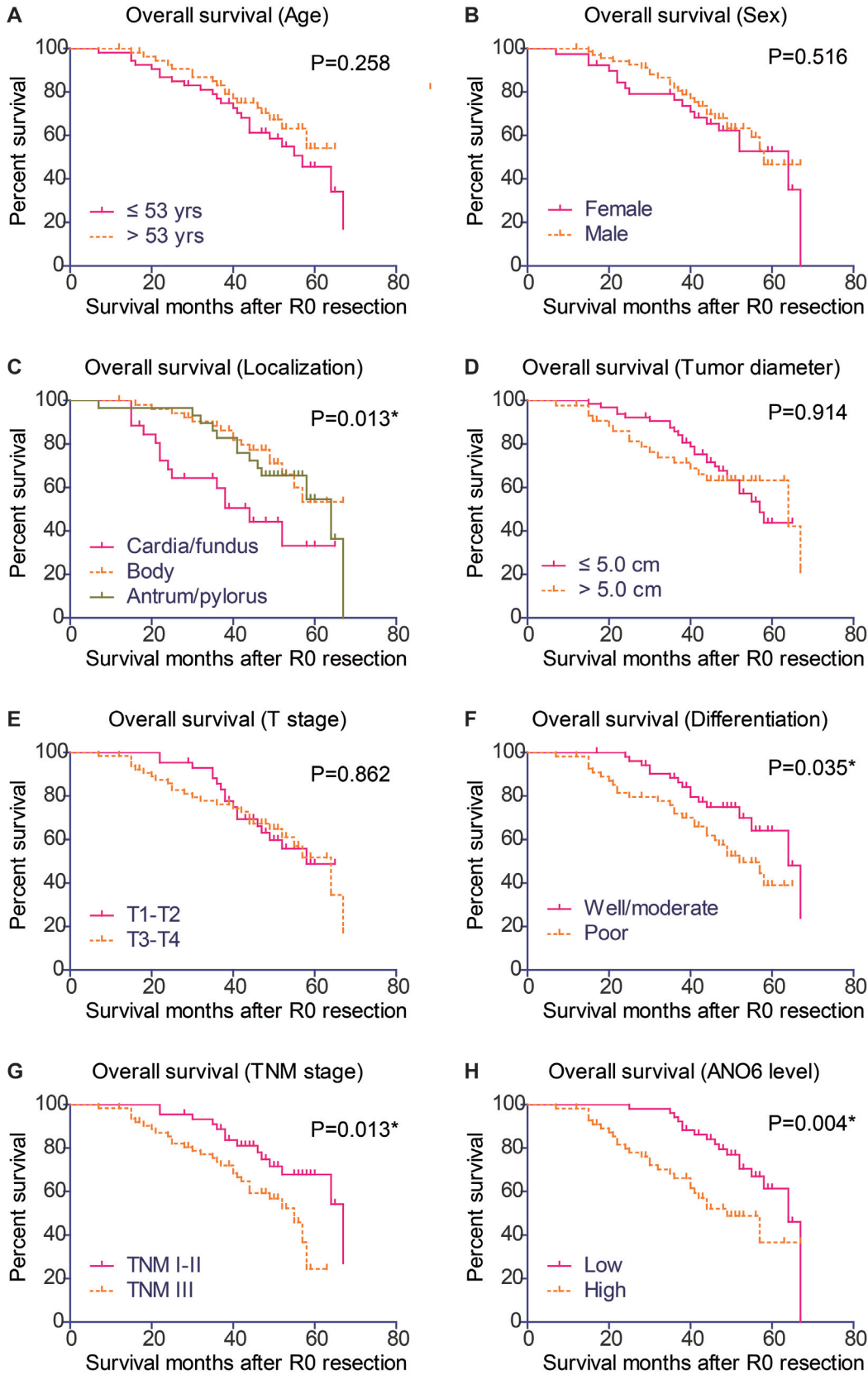*ANO6 promotes gastric cancer proliferation*

Fig. 2. Overall survival of gastric adenocarcinoma patients by Kaplan-Meier test. The overall survival curves were plotted according to different clinicopathological characteristics, including age **(A)**, sex **(B)**, tumor localization **(C)**, tumor size **(D)**, T stage **(E)**, tumor differentiation **(F)**, TNM stage **(G)** and ANO6 expression level **(H)**. Data were compared by log-rank test. * indicates P<0.05.

those in the low-ANO6 group were more prevalent with TNM stage I-II (32/45, 71.1%). Therefore, it is high likely that ANO6 participates in the tumor progression of gastric adenocarcinoma. Nevertheless, our cohort did not reflect any significant correlation between ANO6 level with several well-known factors that related to gastric cancer pathogenesis, including H. pylori infection, TP53 mutation, HER2 status, and KI67 level (Table 2, all P>0.05).

Prognostic significance of ANO6 and clinical variables in gastric adenocarcinoma

The prognostic effects of all retrieved clinicopathological factors were analyzed by Kaplan-Meier methods (Fig. 2). Accordingly, patients with cardia or fundus tumor location exhibited poorer overall survival compared to those with gastric body or antrum location (P=0.013). Meanwhile, tumor differentiation and TNM stage were also significant factors affecting patients' overall survival (P=0.035 and P=0.013, respectively). Of note, we found that high-ANO6 expression patients showed shorter overall survival time than those with low-ANO6 expression (46.8±2.9 vs 58.5±1.8 months). Consistently, the 5-year overall survival rate was significantly lower in high-ANO6 group compared with that in low-ANO6 group (36.6% vs 61.5%, P=0.004, Table 3).

We next conducted multivariate analysis using the Cox regression model, whose covariates included the location, differentiation, TNM stage, and ANO6 expression level (Table 4). As a result, ANO6 was verified as an independent unfavorable prognostic factor of gastric cancer patients (HR=1.998, 95% CI 1.016-3.929, P=0.045). Advanced tumor TNM stage also exhibited independent prognostic value (HR=2.584, 95% CI 11.175-5.681, P=0.018). Besides, patients with cardia or fundus tumor location were characterized with poorer clinical outcomes (P<0.05, Table 4).

Since our data were based on the protein level of ANO6 with a limited case number from a single medical center, we further assessed its expression difference and prognostic significance based on RNAseq data from online database to avoid bias. We utilized Gene Expression Profiling Interactive Analysis (GEPIA) to show the differential expression of ANO6. GEPIA data revealed that ANO6 mRNA was higher in gastric cancer tissues (n=408) compared with that in normal stomach (n=211) tissues (P<0.001, Fig. 3A). The survival analyses were obtained from the Kaplan–Meier Plotter (https://www.kmplot.com/) online tool based on the mRNA level of ANO6. Accordingly, significantly shorter OS and RFS were observed in patients with high ANO6 when compared to those with low ANO6 level (Fig. 3B,C), which was consistent with our findings based on its protein level.

Expression and function of ANO6 in gastric cancer cells

Additionally, we explored the expression profile of ANO6 in several gastric adenocarcinoma cell lines as well as in nontumorous GES-1 cells. Immunoblotting data demonstrated that ANO6 was remarkably upregulated in all three gastric adenocarcinoma cell lines compared to that in GES-1 cells (Fig. 4A,B; P=0.002).

Knockdown experiments were then conducted with specific siRNAs targeting ANO6 or scrambled siRNAs in both MKN28 and AGS-1 cells. The effects of ANO6-depeletion on tumor progression were tested with CCK-8 assays. As expected, silencing ANO6 significantly

Table 3. Kaplan-Meier univariate analyses of overall survival (OS).

Characteristics	Cases (n=108)	OS months (Mean ± S.D.)	5-year OS (%)	P value
Age				
≤53 yrs	53	50.5 ± 2.7	45.7%	0.258
>53 yrs	55	53.8 ± 2.2	54.2%	
Sex				
Female	39	50.6 ± 3.3	52.7%	0.516
Male	69	53.7 ± 2.1	46.7%	
Localization				
Cardia/fundus	26	40.3 ± 4.1	33.2%	0.013*
Body	53	56.0 ± 2.2	53.4%	
Antrum/pylorus	29	54.7 ± 3.1	54.6%	
Tumor diameter				
≤5.0 cm	64	52.7 ± 1.9	43.8%	0.914
>5.0 cm	44	51.7 ± 3.2	63.3%	
T stage				
T1/T2	43	53.1 ± 2.2	48.8%	0.862
T3/T4	65	51.6 ± 2.5	51.8%	
Differentiation				
Well/moderate	53	57.0 ± 2.2	64.1%	0.035*
Poor	55	48.0 ± 2.5	39.0%	
TNM stage				
Stage I-II	45	57.8 ± 2.3	67.9%	0.013*
Stage III	63	46.8 ± 2.2	24.5%	
ANO6 protein level				
Low	52	58.5 ± 1.8	61.5%	0.004*
High	56	46.8 ± 2.9	36.6%	

Table 4. Multivariate analysis.

Clinicopathologic variables	Hazard ratio	95% CI	P value
Localization			
Cardia/fundus	Reference		
Body	0.244	0.109-0.549	0.001*
Antrum/pylorus	0.311	0.137-0.702	0.005*
Differentiation			
Well/moderate	Reference		
Poor	1.495	0.780-2.865	0.226
TNM stage			
Stage I-II	Reference		
Stage III	2.584	1.175-5.681	0.018*
ANO6 protein level			
Low	Reference		
High	1.998	1.016-3.929	0.045*

attenuated the proliferation capacities of both cell lines (Fig. 4C,D). In contrast, overexpressing ANO6 resulted in enhanced tumor proliferation process (Fig. 4E,F), indicating that ANO6 promotes gastric cancer progression at least partially by accelerating tumor growth.

Discussion

The excitement regarding anoctamin proteins has been enhanced since the finding that ANO1 was linked to cancer. Overexpression of ANO1 is significantly
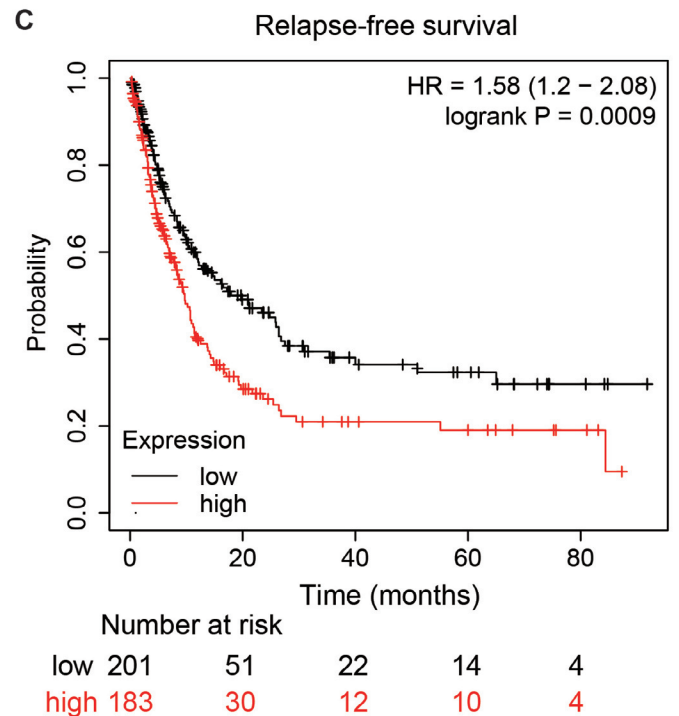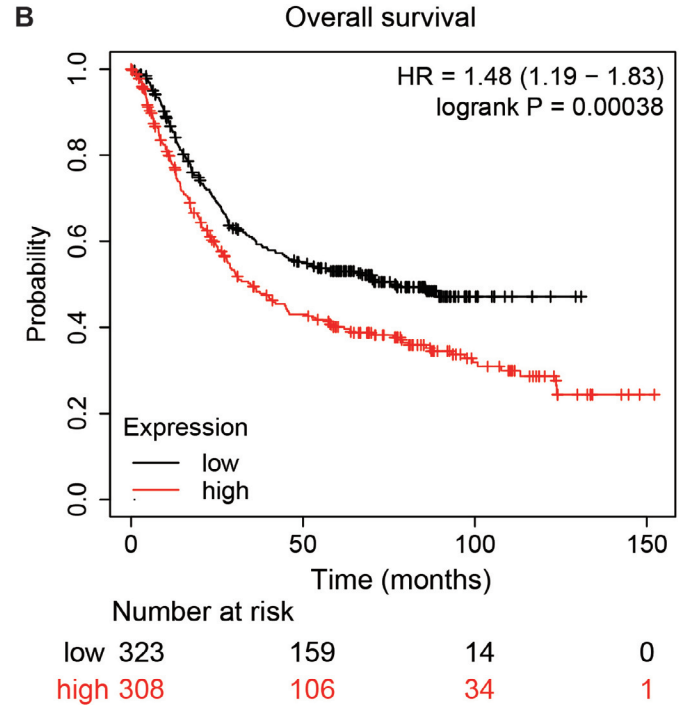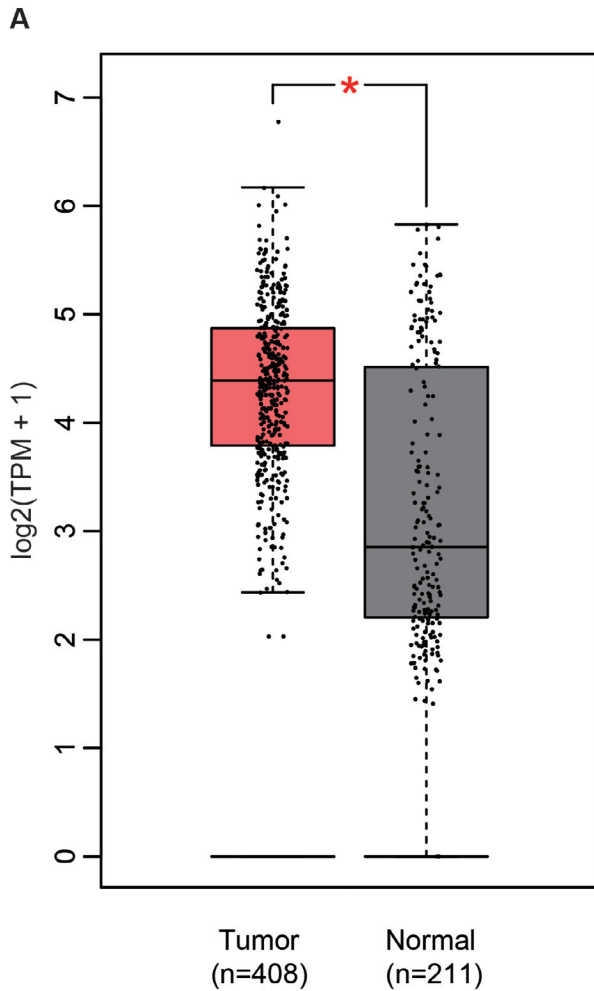

Fig. 3. mRNA level of ANO6 in gastric cancer tissues and its prognostic significance. **A.** Analysis of ANO6 mRNA expression level in human gastric cancer by Gene Expression Profiling Interactive Analysis (GEPIA) website. The y-axis represents transcripts per million (TPM) and is described as log2(TPM+1). Each dot represents a tumor tissue sample (left) or nontumorous stomach tissue (right). **B.** Kaplan–Meier survival statistics analysis for the relationship between overall survival time and ANO6 signature in gastric cancer was performed by using the online tool (https://kmplot.com/analysis/) according to the RNAseq data. Patients were divided into low-ANO6 and high-ANO6 groups based on the median mRNA level. **C.** Kaplan–Meier survival statistics analysis for the relationship between relapse-free survival time and ANO6 signature in gastric cancer was performed by using the online tool (https://kmplot.com/analysis/) according to the RNAseq data. Patients were divided into low-ANO6 and high-ANO6 groups based on the median mRNA level.
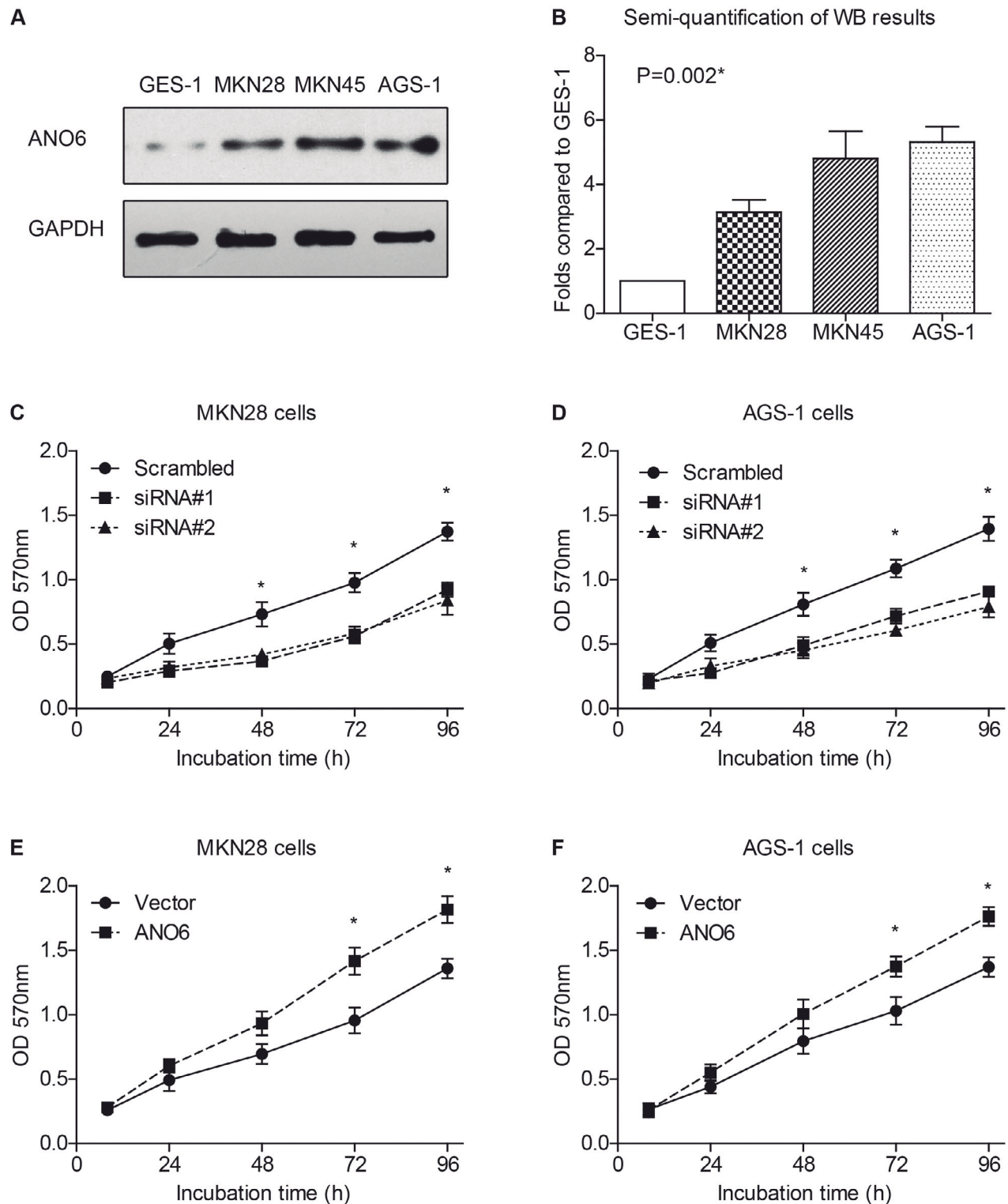
Fig. 4. Expression and function of ANO6 in gastric cancer cell lines. **A.** Representative Western blotting data showed the different endogenous protein levels of ANO6 in nontumorous GES-1 cell line and three gastric adenocarcinoma cell lines (MKN28, MKN45, AGS-1). **B.** Semi-quantification of the immunoblotting results in Fig. 4A. **C, D.** Silencing ANO6 by specific siRNAs resulted in decreased cell proliferation capacities compared to those transfected with nonspecific scrambled siRNA. **E, F.** Cells transfected with pcDNA3.1-ANO6 plasmids showed better proliferation capacities compared to those transfected with pcDNA3.1-vector plasmids. Data were summarized from three independent repeats and compared by One-way ANOVA test.

correlated with poor prognosis of breast cancer, gastric cancer, colorectal cancer, head and neck squamous cell carcinoma, and oral cancer etc. (Huang et al., 2002; Carles et al., 2006; Kulkarni et al., 2017; Park et al., 2019; Zeng et al., 2019). Besides its protein expression level, activation of ANO1 can modulate cell shrinkage at the rear end of migrating cells, thereby facilitating cell movement and metastasis (Ruiz et al., 2012). Although the tumor-related roles of other anoctamins were not well-recognized, they were indeed reported to play completely distinct roles in different tumors. For example, ANO7 was detected in prostate cancer (Bera et al., 2004), which was highly concentrated at cell-cell contact regions, implying its involvement in tumor metastasis and potency as a novel therapeutic target (Das et al., 2007). In contrast, ANO5 exhibited anti-tumor effects in both prostate cancer and thyroid cancer (Chang et al., 2017; Yu et al., 2020), highlighting the complicated involvement of anoctamins in malignancies.

As a transmembrane channel protein, ANO6 is widely expressed in human tissues and cell types such as platelets (Yang et al., 2012) and renal podocytes (Ousingsawat et al., 2018). Dysregulated ANO6 expression or ANO6 mutation had been reported to be correlated with various diseases including Scott syndrome (Millington-Burgess and Harper, 2020), bone dysplasia (Ehlen et al., 2013), as well as cancer (Xuan et al., 2019). However, the only reported ANO6-related malignancy was glioblastoma, while its role in other tumor types remains to be identified. Here we collected 108 gastric adenocarcinoma tissues and tested protein expression pattern of ANO6 using IHC strategy. The increased immunoreactivities in tumors with advanced stages implied that ANO6 may be correlated with gastric cancer progression. Furthermore, TCGA database search demonstrated a significantly higher ANO6-mRNA level in gastric cancer tissues than normal stomach tissues. The clinical relevance encouraged us to further investigate its prognostic significance. As a result, higher ANO6 protein expression was positively correlated with an unfavorable overall survival of gastric patients after curative surgical treatment. Moreover, multivariate Cox analysis verified that ANO6 can serve as an independent prognostic biomarker for gastric cancer patients. Consistent with the clinical significance of its protein level, higher mRNA level of ANO6 also indicated poorer overall survival and relapse-free survival of gastric cancer patients according to the data retrieved from TCGA database. Besides ANO6 expression level, our cohort also identified tumor location and TNM stage as independent prognostic factors. Briefly, cardia tumor location or advanced TNM stages were both hazard factors for the clinical outcomes of gastric adenocarcinoma patients. Tumor differentiation grade exhibited no independent significance although it was correlated with an unfavorable survival by univariate analysis.

Besides the clinical samples, we also assessed the expression of ANO6 in gastric cancer cell lines for the first time. According to the Western blotting data, ANO6 exhibited detectable protein levels in both gastric cancer cells and nontumorous gastric epithelial (GES-1) cells. However, ANO6 showed significantly higher immunoreactivities in gastric cancer cell lines than in GES-1 cells. Since cellular expression data was consistent with the clinical obtained tissue samples, we next selected two gastric cancer cell lines for knockdown assay and overexpression assay. Knockdown of ANO6 by siRNAs resulted in a significant inhibition of the cell proliferation process as revealed by CCK-8 assay. In contrast, transient transfection of MKN28 and AGS-1 cells with pcDNA3.1-ANO6 plasmids led to enhanced cell proliferation capacities. Therefore, our data demonstrated that ANO6 may participate in gastric cancer progression by promoting tumor growth.

Our study has several limitations. Firstly, all the enrolled cases were obtained from a single medical center and may result in regional or racial bias. We tried to make our major conclusion more convincing by retrieving the mRNA level of ANO6 in TCGA database. Secondly, this study mainly focused on exploring the clinical significance of ANO6 in gastric cancer, therefore we did not fully dig into its oncogenic signaling mechanisms. Instead, we validated our clinical results by assessing the effects of ANO6 on cell proliferation in two gastric cancer cell lines. More *in vitro* and *in vivo* assays will be necessary to further illustrate the detailed mechanisms of ANO6 in tumorigenesis and tumor progression.

Conclusion

Here we demonstrated that ANO6 is upregulated in gastric cancer cells, and its high expression is associated with poor prognosis based on both TCGA database and our retrospective cohort. Especially, it can serve as an independent factor of the prognosis of gastric adenocarcinoma according to univariate and multivariate analyses. Furthermore, we have revealed for the first time the role of ANO6 in enhancing proliferation of gastric cancer cells. Taken together, our data described a novel and promising biomarker for prognosis of gastric cancer.

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