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Basic Study

Anoctamin 5 regulates the cell cycle and affects prognosis in gastric cancer

Fukami T *et al*. ANO5 regulates cell cycle in GC.

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Abstract

BACKGROUND

Anoctamin 5 (ANO5)/transmembrane protein 16E (TMEM16E) belongs to the ANO/TMEM16 family of anion channels. ANOs comprise a family of plasma membrane proteins that mediate ion transport, phospholipid scrambling, and the regulation of other membrane proteins in numerous cell types. Previous studies have elucidated the roles of and the mechanisms by which ANO5 is activated in various cancer types. However, it currently remains unclear whether ANO5 acts as a plasma membrane chloride channel, and its expression and functions in gastric cancer (GC) have not been investigated.

AIM

The aim of the present study was to examine the role of ANO5 in the regulation of tumor progression and the clinicopathological significance of its expression in GC.

METHODS

Knockdown experiments using ANO5 small interfering RNA (siRNA) were conducted in human GC cell lines, and changes in cell proliferation, cell cycle progression, apoptosis, and cellular movement were assessed. The gene expression profiles of GC cells were investigated following ANO5 silencing using a microarray analysis. Immunohistochemical (IHC) staining of ANO5 was performed on 195 primary tumor samples obtained from patients with GC who underwent curative gastrectomy at our department.

RESULTS

Reverse transcription-quantitative PCR (RT-qPCR) and western blotting demonstrated high ANO5 expression in NUGC4 and MKN45 cells. In these cells, the ANO5 silencing inhibited cell proliferation and induced apoptosis. In addition, the knockdown of ANO5 inhibited G_1-S phase progression, as well as invasion and migration. The results

of the microarray analysis showed the up- or downregulation of several cyclin-associated genes, such as CDKN1A, CDK2/4/6, CCNE2, and E2F1, in ANO5-depleted NUGC4 cells. The expression of these genes was verified by RT-qPCR. IHC staining revealed that high ANO5 expression levels were associated with a poor prognosis. The multivariate analysis identified high expression of ANO5 as an independent prognostic factor for 5-year survival in GC patients (P = 0.0457).

CONCLUSION

The results of the current study indicate that ANO5 regulates the cell cycle progression by regulating the expression of cyclin-associated genes and affects the prognosis of patients with GC. These results may provide insights into the role of ANO5 as a key mediator in tumor progression of and/or promising prognostic biomarker for GC.

Key Words: Anoctamin 5; Gastric cancer; Cell cycle; G1/S checkpoint; Cell proliferation

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Core Tip: The aim of the present study was to investigate the role of Anoctamin 5 (ANO5) in the regulation of tumor progression and the clinicopathological significance of its expression in gastric cancer (GC). Immunohistochemical staining revealed that high ANO5 expression levels were associated with a poor prognosis. The results of the microarray analysis suggest that ANO5 regulates the cell cycle progression by regulating the expression of cyclin-associated genes. These results may provide insights into the role of ANO5 as a mediator of and/or biomarker for GC.

INTRODUCTION

The anoctamin (ANO)/transmembrane protein 16 (TMEM16) family is present in numerous eukaryotes, and ten ANO paralogs, ANO1-ANO10 (TMEM16A-H, TMEM16J and K), have been identified in vertebrates [1], several of which function as calcium-activated chloride channels (CaCC). ANOs comprise a family of plasma membrane proteins that mediate ion transport, phospholipid scrambling, and the regulation of other membrane proteins in numerous cell types [2-12]. Their expression has been detected in both epithelial and non-epithelial tissues types [7]. Although the regulation of ANOs has been extensively examined, the mechanisms through which increased intracellular calcium concentration activates chloride or cation conductance have not been elucidated.

Recent molecular and biochemical studies reported a role for ANOs in human carcinogenesis. For instance, the expression of ANO proteins is upregulated in cancer and associated with a poor patient prognosis [13]. A relationship has been demonstrated between ANO1 and patient prognosis in various cancer types, including gastric, esophageal, breast, lung, prostate, and head and neck cancer [14-21]. The upregulation of the genes encoding ANO1 and ANO3 has been associated with several cancer types, specifically gastrointestinal stromal tumors, breast cancer and squamous cell carcinoma [21,22]. Furthermore, ANO6 has been strongly implicated in the metastatic potential of breast cancer [23]. The expression levels of other members of the ANO family are also associated with cell proliferation and cancer development [24-26].

Our previous studies identified a crucial role for several chloride ion channels and transporters in patients with gastric cancer (GC); proliferation and the cell cycle progression were regulated by intracellular chloride [27,28], while G_0/G_1 arrest was induced by furosemide, a potent inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter [29]. Furthermore, leucine rich repeat containing 8 VRAC subunit A (LRRC8A) regulated the proliferation, apoptosis, migration, and invasion of GC cells [30]. Our previous work also demonstrated the clinicopathological and prognostic significance of the expression of chloride ion channels and transporters, such as ANO9, LRRC8A, CFTR, CLCN2, and CLIC1, in human esophageal squamous cell carcinoma [31-35].

ANO5 has recently been implicated in various cancers, such as thyroid cancer [36] and pancreatic cancer [37]; however, limited information is currently available on its involvement in tumor progression in patients with GC or the clinical significance of its expression. Therefore, the aim of the present study was to investigate whether ANO5 contributes to the regulation of cancer growth and attempted to clarify its clinicopathological significance in GC.

MATERIALS AND METHODS

Cell lines, antibodies

The MKN7, MKN45, MKN74, HGC27 and NUGC4 human GC cell lines were purchased from the Riken Cell Bank (Tsukuba, Japan). They were cultured in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) containing 100 μg/mL of streptomycin, 100 U/mL penicillin, and 10% FBS at 37°C in a 5% CO_2 incubator. A rabbit polyclonal anti-ANO5 antibody was obtained from Funakoshi (GTX81161) for use in immunohistochemistry (IHC) and Western blotting. A mouse monoclonal anti-β-actin (ACTB) antibody was provided by Sigma-Aldrich (St. Louis, USA) and HRP-conjugated anti-rabbit and mouse secondary antibodies by Cell Signaling Technology (Beverly, MA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from cancer cells using an RNeasy kit (Qiagen, Valencia, USA). The Step One plus Real-Time PCR System (Applied Biosystems, Foster city, CA) and TaqMan Gene Expression Assays (Applied Biosystems) were employed for RT-qPCR analysis using the following PCR thermocycling conditions: Initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The expression levels of the following genes were assessed: ANO5 (Hs01381106_m1), cyclin dependent kinase inhibitor 1A (CDKN1A; Hs00355782_m1), cyclin-dependent kinase (CDK) 2 (Hs00608082_m1), CDK4 (Hs00175935_m1), CDK6 (Hs00608037_m1), cyclin E2 (CCNE2; Hs00180319_m1), and E2F1 (Hs00153451_m1) (all from Applied Biosystems). The expression of each gene was normalized using the housekeeping gene ACTB (Hs01060665_g1; Applied Biosystems). All assays were conducted three times.

Western blotting

Cells were washed twice with ice-cold PBS and harvested in M-PER lysis buffer (Pierce, Rockford, USA,) supplemented with protease inhibitors (Pierce). Protein concentrations were measured using a modified Bradford assay (Bio-Rad, Hercules, USA). Cell lysates containing equal amounts of total protein (10 mg/Lane) were resolved using 10% SDS-PAGE gels, then transferred to PVDF membranes (GE Healthcare, Piscataway, USA). Membranes were incubated with antibodies for 24h at 4°C. Band densities were quantified using ImageJÒ (version 1.52; National Institutes of Health).

Small interfering RNA (siRNA) transfection

All siRNA reverse transfection procedures were performed using Lipofectamine® RNAiMAX reagent (Invitrogen, Carlsbad, USA) with a final siRNA concentration of 20 nmol/L, according to the manufacturer's instructions. ANO5 siRNA (Stealth RNAi siRNA; HSS137119, Stealth RNAi siRNA; HSS137120) and control siRNA (Stealth RNAiTM siRNA Negative Control) were obtained from Invitrogen.

Overexpression study

Control-HaloTag^{R} plasmid (Promega, G6591) and ANO5-HaloTag^{R} plasmid (pFN21AE5809) were transfected using P3000TM (Invitrogen) and lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. After passaging cells, ANO5-expressing cells were used for cell proliferation assay.

Cell proliferation assay

NUGC4 and MKN45 cells were seeded at densities of 1.0 and 2.0×10^{5} cells/well, respectively, on 6-well plates and incubated at 37°C in a 5% CO_2 incubator. siRNA was transfected 24 h after seeding. The cells were detached from the plates with trypsin-EDTA 48 and 72 h after siRNA transfection and counted using a hemocytometer.

The cell proliferation activity was measured using the water-soluble tetrazolium salts-8 assay with Cell Count Reagent SF (Nacalai Tesque). NUGC4, MKN45, and MKN7 cells were seeded at density of 1.0×10^{4}, 1.0×10^{4}, 1.5×10^{4} cells/well, respectively, on 24-well plates and incubated at 37°C in a 5% CO_2 incubator. siRNA was transfected

24 h after seeding. Cell proliferation was evaluated every 24 h by measuring the absorbance at 450 nm using Thermo Scientific Multiskan FC (Thermo Fisher Scientific).

Cell cycle assay

Cell cycle progression was assessed 48 h after siRNA transfection using flow cytometry. Trypsin-EDTA was used to detach the cells from the plates, then treated with 0.2% Triton X-100 and stained with propidium iodide (PI) with RNase staining buffer (BD Biosciences, San Jose, USA). Flow cytometry data were acquired from a BD Accuri C6 plus flow cytometer (BD Biosciences) assess the content of DNA in at least 10,000 cells.

Apoptosis assay

Cells were evaluated 72 h after transfection and stained using the ANNEXIN V-FITC Kit (Beckman Coulter, Brea, USA). Early and late apoptotic cell frequencies among at least 10,000 cells were assessed using a BD Accuri C6 plus flow cytometer.

Migration and invasion assays

Migration assays were performed using 24-well Cell Culture Inserts with 8-μm pores (BD Biosciences). Invasion assays were conducted using Biocoat Matrigel® (BD Biosciences). At 48 h post-transfection, NUGC4 and MKN45 cells were seeded at a density of 3.0×10^5 cells/well in serum-free RPMI-1640 in the upper chamber, while the lower chamber contained RPMI-1640 with 10% FBS. Matrigel and cells remaining in the upper chamber after a 48-h incubation were removed. Diff-Quick staining reagents (Sysmex) were used to stain migrated or invaded cells, which were counted in four independent fields of view. The assays were conducted three times.

Microarray analysis

NUGC4 and MKN45 cells were transfected with control and ANO5 siRNA. At 48 h after siRNA transfection, total RNA extraction was performed using RNeasy kit. Cyanine 3 (Cy3)-labeled cRNA was prepared from 0.1 μg total RNA using the Low Input Quick Amp Labeling Kit (Agilent Technologies, USA), then subjected to RNeasy column purification (Qiagen). Dye incorporation and cRNA yields were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Subsequently, 0.6 μg

Cy3-labeled cRNA was fragmented in a 25-μl reaction volume containing 1×Agilent fragmentation buffer and 2×Agilent blocking agent at 60°C for 30 min. The 2×Agilent hybridization buffer (25μl) was then added and hybridization to SurePrint G3 Human GE 8×60K Microarray Ver3.0 (Agilent Technologies) was conducted at 65°C for 17 h in a rotating Agilent hybridization oven. Microarrays were then washed with GE Wash Buffer 1 (Agilent Technologies) at room temperature for 1 min followed by GE Wash buffer 2 (Agilent Technologies) at 37°C for 1 min.

Processing of microarray data

Slides were scanned using the Agilent SureScan Microarray Scanner (G2600D) with the one color scan setting for 8×60k array slides. The scanned images were analyzed with Feature Extraction Software (Agilent Technologies) using default parameters to obtain background-subtracted and spatially-detrended processed signal intensities. Microarray data were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood city, CA).

Patients and primary tissue samples

Histologically proven primary GC tumor samples were obtained from 195 consecutive patients who underwent curative gastrectomy between 2011 and 2013 at Kyoto Prefectural University of Medicine (Japan). For mRNA analysis, frozen tissue samples of normal stomach and tumor, respectively, were collected from surgical specimens and stored at -80°C. Written informed consent was obtained from all patients prior to their enrollment. Exclusion criteria were as follows: non-curative resection or preoperative chemotherapy. Tumor staging was conducted according to the International Union Against Cancer (UICC)/TNM Classification of Malignant Tumors (8th edition) [38]. The present study received approval from the Institutional Review Board of the Kyoto Prefectural University of Medicine (ERB-C-1195).

IHC

The Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, USA) was employed for IHC staining using the avidin-biotinylated peroxidase complex (ABC) method. After deparaffinization in xylene, sections were rehydrated in a graded series

of ethanol. Sections were then incubated in 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. Endogenous biotin, biotin receptors, and avidin binding sites were also blocked using an Avidin/Biotin Blocking Kit (Vector laboratories). Sections were incubated with the ANO5 antibody diluted 1:100 at 37°Cfor 1 h, then at 4°C overnight. The cells were visualized using the standard ABC method, with hematoxylin as the counterstain.

ANO5 expression levels in immunohistochemically stained samples were semi-quantitatively graded based on the staining intensity and proportion of cytoplasm in stained cancer cells. Staining intensity was scored as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). The proportion of stained tumor cells as a percentage of the stained area in the cancer area was scored from 0 to 1.0. IHC scores were calculated as the maximum multiplied product of intensity and proportion scores (0 to 3.0). The decision of IHC diagnosis based on the assessment of tumor ANO5 expression and other IHC parameters was performed by at least two physicians, including an experienced pathologist.

Quantification of intracellular chloride concentration and low chloride stimulation

MQAE reagent, a chloride-sensitive fluorescence probe (Dojindo Laboratories, Kumamoto, Japan) was employed to assess intracellular chloride concentrations. NUGC4 and MKN45 cells were seeded on 24-well plates at a density of 3.0×10^4 cells/well and then incubated in normal medium at 37°C with 5% CO_2. The medium was then replaced with standard and low chloride medium, in which MQAE was dissolved, and cells were incubated at 37°C in a CO_2 incubator for a further 12 h. Following washing with PBS five times, the fluorescence intensity of MQAE was evaluated under a fluorescence microscope (BZ-X800; Keyence, Osaka, Japan). Three fields of view were analyzed per sample at ×100 magnification. Quantification was performed using a BZ-X800 analyzer and accompanying software (BZ-H4C, v.1.1.1.8; Keyence).

A low chloride stimulation experiment was conducted to examine the effects of changes in intracellular chloride concentrations on GC cells. Low-chloride medium

supplemented with 10% FBS was prepared in chloride-free RPMI-1640 (chloride was replaced with NO_3^-) (Nacalai Tesque).

JNK signaling pathway inhibitor treatment

To block JNK signaling pathway, the NUGC4 and MKN45 cells were incubated with JNK inhibitor SP600125 (10μm, ab120065, Abcam) according to manufacturer's instructions. The cells were divided into 3 groups: control group, ANO5 siRNA group, JNK inhibitor group (ANO5 siRNA + SP600125). The cell proliferation was detected every 24 h after ANO5 silencing.

Statistical analysis

Statistical analysis was performed using the Mann-whitney u test for two-group comparisons. Categorical data were analyzed using Fisher's exact test. The Kaplan–Meier method was used to construct survival curves, and differences in survival were examined using the log-rank test for equality. Prognostic factors were identified using a Cox proportional hazard model. These analyses were performed using the statistical software JMP (version 15; SAS Institute, Cary, NC, USA). Data are presented in the graphs as the mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Expression of ANO5 in GC cells

The expression of ANO5 was first examined in five human GC cell lines, MKN7, MKN45, MKN74, HGC27, and NUGC4, using RT-qPCR and western blotting. The expression of ANO5 in the 5 GC cell lines was detected in a number of cells (Figure 1A, B). Compared with paired adjacent normal tissue, ANO5 expression was significantly upregulated in GC tissue ($P = 0.004$; $n = 12$; Supplementary Figure 1).

ANO5 expression was knocked down using siRNA in NUGC4 and MKN45 cells, and the effects on tumor progression were assessed. ANO5 mRNA (Figure 1C) and protein levels (Figure 1D) were downregulated in NUGC4 and MKN45 cells. We also

conducted an overexpression study in MKN7 cell. ANO5 plasmid increased ANO5 mRNA levels (Supplementary Figure 2A, left panel).

ANO5 regulates cell growth and survival in GC cells

The effect of ANO5 siRNA transfection on the proliferation and cell cycle progression of NUGC4 and MKN45 cells was then examined. Compared with the control siRNA, the number of NUGC4 and MKN45 cells was significantly reduced at 48 and 72 h after the transfection of ANO5 siRNA (Figure 2A, left panel). The results of the cell proliferation assay showed that the relative absorbance of GC cells with the control siRNA (NUGC4 and MKN45) was significantly lower than that of GC cells with ANO5 siRNA (HSS137119) (NUGC4 and MKN45) (Figure 2A, right panel). Whereas, ANO5 plasmid increased the relative absorbance of MKN7 cell (Supplementary Figure 2B). Moreover, ANO5 silencing increased the numbers of NUGC4 and MKN45 cells in the G_0/G_1 phase (Figure 2B). These results indicated that ANO5 regulated the proliferation and cell cycle of GC cells.

To further clarify the role of ANO5, apoptosis assays were performed on NUGC4 and MKN45 cells. ANO5 silencing significantly increased the frequency of early and late apoptotic NUGC4 cells and the frequency of early apoptotic MKN45 cells 72 h after siRNA transfection (Figure 3A). These results indicate that the apoptosis of NUGC4 and MKN45 cells is regulated by ANO5 expression. Another ANO5 siRNA (HSS137120) was used to assess the impact on cell growth and survival, with similar results to HSS137119 (Supplementary Figure 3).

ANO5 promotes the migration and invasion of GC cells

The effects of the ANO5 silencing on NUGC4 and MKN45 cell migration and invasion were examined using a Boyden chamber assay. The results demonstrated that the knockdown of ANO5 in NUGC4 and MKN45 cells significantly reduced their migration and invasion (Figure 3B).

Gene expression profiling in ANO5 siRNA-transfected NUGC4 cells

To elucidate the molecular mechanisms underlying the regulation of cellular functions by ANO5, the gene expression profiles of NUGC4 cells transfected with

ANO5 siRNA were investigated using a microarray. The results obtained revealed that the expression levels of 3,491 genes in NUGC4 cells following ANO5 knockdown exhibited fold-changes > 1.8 compared with those in negative control. Among these, the pression levels of 1,802 genes were upregulated, whereas the levels of 1,689 genes were downregulated in NUGC4 cells following ANO5 knockdown. The top 20 genes with significant changes in expression in ANO5-depleted NUGC4 cells are listed in Table 1. IPA showed that 'Cancer' was the top-ranked disease and disorder, while 'DNA Replication, Recombination, and Repair' and 'Cell Cycle' were the two top-ranking molecular and cellular functions (Table 2).

Validation of gene and protein expression

The microarray analysis identified 'Cell Cycle: G1/S Checkpoint Regulation' as one of the top-ranking canonical pathways in ANO5-depleted NUGC4 cells (Figure 4A). To confirm these results, seven genes were selected (CDK2, CDK4, CDK6, CDKN1A/p21, CCNE2, E2F1, and Rb). These genes were included in 'Cell Cycle: G1/S Checkpoint Regulation', and CDK2 and CDK6 were the two top-ranking downregulated genes in NUGC4 cells following ANO5 knockdown (Table 1). RT-qPCR confirmed the expression levels of six genes. NUGC4 and MKN45 cells transfected with ANO5 siRNA had significantly lower CDK2, CDK4, CDK6, CDKN1A, CCNE2, and E2F1 expression levels and significantly higher CDKN1A expression levels than cells transfected with the control siRNA (Figure 4B). Further, ANO5 plasmid decreased CDKN1A/p21 mRNA levels (Supplementary Figure 2A, right panel). Western blotting revealed that phosphorylated Rb was inhibited following the knockdown of ANO5 in NUGC4 and MKN45 cells (Figure 4C). Since the CDK2 gene is located upstream of Rb and downstream of p21 in the cell cycle transition from G_1 phase to S phase, the downregulation of ANO5 GC cells appears to affect the transition from the G1 phase to the S phase by regulating the expression of p21 and its downstream genes in signal pathways.

In addition, the gene expression profile of MKN45 cells transfected with ANO5 siRNA were also investigated using a microarray. Gene expression changes in ANO5-

depleted NUGC4 and MKN45 cells are shown in Supplementary Figure 4. Among the 21,440 genes, 7,246 genes were upregulated and 6,622 genes were downregulated in both cell lines, for a total of 13,868 genes (64.7%) with identical expression direction in NUGC4 and MKN45 cells. The direction of gene expression changes of gene related to "Cell Cycle: G_1/S Checkpoint Regulation" was consistent in both cell lines (Supplementary Table 1). Furthermore, all 40 genes displayed in Table 1 showed the identical expression direction in ANO5-depleted MKN45 cells (Supplementary Table 2). These results support that ANO5 affects the cell cycle by similar mechanisms in both NUGC4 and MKN45 cell lines.

ANO5 inhibits the JNK pathway

The activation of the JNK and p38 MAPK classes of protein kinases mediates cellular responses, such as apoptosis and the maturation of some cell types. JNK has been shown to stabilize the p21 protein through phosphorylation [39]. To elucidate the regulatory role of ANO5 on JNK signaling pathway in GC cells, the phosphorylation of JNK protein was examined. ANO5 silencing significantly increased JNK phosphorylation levels in NUGC4 and MKN45 cells (Figure 4D). Furthermore, the increase of CDKN1A/p21 mRNA expression induced by ANO5 silencing in NUGC4 and MKN45 cells was suppressed by JNK inhibition (Supplementary Figure 5, lower panel). Whereas, treatment with JNK inhibitors made no difference in ANO5 mRNA expression (Supplementary Figure 5, upper panel). These results indicate that ANO5 expression regulates the cell cycle *via* the upregulation of p21 through the activation of JNK cascades in GC cells.

Effects of low-chloride conditions

To elucidate the molecular mechanisms by which ANO5 affects the cell cycle transition from G_1 phase to S phase, changes in the intracellular ion environment were examined. Previous study reported that cancer growth was affected by intracellular chloride through its phosphorylation of several key molecules in signal transduction pathways [29]. We previously reported that the culture in the Cl-replaced medium (replacement of Cl^- by NO_3^-) decreased the intracellular chloride concentration ($[Cl^-]_i$)

and inhibited cell growth in GC cells [28]. Our previous study also demonstrated that JNK activation under low-chloride conditions inhibited the growth of GC cells by upregulating p21 expression [27]. Intracellular chloride concentrations in cells were measured based on the fluorescence intensity of MQAE, a chloride-sensitive fluorescence probe. The results obtained revealed increases in the fluorescence intensity of MQAE in NUGC4 and MKN45 cells following the knockdown of ANO5 (Figure 5). Therefore, the knockdown of ANO5 altered intracellular chloride concentrations in GC cells. Furthermore, low-chloride conditions effectively increased JNK phosphorylation and reduced Rb phosphorylation (Supplementary Figure 6). These results indicated that ANO5 regulated the cell cycle *via* JNK signaling by controlling intracellular chloride levels.

IHC of ANO5 expression in human GC tissue

IHC detected the expression of ANO5 in non-cancerous gastric (Figure 6A) and cancerous epithelia (Figure 6B). ANO5 was expressed in the cell membranes and cytoplasm of GC tissue. The criteria for staining intensity score were defined as 0 (no staining; Figure 6C), 1 (weak; Figure 6D), 2 (moderate; Figure 6E), or 3 (strong; Figure 6F). The median and mean scores for the expression of ANO5 were 0.9 (range, 0-2.1) and 0.97 (SD=0.53), respectively. A cut-off value of 1.3 was used to obtain the smallest P-value in comparisons of 5-year overall survival rates [41]. The 5-year OS rates with each cut-off value are shown in Table 3.

Patients with GC were divided into low- (ANO5 scores <1.3, n = 137) and high-ANO5 (ANO5 scores ≥1.3, n = 58) expression groups based on the cut-off value of 1.3 (Figure 6G). An analysis of clinicopathological features revealed that ANO5 expression levels were not associated with any of the variables (Table 4). To evaluate the prognostic significance of ANO5 after surgery, the following 10 variables were compared: Sex, age, tumor location, tumor length, histological type, lymphatic invasion, venous invasion, the pathological T stage, pathological N stage and ANO5 IHC scores. The univariate analysis showed that patient prognosis correlated with tumor length, lymphatic invasion, venous invasion, the pathological T stage, pathological N stage and

ANO5 IHC scores (P = 0.0020, 0.0002, 0.0126, <0.0001, <0.0001, and 0.0104, respectively). The multivariate analysis identified high ANO5 expression (≥1.3) as an independent prognostic factor (P = 0.0457) (Table 5). Furthermore, the 5-year OS rate was significantly lower in the high-expression group (73.9%) than in the low-expression group (89.6%). Data obtained from the Kaplan-Meier plotter database also revealed a relationship between high ANO5 expression and poor prognosis in GC (Supplementary figure 7), that was consistent with the present results.

DISCUSSION

The ANO family of membrane proteins, also known as TMEM16, has been reported to play key roles in several physiological functions ranging from ion transport to phospholipid scrambling [40] and ion channel regulation [42]. While the roles of ANO1 (TMEM16A) and ANO2 (TMEM16B) as CaCC have been firmly established [43-46], the functions of other family members remain unclear.

Previous studies evaluated the expression and role of ANO5 during tumor development in various cancer types. Song *et al* [47] demonstrated that ANO5 (TMEM16E) was widely expressed in the epithelial cells of the human gastrointestinal tract. ANO5 is also expressed in human pancreatic cancer tissues, but not in normal pancreatic tissue [37]. Chang *et al* [36] reported that the expression of ANO5 was downregulated in thyroid cancer, which promoted thyroid cancer cell migration and invasion. However, the expression of ANO5 in human GC tissue and the pathophysiological role of its expression in GC cells have not been demonstrated.

The present study revealed that the downregulation of ANO5 in GC cells regulated the cell cycle and induced apoptosis, whilst inhibiting proliferation, migration and invasion. These results highlight the potential of inhibitors of ANO5 as therapeutic agents for the treatment of GC or other cancer types with high ANO5 expression levels. The present study also indicated that ANO5 played a key role in the proliferation of GC cells. Cell cycle analysis showed that the population of cells remaining in the G_0/G_1 phase was significantly increased and that the number of cells in the S or G_2/M phase

decreased in ANO5-depleted NUGC4 and MKN45 cells, suggesting that the downregulation of ANO5 inhibits the proliferation of GC cells *via* cell cycle arrest at the G_0/G_1 phase.

The induction of p21 is dependent on the tumor suppressor protein, p53. However, chloride ions have been shown to play important roles in cell-cycle progression by regulating the expression of p21 through a p53-independent pathway in GC cells [28]. It was also demonstrated that a decrease in chloride induced G_0/G_1 phase arrest by downregulating the expression of CDK2 and phosphorylated Rb through the upregulation of p21. Furthermore, the activation of p38 and JNK under low-chloride conditions inhibited the viability of GC cells *via* the up-regulated expression of p21 [27].

Moreover, ANO5 expression in GC cells acted on the transition from the G_1 to the S phase of the cell cycle by regulating the expression of p21 and its downstream genes through the activation of the JNK signaling. To the best of our knowledge, the chloride channel activity of ANO5 has not been confirmed. To elucidate the molecular mechanisms underlying the effects of ANO5, the present study evaluated changes in the intracellular chloride ion environment. A quantitative analysis of intracellular chloride ion concentrations was conducted based on the fluorescence intensity of MQAE. The results of the immunofluorescent analysis showed that the fluorescence intensity of MQAE increased following ANO5 silencing, indicating a decrease in intracellular chloride concentration. These results suggest that the downregulation of ANO5 induces G_0/G_1 phase arrest by altering the expression of G_1/S checkpoint-related genes through the intracellular chloride environment of GC cells. Furthermore, since ANO5 functioned as a chloride channel, it may have the potential to inhibit tumor growth by regulating intracellular chloride concentrations in a therapeutic setting.

Although ANO5 was recently implicated in various cancers, its role in tumor progression in patients with GC has not yet been clarified. To demonstrate the clinical significance of the expression of ANO5, the survival rate of 195 patients who underwent curative resection for primary GC was investigated. IHC showed that high ANO5 expression levels were a poor prognostic factor in patients with GC. Under low-chloride

conditions, ANO5 appeared to function as a chloride channel in GC cells. Previous findings showed that various ion transporters function as biomarkers and therapeutic targets [48, 49]. The targeting of ion channels that are activated in cancer cells may be an important strategy for cancer therapy. To our knowledge, the present study is the first to report a relationship between ANO5 expression levels and the prognosis of patients with GC. Additional functional studies will provide insights into the role of ANO5 in the progression of GC.

There are a number of limitations that need to be addressed. Firstly, this was a retrospective study. Due to the limited sample size, pN classification factors were not correlate with the 5-year OS rate. Therefore, larger studies are needed to confirm the present results. Secondary, in the selection of gastric cancer cell lines, we selected five cell lines, but only three of the them were used in the study.

CONCLUSION

In conclusion, the present study revealed that ANO5 plays a significant role in cell cycle progression in human GC cells. The results of the microarray analysis showed the impact of ANO5 on the expression of G_1/S checkpoint-related genes. Furthermore, the expression of ANO5 exerted significant effects on the JNK signaling. Collectively, these results indicate that ANO5 plays an important role in cell cycle progression by regulating the expression of p21 through JNK signaling in human GC cells. The results of the IHC analysis also suggest that high ANO5 expression levels are a poor prognostic factor in patients with GC. The present study may contribute to the identification of ANO5 as a key mediator in tumor progression, with it ultimately being a promising prognostic biomarker or a novel therapeutic target of GC.

ARTICLE HIGHLIGHTS

Research background

Anoctamin 5 (ANO5) is a family of calcium-activated chloride channels (CaCCs) containing 10 (ANO1-10) members, also known as transmembrane proteins, and has been reported to be associated with various cancers.

Research motivation

The role of ANO5 in gastric cancer (GC) remains poorly understood. In the present study, we analyzed the relationship between ANO5 expression and tumor progression in GC.

Research objectives

The objectives of the present study was to investigate whether ANO5 contributes to the regulation of cancer growth and attempted to clarify its clinicopathological significance in GC.

Research methods

Knockdown (KD) experiments were performed by transfecting human GC cell lines with ANO5 siRNA. Gene expression was then assessed using microarray analysis. Samples from 195 patients with GC were subjected to immunohistochemistry (IHC) for ANO5, and its relationship with clinicopathological factors and prognosis were examined.

Research results

ANO5-KD suppressed the proliferation, migration and invasion of cells and enhanced apoptosis. Cell cycle analysis showed that ANO5-KD suppressed the progression of G1-S phase. The results of microarray analysis showed the up- or down- regulated expression of genes related to "Cell Cycle: G1/S Checkpoint Regulation" in ANO5-KD NUGC4 cells. Survival analysis showed significantly poorer 5-year survival in the ANO5 high expression group (high *vs* low; 73.9 *vs* 89.6%, $P = 0.0104$). IHC multivariate

analysis identified the high expression of ANO5 as an independent prognostic factor for 5-year survival in GC patients (P = 0.0457).

Research conclusions

ANO5 plays a significant role in cell cycle progression in human GC cells. The results of the IHC analysis suggest that high ANO5 expression levels are a poor prognostic factor in patients with GC.

Research perspectives

The present study may contribute to the identification of ANO5 as a key mediator in tumor progression, with it ultimately being a promising prognostic biomarker or a novel therapeutic target of GC.

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