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Over-expression of Anterior Gradient 3 Is Associated With Tumor Progression and Poor Survival in Gastric Cancer

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Abstract. *Background/Aim: Anterior gradient (AGR) proteins, including AGR1, AGR2, and AGR3, which are members of the protein disulfide isomerase family, have been reported as biomarkers for various carcinogenesis processes. Although AGR2 and AGR1 have been demonstrated to be associated with gastric cancer (GC) progression and poor survival, the effect of AGR3 on the progression and prognosis of GC remains unknown. Therefore, our study aimed to examine the expression and prognostic significance of AGR3 in patients with GC. Patients and Methods: We investigated 271 GC patients receiving curative surgery. Formalin-fixed and paraffin-embedded tissue blocks were obtained, and long-term survival analysis was performed. The expression of AGR3 in GC tissues was investigated by quantitative reverse transcription-polymerase chain reaction, western blotting, and immunohistochemistry. Results: AGR3 was over-expressed in GC tissue compared with paired normal tissue at the mRNA and protein levels. AGR3 over-expression was significantly associated with larger tumor size, deeper tumor invasion, lymph node metastasis, and advanced tumor stage. The overall survival of patients with positive AGR3 expression was significantly lower than that of patients without positive AGR3 expression. Multivariate analysis demonstrated that AGR3 and age were independent prognostic factors associated with overall survival. Conclusion: Over-expression of AGR3 was significantly associated with tumor progression and poor survival of GC patients. Therefore, AGR3 may be a novel biomarker and prognostic factor for GC.*

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Gastric cancer (GC) is one of the most prevalent cancer types and the leading cause of cancer-related mortality worldwide, especially in East Asia. Despite recent advances in therapeutic modalities, patients with advanced GC experience rapid disease progression with high morbidity, mortality, and poor survival (1-3). Early diagnosis is crucial to improve the prognosis of GC. Therefore, the search for reliable and practical molecular biomarkers for the early detection and prediction of cancer progression in GC has attracted much attention among clinicians.

The protein disulfide isomerase (PDI) family includes thiol oxidoreductase, which mediates the formation, folding, and structural maturation of several substrate proteins in the endoplasmic reticulum (ER) (4-7). Its expression is elevated in a variety of cancers, which is closely related to the invasion and oncogenic phenotypes of tumor cells, and plays a significant role in the progression and prognosis of cancers (8-13).

Anterior gradient (AGR) proteins are members of the PDI family involved in secretory and transmembrane proteostasis in the ER (14-16). AGR proteins are composed of three PDI-like proteins including AGR1, AGR2, and AGR3 (14-16). Several studies have suggested that AGR proteins exhibit various cellular functions (*e.g.*, cell migration, differentiation, and proliferation) and pro-oncogenic functions in different types of cancers (14-16).

AGR2 is the most described PDI family member and is significantly over-expressed in ovary, breast, cervix, prostate, and gastric cancers (17-21). In addition, over-expression of AGR2 is associated with tumor initiation, progression, and metastasis, as well as poor survival, acting as a pro-oncogenic protein (22-26).

AGR1 is the founding gene of AGR proteins and exhibits oxidase and isomerase activities. AGR1 has been reported to promote the proliferation, migration, and invasion of GC cells (27) and the epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma cells (28).

AGR3 is also an ER-resident protein involved in the formation of disulfide bonds and a highly related homolog of pro-oncogenic AGR2 (14-16). In comparison with AGR2 and AGR1, less is known about the role of AGR3 in cancer. Previously, AGR3 was found to be over-expressed in human

cancers of the breast, ovary, prostate, and liver (29-32). In addition, over-expression of AGR3 was associated with patient survival in breast and ovarian cancers (29, 30). However, some studies reported conflicting results. Thus far, there is a lack of data on the role of AGR3 in the progression and prognosis of GC.

The aim of this current study was to examine the expression and prognostic significance of AGR3 in a well-defined series of human GCs with a focus on long-term patient survival.

Patients and Methods

Patients and tumor specimens. From January 2009 to December 2009, 271 patients (173 males, 98 females) who underwent GC surgery at Chonnam National University Hwasun Hospital were retrospectively selected. Patients who received preoperative chemotherapy or irradiation prior to surgery were excluded from this study. The histologic grade was classified according to the criteria of Lauren and the World Health Organization (33, 34). The status of the tumors was determined by TNM staging using the American Joint Committee on Cancer (AJCC) system (35). Overall survival (months) was determined from the time of the first surgery to the follow-up on December 31, 2020. The median age was 59.3±11.0 years [mean±standard deviation (SD)] and ranged from 25.0 to 83.0 years. The mean size of the tumors was 3.9±2.8 cm (mean±SD) and ranged from 0.2 to 20.2 cm. To evaluate the RNA and protein expression levels of AGR3, 20 GC and paired normal gastric mucosa tissues from the same patient were collected by endoscopic biopsy at Chonnam National University Hwasun Hospital (Gwangju, Republic of Korea). To maintain freshness, biopsy tissue samples were frozen using liquid nitrogen and stored in a deep freezer until use. All samples were obtained with the consent of the patient, and this study was conducted with the approval of the Ethics Committee of Chonnam National University Hwasun Hospital (IRB No.CNUHH-2017-170).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA was isolated from the cancer tissue using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, New York, NY, USA). The cDNA was synthesized using 1 μg of RNA and reverse-transcribed with Moloney murine leukemia virus (MMLV) transcription reagents (Invitrogen, Carlsbad, CA, USA). The amplification of the *AGR3* gene was performed with specific primers and the Go Taq® DNA polymerase (Promega, Madison, WI, USA). The following primers were used: AGR3 5'-TCAGCTTTGGGTCTCTGCCTC-3'/5'-CAATA GGGGTAAATCCCGAG-3'; GAPDH 5'-ACCACAGTCCATGCC ATCAC-3'/5'-TCC ACC ACC CTG TTG CTG TA-3'. The following cycling conditions were used for PCR: denaturation at 95˚C for 10 min; 35 cycles at 95˚C for 10 s, 60˚C for 15 s, and 72˚C for 20 s. PCR products were separated in 1% agarose gel, and PCR bands were quantified using Multi-Gauge gel analysis software (ver 3.0; Fujifilm, Tokyo, Japan).

Western blotting. Proteins were extracted with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) and quantified by BCA protein assay (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein samples (10 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA, USA).

To block non-specific antigen binding, the membrane was treated with 5% bovine serum albumin (BSA) solution at room temperature for 30 min. Subsequently, the membrane was incubated overnight with polyclonal rabbit anti-human AGR3 antibody (1:1,000) against human AGR3 protein and polyclonal rabbit anti-human GAPDH antibody (1:1,000). Antibodies against AGR3 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The bands of AGR3 and GAPDH were detected using LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan) and an enhanced chemiluminescence detection system for HRP. Protein bands were quantified using the Multi-Gauge gel analysis software (ver 3.0; Fujifilm).

Immunohistochemistry. The 271 specimens from GC patients were formalin-fixed and paraffin-embedded. The specimens were sectioned (4 μm in thickness), and the sections were deparaffinized with xylene, rehydrated with graded alcohol solution, and retrieved with citrate buffer (pH 6.0; Dako, Carpentaria, CA, USA). To block endogenous peroxidase activity, the sections were treated with Dako REAL™ peroxidase blocking solution (Dako) and incubated at room temperature for 10 min. Then, the sections were incubated with 5% bovine serum albumin (BSA) solution for 30 min to block non-specific antigen binding and subsequently incubated overnight with polyclonal rabbit anti-human AGR3 antibody (1:100; Santa Cruz Biotechnology) against human AGR3 protein. After washing with washing buffer, the sections were visualized using the Dako Real™ Envision HRP/DAB detection system (Dako) and stained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA).

Evaluation of AGR3 expression. The immunoreactivity of stained tissue samples, including the intensity, area, and pattern of immunostaining, was examined independently by two observers without knowledge of the clinical outcome. In cases of discrepancy, an agreement was reached after further evaluation. The immunoreactive intensity was scored on a scale of four grades: no staining of cancer cells [0]; weak staining [1]; moderate staining [2]; strong staining [3]. The percentage of the immunoreactive area was also divided into four grades: none [1]; <10% [2]; 10-50% [3]; >50% [4]. The overall score was calculated as the product of immunoreactive intensity and immunoreactive area. Theoretically, overall scores could range from 0 to 12. The mean overall score for the 271 specimens was 4. Specimens with a score >4 were regarded as having a positive expression, and those with a score ≤4 were regarded as having a negative expression.

Statistical analysis. Student's *t*-test was performed to compare RNA and protein expression levels of AGR3 between GC and paired normal gastric mucosa tissues. The association of AGR3 expression with the various clinicopathological parameters of GC patients was analyzed using the χ^2 test and Fisher's exact test. Survival rates were estimated using the Kaplan–Meier method, and the statistical significance of differences was analyzed by using the log-rank test. Multivariate Cox proportional hazards regression analysis was performed to identify prognostic factors. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 20.0 software (IBM Corporation, Armonk, NY, USA). A *p*-value <0.05 was considered statistically significant.

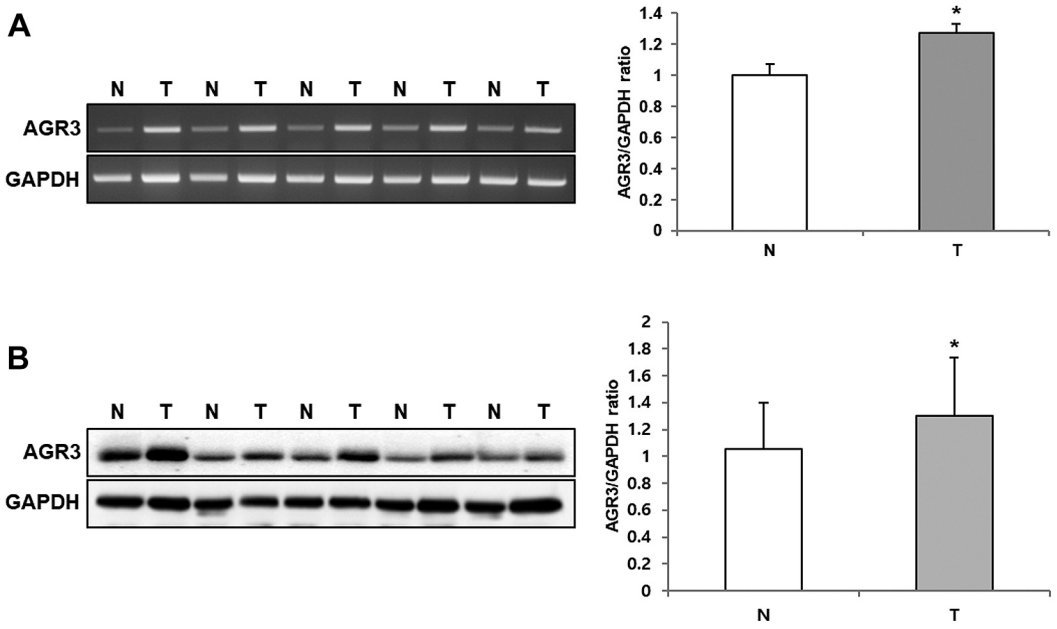

Figure 1. *AGR3 expression in gastric cancer tissue analyzed by (A) quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and (B) western blotting. AGR3 expression was significantly up-regulated in cancer tissues compared with paired normal tissues at the (A) mRNA level and (B) protein level. Each bar represents the mean±standard deviation of 20 cases. *p<0.05 vs. normal gastric mucosa tissue. N: Normal gastric mucosa tissue; T: gastric cancer tissue; AGR3: anterior gradient 3.*

Results

Up-regulation of AGR3 expression in GC tissue. We measured the expression of AGR3 at the mRNA and protein levels by qRT-PCR, western blotting, and immunohistochemistry in GC and paired normal gastric mucosa tissues from the same patients obtained by endoscopic biopsy. AGR3 expression was up-regulated in GC tissue compared with paired normal gastric mucosa tissue at the mRNA and protein levels (Figure 1A and B). Densitometric analyses of the mRNA and protein expression levels of AGR3 showed that they were significantly higher in GC tissue than in normal gastric mucosa tissue ($p<0.001$ and $p=0.032$, respectively) (Figure 1A and B). In the normal gastric epithelia, no or weak AGR3 immunoreactivity was observed (Figure 2A). In contrast, immunoreactivity for AGR3 protein was predominantly observed in the cytoplasm of tumor cells but not in the surrounding stroma. Immunoreactivity in GC cells was graded as follows: no, weak, moderate, and strong immunostaining (Figure 2B-E).

Association of AGR3 over-expression with tumor progression and poor prognosis in GC. To investigate the prognostic role of AGR3 in GC progression, we examined the expression of AGR3 protein in formalin-fixed and paraffin-embedded tissue blocks obtained from 271 GC patients based on clinicopathological data, including long-term survival. The correlation between the expression of AGR3 and clinicopathological parameters was analyzed. Based on the study criteria, the expression of AGR3 was detected in 129 of the 271 (47.6%) GC tissue samples (Table I). The positive expression of AGR3 was significantly associated with larger tumor size, deeper tumor invasion, lymph node metastasis, and advanced tumor stage ($p<0.001$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively) (Table I). Moreover, the overall survival of patients with positive AGR3 expression was significantly lower than that of patients with negative AGR3 expression ($p=0.004$) (Figure 3). Following Cox multivariate regression analysis, AGR3 and age were independent prognostic factors associated with overall survival when adjusted for several covariates, including age and sex with hazard ratios of 1.697 (95% CI=1.205-2.388; $p=0.002$) and 2.074 (95%CI=1.436-2.997; $p<0.001$), respectively (Table II).

Discussion

AGR proteins including AGR1, AGR2, and AGR3 are members of the PDI family involved in oxidative protein folding in the ER (14-16). AGR proteins play a critical role in embryonic development, tissue regeneration, and tumor development and progression (14-16).

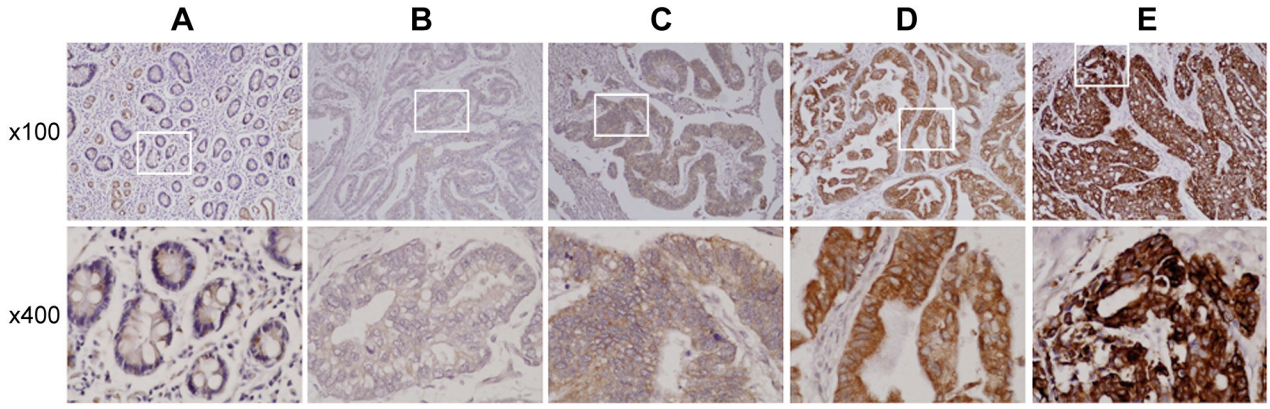

Figure 2. *Representative images of immunohistochemical staining of AGR3. (A) Non-stained or weakly stained AGR3 protein in the normal gastric mucosa. (B) A score of 0 indicates no immunostaining for AGR3. (C) A score of 1 indicates weak immunostaining for AGR3. (D) A score of 2 indicates moderate immunostaining for AGR3. (E) A score of 3 indicates strong immunostaining for AGR3. Original magnification, ×100, ×400. AGR3: Anterior gradient 3.*

Previously, AGR2 has been found to be over-expressed in a variety of cancers including ovary, breast, cervix, prostate, and gastric cancers (17-21). In addition, AGR1 is over-expressed in GC and hepatocellular carcinoma (27, 28). These findings suggest that AGR2 and AGR1 may be closely related to carcinogenesis.

AGR 3 is a highly related homolog of pro-oncogenic AGR2 and belongs to the PDI family (14-16). It is over-expressed in several cancers including breast, ovary, prostate, and liver cancers (19-23). However, thus far, there is a lack of data on the expression of AGR3 in GC. Therefore, we measured the expression of AGR3 in GC and paired normal gastric mucosa tissues by qRT-PCR, western blotting, and immunohistochemistry. Its expression was significantly higher in GC tissue than that in normal tissue at the mRNA and protein levels. These observations suggest that AGR3 may play a critical role in human GC development.

Particularly, the expression of AGR2 has a prognostic value in a variety of cancers including GC (14-26). AGR2 expression was observed to be significantly associated with tumor location and size, depth of invasion, stage, lymphatic metastasis, vessel invasion, distant metastasis, Lauren's classification, and poor prognosis among 436 GC patients. Furthermore, multivariate survival analysis demonstrated that AGR2 was an independent prognostic factor in GC (21). However, a study showed that the expression of AGR2 was not significantly different between GC and normal tissues based on western blot results and not associated with poor survival (36).

The expression level of AGR1 was previously reported to be higher in GC tissue than in non-tumor tissue, and AGR1 expression was correlated with tumor size, lymph node involvement, and poor clinical prognosis. Furthermore, in a GC cell line study, AGR1 over-expression promoted the growth, migration, and invasion of GC cells, whereas AGR1 knockdown reversed these changes, thus indicating its oncogenic role (27).

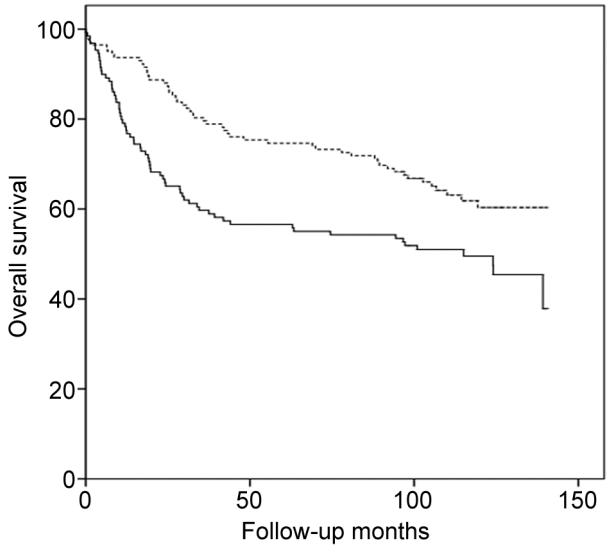

Figure 3. *Kaplan–Meier survival curve of the correlation of overall survival with the positive expression (solid line) and negative expression (dotted line) of AGR3. The overall survival of patients with AGR3-positive tumors was significantly lower than that of patients with AGR3-negative tumors (p=0.004). AGR3: Anterior gradient 3.*

AGR3 has been found to be over-expressed in various cancers (19-23) and associated with poor prognosis in breast and ovarian cancers (19, 20). However, some studies have revealed contrasting findings. Prihantono *et al.* reported that

Table I. *Correlation between AGR3 expression and the clinicopathological parameters of patients with gastric cancer.*

Parameter	Total (n=271)	AGR3 Negative (n=142)	AGR3 Positive (n=129)	*p*-Value
Age (years)				0.313
<59.3	120	67	53	
≥59.3	151	75	76	
Sex				0.869
Male	173	90	83	
Female	98	52	46	
Tumor size (cm)				<0.001
<3.9	153	97	56	
≥3.9	118	45	73	
Histologic type				0.115
Differentiated	123	58	65	
Undifferentiated	148	84	64	
Stage				<0.001
I	137	94	43	
II	35	16	19	
III	61	23	38	
IV	38	9	29	
Depth of invasion (T)				<0.001
T1	123	84	39	
T2	30	14	16	
T3	97	39	58	
T4	21	5	16	
Lymph node metastasis (N)				<0.001
N0	155	101	54	
N1	66	27	39	
N2	29	8	21	
N3	21	6	15	

AGR3: Anterior gradient 3.

Table II. *Cox multivariate regression of the association between AGR3 immunoreactivity and survival in gastric cancer with adjustment for clinicopathological parameters.*

Covariate	HR	95%CI	*p*-Value
AGR3 expression			
Negative	1.000	1.205-2.388	0.002
Positive	1.697		
Age (years)			
<59.3	1.000	1.436-2.997	<0.001
≥59.3	2.074		
Sex			
Male	1.000	0.568-1.165	0.261
Female	0.814		

AGR3: Anterior gradient 3; HR: hazard ratio; CI: confidence interval.

AGR3 mRNA expression was higher in benign than in malignant breast tumors and associated with non-aggressive tumors, which could be used as a marker for less aggressive breast tumors (37). Another study reported that AGR3 was associated with the level of differentiation, slowly proliferating tumors, and more favorable prognosis among breast cancer patients, suggesting its tumor suppressive role (38). Therefore, information on the function of AGR3 in breast cancer is different depending on the study. Moreover, the precise role of AGR3 in GC progression and prognosis remains unknown.

To clarify the prognostic significance of AGR3 expression, we examined the correlation between AGR3 expression and various clinicopathological parameters including the survival of GC patients, whose survival rate could be analyzed after more than 10 years of follow-up. Our study showed that positive AGR3 expression was significantly associated with tumor size, depth of invasion, lymph node metastasis, tumor stage, and poor survival. Furthermore, in Cox multivariate regression analysis, AGR3 was an independent prognostic factor associated with overall survival when adjusted for several covariates, including age and sex.

Taken together, the results indicated that AGR3 expression was significantly associated with the tumor progression and survival of GC patients. Therefore, AGR3 may be a novel biomarker and prognostic factor for GC.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: Wan-Sik Lee and Young-Eun Joo. Performed most of the experiments: Young-Lan Park, Sun-Young Park. Designed the experiments and drafted the article: Wan-Sik Lee and Young-Eun Joo. Supervision: Young-Eun Joo. Approval of final article: all Authors.

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