Tohoku J. Exp. Med., 2014, **234**, 83-88

Expression of Anterior Gradient 2 Is Decreased with the Progression of Human Biliary Tract Cancer

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Biliary tract cancers include cancers of the gallbladder and extrahepatic bile ducts, and its prognosis is poor. The anterior gradient 2 (AGR2) is a protein disulfide isomerase and is highly expressed in various human cancers, such as breast, prostate and pancreas cancers. AGR2 is expressed in normal cholangiocytes and its expression is maintained during biliary carcinogenesis. However, the clinical significance of AGR2 expression in biliary tract cancer has not yet been assessed. Thus, we examined the expression of AGR2 protein in biliary tract tumors using immunohistochemistry and its association with various clinicopathologic parameters. This study included 100 patients who underwent surgery for biliary tract cancers: 46 men and 54 women with a mean and median age of 64.2 and 65.0 years, respectively. AGR2 expression was detected in ductal epithelial cells of the normal biliary tract and in 95% of biliary tract cancer tissues. While the AGR2 expression was not associated with cancer location, patient age, patient sex, degree of regional lymph node metastasis (N-status), or residual status, the AGR2 expression level was decreased with increased tumor size (T-status, $p = 0.006$) and progression of tumor stage ($p = 0.009$). Moreover, well-differentiated cancers tended to show higher AGR2 expression than poorly differentiated cancers ($p = 0.068$); in fact, AGR2 expression was not associated with patient survival (Kaplan-Meier analysis, $p = 0.415$). Thus, AGR2 is of limited value as a prognostic marker for biliary tract cancer. In conclusion, the expression of AGR2 is decreased with the progression of biliary tract cancer.

Keywords: anterior gradient 2; biliary tract cancer; clinical parameters; immunohistochemistry; prognosis
Tohoku J. Exp. Med., 2014 September, **234** (1), 83-88.

Introduction

Biliary tract cancers include cancers of the gallbladder, the intrahepatic region, perihilar region (Klatskin tumor), distal extrahepatic bile ducts, and ampulla of Vater (de Groen et al. 1999). The prognosis of biliary tract cancers is very poor, with 5-year survival rates of only 25-30% after curative resection and 5-10% for all patients (Ramirez-Merino et al. 2013). Moreover, biliary tract cancers are often diagnosed at an advanced stage, limiting the possibility of curative resection. Treatment options for patients with unresectable or metastatic disease include fluoropyrimidine-, platinum- or gemcitabine-based chemotherapy regimens (Sasaki et al. 2013). However, combined treatment with gemcitabine and cisplatin has been demonstrated to extend the median overall survival by no more than 11.7 months (Valle et al. 2010). Thus, understanding the biological and molecular mechanisms behind the development and progression of these tumors is essential for improving the prognosis of patients with biliary tract cancer.

Anterior gradient 2 (*AGR2*, also known as *hAG-2*) is a human orthologue of the *Xenopus laevis* cement gland-specific gene *XAG-2* (Thompson and Weigel 1998). The XAG-2 protein is involved in specifying the dorsoanterior ectodermal fate, including the formation of cement glands and induction of forebrain fate in *Xenopus* (Aberger et al. 1998). Murine AGR2 (*Gob-4*) expression has been observed in mucin-secreting goblet cells in the stomach, small intestine, and colon (Komiya et al. 1999). In humans and mice, AGR2 expression has been reported in normal

Received April 4, 2014; revised and accepted August 19, 2014. Published online September 3, 2014; doi: 10.1620/tjem.234.83.
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tissues containing mucin-secreting cells and in endocrine tissues, including the lungs, stomach, colon, prostate, and small intestine (Thompson and Weigel 1998; Komiya et al. 1999). AGR2 is a protein disulfide isomerase residing in the endoplasmic reticulum of intestinal secretory epithelial cells. AGR2 forms mixed disulfide bonds with mucin 2 (MUC2), suggesting a direct role of AGR2 in MUC2 production (Park et al. 2009).

In humans, AGR2 expression was first detected in breast cancer cells by a suppression subtractive hybridization approach that compared the gene expression patterns between estrogen receptor (ER)-positive and ER-negative breast cancer cells (Thompson and Weigel 1998). AGR2 was found to be co-expressed with ER in breast cancer cell lines, suggesting that it may play a role in the pathogenesis of ER-responsive cancers. Subsequent studies have demonstrated that AGR2 is highly expressed in several human cancers, including adenocarcinomas of the esophagus (Pohler et al. 2004), lung (Fritzsche et al. 2007), pancreas (Iacobuzio-Donahue et al. 2003; Ramachandran et al. 2008), ovary (Park et al. 2011), and prostate (Kristiansen et al. 2005). The tumor-promoting potential of AGR2 is apparent in the augmentation of various tumor phenotypes, including proliferation, survival, metastasis, and drug resistance (Brychtova et al. 2011; Chevet et al. 2013).

Since AGR2 is reportedly up-regulated in various types of cancers, its utility as a cancer biomarker and therapeutic target has been investigated by numerous researchers (Brychtova et al. 2011; Chevet et al. 2013; Salmans et al. 2013). Lepreux et al. (2011) reported that AGR2 expression was high in normal columnar cholangiocytes and that its expression pattern appeared to be conserved during cholangiocarcinoma development. Despite these recent discoveries, the AGR2 protein expression in biliary tract cancers and its association with the clinicopathologic characteristics of biliary tract cancer patients has not been extensively investigated to date. Hence, in this study, we investigated the expression of AGR2 in human biliary tract tumors using immunohistochemistry (IHC) and assessed the relationship between AGR2 protein expression and clinicopathologic findings, including prognosis.

Materials and Methods

Patients and specimens

One hundred patients who underwent surgery for biliary tract cancer (ampulla of Vater, extrahepatic bile duct, hilar, intrahepatic bile duct, and gallbladder cancers) in the Hallym University Sacred Heart Hospital (Anyang, Korea) between May 1999 and December 2011 were included in this study. No patients had received preoperative radiotherapy or chemotherapy. The patients included 46 men and 54 women with a mean and median age of 64.2 and 65.0 years, respectively. The TNM stage was determined according to the hepatobiliary tract cancer TNM standard from the Union for International Cancer Control (American Joint Committee on Cancer [AJCC], 7th edition). The patients were followed-up for between 0.5 and 150 months.

This study was approved by the Institutional Review Board/ Ethics Committee of Hallym University Sacred Heart Hospital (HUSHHIRB No. 2012-1058).

Immunohistochemical staining

The tissue samples were fixed in 10% buffered formalin and embedded in paraffin as per standard protocol. Unstained 4-μm sections of clinical specimens were deparaffinized and rehydrated with 100%, 90%, 80%, and 70% ethanol. IHC was performed using the Ultraview DAB Kit (Ventana Medical Systems, Tucson, AZ). A primary antibody against AGR2 (1:100 dilution, Imgenex Corp., San Diego, CA) was added and the samples were incubated at 42°C for 32 min, followed by addition of the secondary antibody (Universal HRP Multimer) for 8 min. Finally, the slides were developed using 3,3-diaminobenzidine, counterstained with Mayer's hematoxylin and bluing reagent, dehydrated with ethanol, fixed with xylene, and mounted. The staining was first assessed according to the proportion of positive cells (1-3; 1 [0-10%], 2 [11-50%], 3 [> 50%]); and subsequently according to the intensity of the stain in order to obtain an intensity score (1-4; 1 [−], 2 [+], 3 [++], and 4 [+++]). The final scores (1-12) were obtained by multiplying the percentage score and the intensity score of each sample. The immunohistochemical stains were classified into three categories: negative (1) and weakly positive (2-4), moderately positive (5-8), and strongly positive (9-12).

Statistical analysis

Statistical analysis of quantitative data was performed with linear by linear association for categorical data. Survival curves were constructed using the Kaplan-Meier method. Statistical significance was defined as $p < 0.05$.

Results

AGR2 expression in biliary tract cancer

We examined the expression of AGR2 in the resected tumor specimens using IHC. AGR2 was expressed in the ductal epithelia of normal biliary tract tissues, including the intrahepatic and extrahepatic ducts, gallbladder, and ampulla of Vater (Fig. 1, arrows). Overall, 95% (95/100) of the biliary tract cancer tissues, including the ampulla of Vater (Fig. 1A-D), extrahepatic duct (Fig. 1E-H), intrahepatic duct (Fig. 1I-L), and gallbladder (Fig. 1M-P), stained positive for AGR2. In total, 20% (20/100), 22% (22/100), and 58% (58/100) of the tissues stained negative and weakly positive (scores 1-4), moderately positive (scores 5-8), and strongly positive (scores 9-12), respectively (Table 1).

AGR2 expression and clinicopathologic findings

The IHC scores of AGR2 expression were retrospectively analyzed against the clinical findings. The AGR2 expression score was not found to be significantly associated with any of the categorical variables, including cancer location, patient age, patient sex, N-status, and residual status. However, AGR2 expression was found to decrease with increased tumor size (T-status) and progression of tumor stage significantly (linear by linear association, $p = 0.006$ and $p = 0.009$, respectively; Table 1). Moreover,

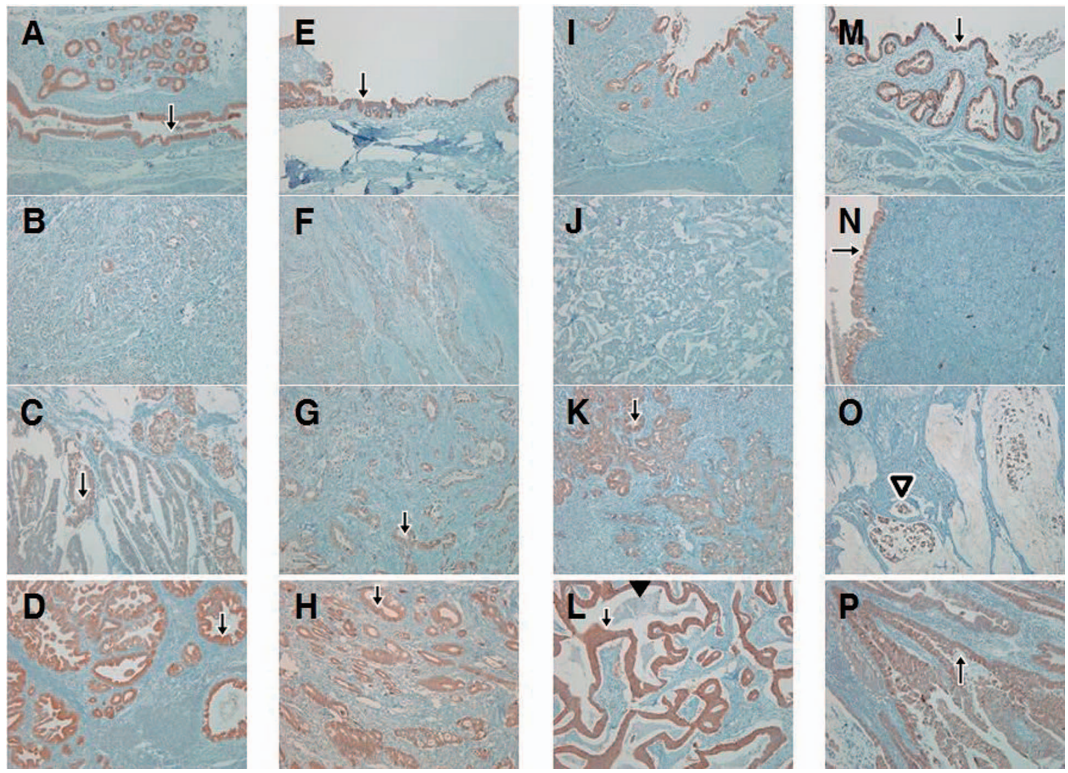

Fig. 1. Representative immunohistochemical staining of anterior gradient 2 (AGR2) expression.
(A-D) Ampulla of Vater (AOV) tissue. (A) Normal AOV: the epithelial cells displayed positive AGR2 expression. (B) AOV cancer (poorly differentiated): cytoplasmic staining 60%, staining intensity 1+. (C) AOV cancer (moderately differentiated): cytoplasmic staining 80%, staining intensity 2+. (D) AOV cancer (well differentiated): cytoplasmic staining 90%, staining intensity 3+. **(E-H)** Extrahepatic duct tissue. (E) Normal common bile duct: the epithelial cells displayed positive AGR2 expression. (F) Common hepatic duct cancer (moderately differentiated): cytoplasmic staining 80%, staining intensity 1+. (G) Klatskin tumor (moderately differentiated): cytoplasmic staining 90%, staining intensity 2+. (H) Common bile duct cancer (well differentiated): cytoplasmic staining 100%, staining intensity 3+. **(I-L)** Intrahepatic duct (IHD) tissue. (I) Normal IHD: the epithelial cells displayed positive AGR2 expression. (J) IHD cancer (moderately differentiated): cytoplasmic staining 0%, staining intensity 0. (K) IHD cancer (moderately differentiated): cytoplasmic staining 80%, staining intensity 3+. (L) Mucinous IHD cancer showing prominent intraluminal mucin (filled arrowhead): cytoplasmic staining 80%, staining intensity 3+. **(M-P)** Gallbladder tissue. (M) Normal gallbladder: the epithelial cells displayed positive AGR2 expression. (N) Gallbladder cancer (undifferentiated): cytoplasmic staining 0%, staining intensity 0. The normal epithelial cells were positive for AGR2. (O) Gallbladder cancer (signet ring carcinoma): cytoplasmic staining 100%, staining intensity 3. The scattered tumor cells displayed strong AGR2 expression (blank arrowhead). (P) Gallbladder cancer (moderately differentiated): cytoplasmic staining 100%, staining intensity 3+. Arrows mark the luminal epithelia of the biliary ducts. Original magnification, × 100.

AGR2 expression tended to be positively associated with differentiation status, as defined by the histologic grade (linear by linear association, $p = 0.068$; Table 1) and to be weakly inversely associated with metastatic status ($p = 0.109$). In general, the AGR2 expression score was lower in tumors that progressed.

AGR2 expression and prognosis

Next, we analyzed the associations between AGR2 expression and survival by conducting a retrospective review of the records of the 100 patients included in the immunohistochemical analysis. The mean survival times were 51.39 months (range, 31.15-71.64 months), 58.80 months (range, 39.60-77.99 months), and 104.45 months (range 80.36-128.54 months) in the negative and weakly positive, moderately positive, and strongly positive staining groups, respectively. Although patients with AGR2-positive biliary tract cancer tended to survive longer than patients with ARG2-negative cancer, AGR2 expression was not significantly correlated with the overall survival of biliary tract cancer patients (Fig. 2; Kaplan-Meier analysis, $p = 0.415$).

Discussion

AGR2 is highly expressed in numerous human cancers and is associated with the development of various tumor characteristics, including increased proliferation, survival, metastasis, and drug resistance (Brychtova et al. 2011; Chevet et al. 2013). Increased AGR2 expression has previously been shown to be associated with metastasis and a poor prognosis in breast cancer patients (Barraclough et al. 2009), and to correlate with high-grade serous carcinoma

Table 1. Clinicopathological parameters and anterior gradient 2 (AGR2) expression of biliary tract cancer (linear by linear association).

Variable		Total	No. of patients (%)			*p* value
			AGR2 negative and weakly positive	AGR2 moderately positive	AGR2 strongly positive	
Cancer location	Ampulla of Vater	24	6 (1*) (25.0%)	7 (29.2%)	11 (45.8%)	0.488
	Extrahepatic duct	32	5 (0*) (15.6%)	8 (25.0%)	19 (59.4%)	
	Intrahepatic duct	11	2 (2*) (18.2%)	1 (9.1%)	8 (72.7%)	
	Gallbladder	33	7 (1*) (21.2%)	6 (18.2%)	20 (60.6%)	
Patient age	< 65 years	43	8 (18.6%)	14 (32.6%)	21 (48.8%)	0.400
	≥ 65 years	57	12 (21.1%)	8 (14.0%)	37 (64.9%)	
Sex	Male	46	9 (19.6%)	10 (21.7%)	27 (58.7%)	0.896
	Female	54	11 (20.4%)	12 (22.2%)	31 (57.4%)	
T-status[1]	T1	25	0 (0.0%)	6 (24.0%)	19 (76.0%)	0.006
	T2-4	75	20 (26.7%)	16 (21.3%)	39 (52.0%)	
N-status[1]	N0	68	13 (19.1%)	12 (17.6%)	43 (63.2%)	0.379
	N1	28	5 (17.9%)	10 (35.7%)	13 (46.4%)	
M-status[1]	M0	96	19 (19.8%)	19 (19.8%)	58 (60.4%)	0.109
	M1	4	1 (25.0%)	3 (75.0%)	0 (0.0%)	
Stage[2]	1-2	73	11 (15.1%)	14 (19.2%)	48 (65.8%)	0.009
	3-4	27	9 (33.3%)	8 (29.6%)	10 (37.0%)	
Histologic grade[3]	G1	47	6 (12.8%)	10 (21.3%)	31 (66.0%)	0.068
	G2/3	47	12 (25.5%)	12 (25.5%)	23 (48.9%)	
Residual status[4]	R0	77	15 (19.5%)	18 (23.4%)	44 (57.1%)	0.791
	R1/2	21	4 (19.0%)	4 (19.0%)	13 (61.9%)	

*Other histology: ampulla of Vater cancer (mucinous), gallbladder cancer (signet ring cell, undifferentiated), intrahepatic duct cancer (two cases of mucinous cancer, sarcomatoid cancer).

[1]TNM status is based on hepatobiliary tract cancer TNM standard of Union for International Cancer Control (American Joint Committee on Cancer, 7th edition). The data for N-status were not available from four patients.

[2]Tumor stage is based on the TNM status determination collectively.

[3]Histologicgrade: G1, G2, and G3 represent well differentiated, moderately differentiated, and poorly differentiated tumors, respectively. The relevant data were not available from six patients.

[4]Residual status: R0, R1, and R2 represent no residual tumor, microscopic, and macroscopic residual tumors following treatment, respectively. The relevant data were not available from two patients.

and shortened overall survival of ovarian cancer patients (Innes et al. 2006). In addition to the tumor-promoting roles of AGR2, tamoxifen-induced AGR2 expression has also been implicated in the development of resistance of tumors against tamoxifen and cisplatin treatments (Hrstka et al. 2010).

In contrast to the results demonstrating the aggressiveness and poor prognosis of AGR2-expressing tumors, our results indicated that AGR2 expression in biliary tract cancer tissues is associated with lower-stage and well-differentiated tumors. Similarly, in a previous study, co-expression of AGR2 and MUC4 was also found to be higher in well-differentiated pancreatic ductal carcinoma than in poorly differentiated tumors (Brychtova et al. 2014), and survival analyses of pancreatic and prostate cancer patients revealed that ARG2 expression in the tumor tissue had no prognostic significance (Kristiansen et al. 2005; Riener et al. 2009; Maresh et al. 2010; Ho et al. 2013). However, in a previous study on pancreatic cancer, aberrant AGR2 expression in poorly differentiated pancreatic cancer was found to be associated with a worse prognosis (Brychtova et al. 2014). In addition, it has also been reported that AGR2 expression is positively correlated with the aggressiveness of prostate cancers (Zhang et al. 2007). Whether or not increased AGR2 expression in poorly differentiated cancer is associated with an unfavorable prognosis of biliary tract cancer patients remains unclear. It is of considerable interest to determine whether increased AGR2 expression in poorly differentiated biliary tract cancers correlates with poor patient prognosis, as recently demonstrated in the study by Brychtova et al. (2014) in pancreatic cancer.

AGR2 expression has been reported to be high in both normal and cancerous mucinous tissues and endocrine glands (Thompson and Weigel 1998; Komiya et al. 1999), and higher AGR2 expression is found in mucinous colorectal cancer cells compared to in non-mucinous cells (Kim et al. 2011). The morphology of the ductal epithelial cells shifts from a cuboidal to columnar shape along the biliary

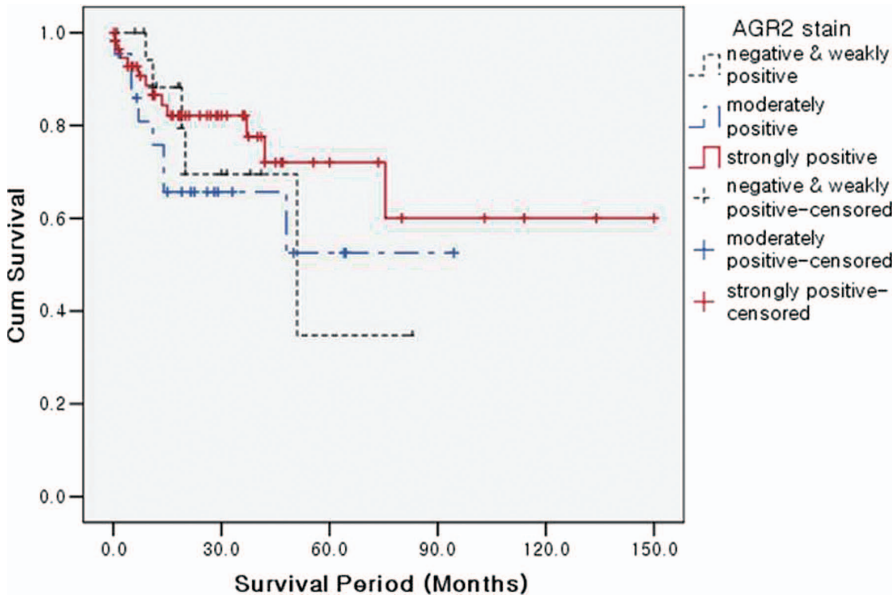

Fig. 2. Cumulative survival of biliary tract cancer patients.
Kaplan-Meier survival curves for biliary tract cancer patients (n = 100). AGR2 expression was not significantly associated with the overall 5-year patient survival (Kaplan-Meier analysis, p = 0.415).

tract. Constant mucin production is observed in the columnar cells, which are present from the large intrahepatic duct to the ampulla of Vater (Sasaki et al. 2007). Therefore, mucin-secreting cells of both the normal and cancerous biliary tract, except the small intrahepatic duct, are anticipated to express AGR2. Accordingly, immunohistochemical analyses of AGR2 expression in normal and cancer cells of the biliary tract have shown that AGR2 expression is not tumor-specific, but is rather associated with a mucin-secreting phenotype (Lepreux et al. 2011). In the present study, excessive intraluminal mucin deposition was found in two cases of intrahepatic duct cancers (Fig. 1L) and in one case of ampulla of Vater cancer, and all these cases showed strong AGR2 expression, supporting the reported AGR2 expression pattern in normal and cancer tissues of the biliary tree (Lepreux et al. 2011). However, whether there is a direct correlation between AGR2 expression and mucin production in biliary tract cancer remains to be determined.

In summary, AGR2 protein expression does not appear to be tumor-specific in biliary tract cancers. Rather, its expression was found to decrease with increased tumor size and progression of tumor stage significantly, but not associated with patient survival. Taken together, these observations cast doubt on the utility of AGR2 as a bona fide prognostic marker for human biliary tract cancers.

Acknowledgments

This study was supported by grant HRF-G-2012-6 from Hallym University and a grant from the Next Generation BioGreen 21 Program (No. PJ009051), Rural Development Administration of Republic of Korea to D. Kang and by grant HUMCRF 01-2009-16 from Hallym University Medical Center to J.H. Kim.

Conflict of Interest

The authors declare no conflict of interest.

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