Aquaporin 1 expression is associated with response to adjuvant chemotherapy in stage II and III colorectal cancer

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Abstract. Aquaporin 1 (AQP1), which functions as a water transporter, is associated with cancer cell proliferation, invasion, metastasis and angiogenesis in numerous types of solid cancer, including colorectal cancer (CRC). The focus of the present study was to address the potential clinical use of AQP1 expression in CRC as a prognostic and predictive biomarker for disease recurrence and therapeutic outcomes. The current study investigated the expression of AQP1 in surgically resected specimens from 268 patients with stage 0-IV CRC. AQP1 expression was positive in 112 (41.8%) patients, and was significantly associated with left-sided tumors (P<0.01) and with aggressive tumor phenotypes, including depth of invasion (P=0.03), lymph node metastasis (P=0.03), lymphatic invasion (P<0.01) and venous invasion (P<0.01). However, AQP1 expression had no significant prognostic effect on disease-free survival (DFS) in patients with stage II and III CRC following curative surgery. In 84 stage II and III patients who were administered 5-fluorouracil-based adjuvant chemotherapy, positive AQP1 expression was associated with an increased DFS rate compared with that of AQP1-negative patients (P=0.05). Additionally, these results identified that receiving adjuvant chemotherapy was not beneficial to patients with AQP1-negative tumors. This suggests that the expression of AQP1 may be a candidate biomarker predictive of response to 5-fluorouracil-based adjuvant chemotherapy following surgery in patients with stage II and III CRC.

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Abbreviations: CRC, colorectal cancer; AQP1, aquaporin 1; IHC, immunohistochemistry; 5-FU, 5-fluorouracil; DFS, disease-free survival

Key words: aquaporin 1, colorectal cancer, 5-fluorouracil, adjuvant chemotherapy, chemosensitivity, predictive biomarker

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer globally, and remains associated with a high mortality rate (1,2). Although surgical resection is effective for patients with localized disease, ~20% of patients with stage II and 40% with stage III CRC develop recurrence within 5 years following surgery (3). This high probability of postoperative recurrence provides the rationale for adjuvant chemotherapy following curative resection. 5-fluorouracil (5-FU)-based chemotherapy is the standard adjuvant treatment for patients with stage III CRC, and this has been established using large randomized clinical trials (3,4). Although the routine use of adjuvant chemotherapy for patients with stage II CRC is not recommended, a subset of stage II patients with high-risk characteristics for relapse may benefit from adjuvant therapy. In addition, there is considerable heterogeneity among tumors which may lead to differing clinical outcomes and chemotherapeutic responses. Therefore, it is important to identify biomarkers associated with differential risk of relapse and sensitivity to adjuvant chemotherapy.

An aquaporin (*AQP*) gene was identified in 1992 as a water transport channel (5). Since 1992, the existence of 13 *AQP* genes has been confirmed in mammals and these AQP genes are widely expressed in numerous human tissue types (6,7). The primary function of AQPs is to facilitate passive water transport, driven by osmotic gradients, across the plasma membrane of the cell, thus serving an important role in fluid homeostasis and numerous biological functions (6-9). Previous studies have demonstrated that certain classes of AQPs were highly expressed in a variety of cancer types and were associated with tumor biological functions (8,10-14). Particularly, increased expression levels of AQP1 have been identified in numerous types of human cancer, including lung, brain, breast, cervical, renal and CRC (8,11,15-17). Certain previous studies have demonstrated that AQP1 is involved in tumor cell proliferation, invasion, metastasis and angiogenesis, which may contribute to promoting tumor progression (8,9). Additionally, previous *in vitro* studies, which utilized lung or ovarian cancer cell lines, have also suggested the potential role of AQP1

expression in modifying the sensitivity to certain anti-cancer drugs (18,19).

While the clinical and biological relevance of AQP1 has been investigated in numerous types of cancer, including CRC, only a small number of studies have focused on the prognostic role of AQP1 expression in CRC, and these results were conflicting (17,20). Furthermore, it remains to be determined whether the expression of AQP1 is predictive of response to chemotherapy. Therefore, the present study aimed to investigate the association between AQP1 expression and survival outcomes or chemotherapeutic response in CRC. Specifically, the potential clinical utility of AQP1 expression as a predictive molecular marker for disease recurrence was evaluated in patients with CRC who underwent curative surgery followed by 5-FU-based adjuvant chemotherapy.

Materials and methods

Tissue samples. A total of 268 surgical specimens of CRC, obtained at Saitama Medical Center (Kawagoe, Japan) between January 2001 and March 2010 were used for immunohistochemistry (IHC; Table I). Patients who received preoperative chemotherapy and/or radiotherapy prior to surgery were not included in the current study. Tumors were staged at the time of the primary tumor resection according to the International Union Against Cancer classification (21). The present study was performed with the approval of the Ethics Committee of the Saitama Medical Center, Saitama Medical University (Saitama, Japan), in accordance with the ethical guidelines for clinical research (application no. 1143). Written informed consent was gained from all participants.

IHC and evaluation of AQP1 expression. The expression of AQP1 was examined using IHC. Briefly, 4-μm-thick, fixed in 20% formalin for ~24 h at room temperature, paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide. Antigens were retrieved by 10 min autoclaving in 10 mM citrate buffer solution [(pH 6.0) 105˚C]. Primary rabbit polyclonal anti-human AQP1 antibody (cat. no. HPA019206; Atlas Antibodies AB, Bromma, Sweden) was incubated in a 1:1,000 dilution of 10 mM PBS at 4˚C overnight. The sections were the incubated with horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibody polymer for 30 min at room temperature according to the manufacturer's protocol of the EnVision™ HRP system (Dako; Agilent Technologies GmbH, Waldbronn, Germany; dilution, ready-to-use). Following this, sections were incubated with 3,3'-diaminobenzene for 3-5 min at room temperature. Blocking was performed using 0.3% hydrogen peroxide for 30 min at room temperature and then washed in PBS. Sections were counterstained with Carrazzi's hematoxylin for 1 min at room temperature. Immunohistochemical evaluations were performed independently by two histopathologists who were blinded to the clinicopathological characteristics of the patients. In total >200 cancer cells were investigated under high power (x200) magnification using a light microscope for AQP1 expression, following screening for areas with the highest staining intensity under lower power (x40) magnification. AQP1 staining of membranous or cytoplasmic staining within the cancer cells was semi-quantitatively scored as 0 (0-5%), 1 (5-20%), 2 (20-50%) or 3 (>50%). Subsequently, tumors determined to have scores of 2 and 3 were considered to have AQP1-positive expression, whilst tumors with scores of 0 and 1 were considered to be negative for AQP1 expression.

Statistical analysis. Statistical analysis of the associations between AQP1 expression and various clinicopathological characteristics was performed using a Fisher's exact test, χ^2 test or Mann-Whitney U test. Cumulative survival was assessed using the Kaplan-Meier method and the differences between the two groups were analyzed using a log-rank test. All statistical analyses were two-sided. Statistical analyses were conducted using GraphPad Prism v6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS Statistics version 22 (IBM SPSS Corporation, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Association between AQP1 expression and clinicopathological characteristics. Clinical characteristics of patients included in the current study are presented in Table I and representative images of AQP1 staining in non-tumor and tumor tissues are shown in Fig. 1A-C. In normal and cancer stromal areas, AQP1 expression was detected in the endothelial cells of microvessels and erythrocytes, which is consistent with previous studies (9,17,22). AQP1 expression was not detectable in normal epithelial cells (Fig. 1A). In CRC tissues, the expression of AQP1 was identified in the cell membrane and cytoplasm of the cancer cells (Fig. 1B). Among the 268 patients with stage 0-IV CRC, positive AQP1 expression was observed in 112 (41.8%) patients, and the remaining 156 (58.2%) patients were identified to be AQP1-negative (Fig. 1B and C, respectively). The associations between AQP1 expression and the clinicopathological features of the 268 patients with CRC are presented in Table I. Positive AQP1 expression was significantly associated with left-sided tumors vs. right-sided tumors ($P<0.01$). AQP1 expression was also significantly associated with numerous aggressive characteristics, including greater depth of invasion ($P=0.03$), lymph node metastasis ($P=0.03$), lymphatic invasion ($P<0.01$) and venous invasion ($P<0.01$). Degree of differentiation was also associated with AQP1 expression ($P=0.02$), with well-differentiated adenocarcinomas more commonly negative for AQP1 expression. However, there was no significant difference in the proportion of patients with liver metastasis between the AQP1-positive and -negative groups.

Prognostic outcomes of AQP1 expression levels in stage II-III CRC. To determine the prognostic performance of AQP1 expression in patients with stage II-III CRC, 5-year disease-free survival (DFS) data was analyzed. Although this did not reach statistical significance, patients with AQP1-positive CRC had an improved DFS compared with that of AQP1-negative patients [hazard ratio (HR), 0.58; 95% confidence interval (CI) 0.30-1.12; $P=0.11$; Fig. 2A]. Therefore, the prognostic efficacy of AQP1 in stage II-III CRC was not validated in the present study.

Table I. Clinicopathological characteristics of patients with colorectal cancer according to AQP1 expression.

Characteristic	Total, n (%)	AQP1 expression		P-value
		Positive, n (%)	Negative, n (%)	
All patients	268	112	156	-
Age, years				0.09
<65	99 (36.9)	47 (42.0)	52 (33.3)	
≥65	169 (63.1)	65 (58.0)	104 (66.7)	
Gender				0.34
Male	172 (64.2)	74 (66.1)	98 (62.8)	
Female	96 (35.8)	38 (33.9)	58 (37.2)	
Location				<0.01
Right	90 (33.6)	27 (24.1)	63 (40.4)	
Left	178 (66.4)	85 (75.9)	93 (59.6)	
Histology				0.02
Well	124 (46.3)	41 (36.6)	83 (53.2)	
Moderately	130 (48.5)	66 (58.9)	64 (41.0)	
Poorly/others	14 (5.2)	5 (4.5)	9 (5.8)	
Depth of invasion				0.03
Tis	25 (9.3)	4 (3.6)	21 (13.5)	
T1	16 (6.0)	5 (4.5)	11 (7.1)	
T2	25 (9.3)	10 (8.9)	15 (9.6)	
T3	126 (47.0)	58 (51.8)	68 (43.6)	
T4	76 (28.4)	35 (31.3)	41 (26.3)	
Lymph node metastasis				0.03
Absent	136 (50.7)	49 (43.8)	87 (55.8)	
Present	132 (49.3)	63 (56.3)	69 (44.2)	
Lymphatic invasion				<0.01
Negative	77 (28.7)	19 (17.0)	58 (37.2)	
Positive	191 (71.3)	93 (83.0)	98 (62.8)	
Venous invasion				<0.01
Negative	85 (31.7)	26 (23.2)	59 (37.8)	
Positive	183 (68.3)	86 (76.8)	97 (62.2)	
Stage				0.12
0	25 (9.3)	4 (3.6)	21 (13.5)	
I	28 (10.4)	8 (7.1)	20 (12.8)	
II	65 (24.3)	32 (28.6)	33 (21.2)	
III	87 (32.5)	43 (38.4)	44 (28.2)	
IV	63 (23.5)	25 (22.3)	38 (24.4)	
Liver metastasis				0.53
Absent	217 (81.0)	91 (81.3)	126 (80.8)	
Present	51 (19.0)	21 (18.8)	30 (19.2)	

AQP1, aquaporin 1.

Association between AQP1 expression and therapeutic outcomes of patients treated with and without 5-FU-based adjuvant chemotherapy. Of the 152 patients with stage II-III CRC, 84 patients received adjuvant chemotherapy following curative surgery, whilst 67 patients were treated with surgery alone. For the 84 patients with adjuvant therapy, regimens primarily comprised intravenous or oral administration of 5-FU-based drugs; however, 9 (10.7%) were treated with 5-FU-based regimens in combination with oxaliplatin (mFOLFOX6 regimen). Among patients that received adjuvant chemotherapy, positive expression of AQP1 was significantly associated with improved DFS (HR, 0.45; 95% CI, 0.21-1.00; P=0.05; Fig. 2B). By contrast, for patients who were not administered adjuvant chemotherapy,

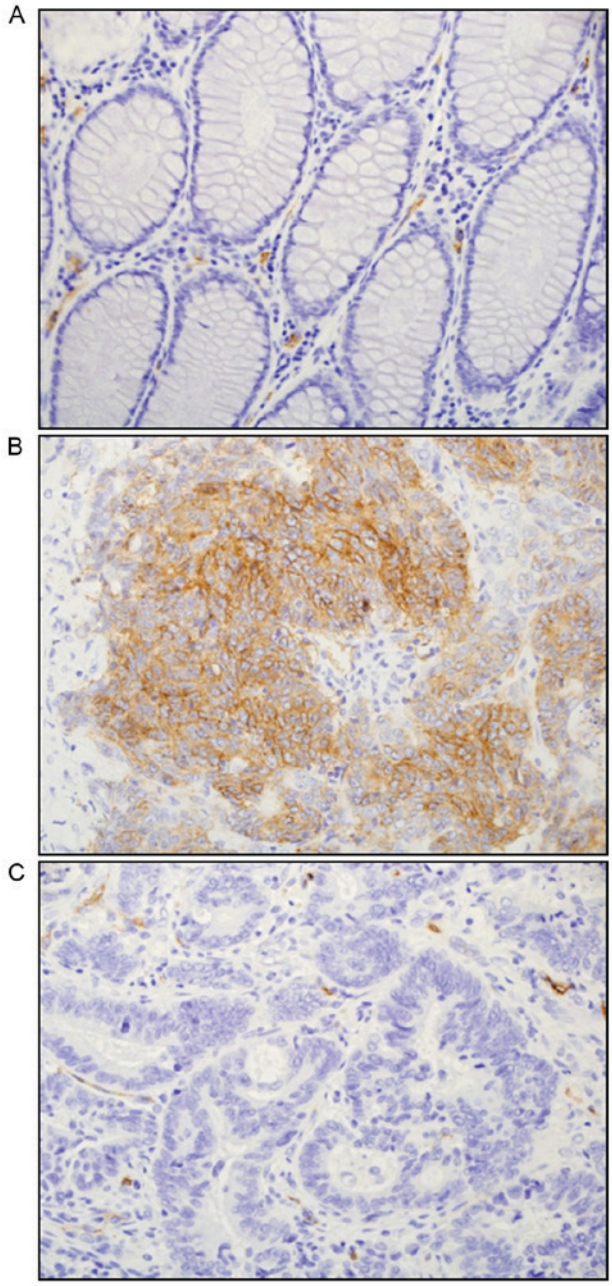

Figure 1. Representative images of immunohistochemical staining for AQP1 expression in non-tumor mucosa and colorectal cancer tissues. (A) Non-tumor epithelial cells with no detectable expression of AQP1. Tumor tissues exhibiting (B) positive and (C) negative AQP1 expression. The membrane and the cytoplasm of cancer cells were stained. Magnification, x400. AQP1, aquaporin 1.

AQP1 expression had no effect on DFS (HR, 1.00; 95% CI, 0.29-3.45; P=0.99; Fig. 2B). As shown in Fig. 2C and D, a similar trend was identified in the stratified analyses of stage II and III patients who received adjuvant chemotherapy, although this did not reach statistical significance due to the small number of patients in each group (HR, 0.22; 95% CI, 0.06-1.42; P=0.13; and HR, 0.53; 95% CI, 0.21-1.29; P=0.16, respectively). In addition, among the 77 patients with stage II and III AQP1-negative CRC, patients who were administered adjuvant chemotherapy had significantly reduced DFS compared with patients treated with surgery alone (P=0.02).

Although an overall survival analysis was conducted in 63 patients with stage IV CRC, the majority of whom were treated with one or more lines of chemotherapy, including 5-FU-based regimens, AQP1 expression had no significant prognostic impact in this subgroup (HR, 0.91; 95% CI, 0.51-1.61; P=0.74; Fig. 3).

Discussion

In the present study, the expression of AQP1 in 268 patients with stage 0-IV CRC was investigated using IHC. AQP1 expression was not detected in normal epithelial cells, whereas ~40% of tumors were identified as AQP1-positive. AQP1 expression was significantly associated with advanced tumor stage, the presence of lymph node metastasis and the presence of lymphatic invasion and venous invasion. In a previous study by Yoshida *et al* (20), tissue microarrays were utilized to evaluate AQP1 expression in 120 patients with stage II and III CRC disease. This study identified that 35.8% of patients were AQP1-positive and that AQP1 expression was significantly associated with lymph node metastasis and lymphovascular and vascular invasion. The results are consistent with the present study, suggesting that a tumor-promoting role of AQP1 may particularly affect lymph node metastasis and vascular invasion. In accordance with this, a previous study (23) demonstrated the involvement of AQP1 in colorectal carcinogenesis, and indicated that AQP1 expression is induced in early-stage disease and maintained throughout the late stages of colon cancer development. *In vitro* studies have also indicated that AQP1 increases the potential of invasion and migration in CRC cell lines (12,22). Notably, a significant association of AQP1 expression with left-sided CRC was demonstrated in the present study, as well as in that by Yoshida *et al* (20). This may suggest that AQP1 has the potential to contribute to tumor progression, particularly in left-sided colorectal tumors. However, Kang *et al* (17) reported that marked positive AQP1 expression was negatively associated with lymph node metastasis in stage I-III CRC. Therefore, there are still conflicting results regarding the role of AQP1 in lymph node metastasis. Further studies are required to specifically elucidate the clinical and biological significance of AQP1 in lymph node metastasis in CRC.

In terms of the prognostic significance of AQP1 expression, Yoshida *et al* (20) demonstrated positive AQP1 as a poor prognostic factor for overall survival (OS) in patients with stage II-III CRC using multivariate analysis, whereas Kang *et al* (17) identified that AQP1 expression had no significant survival impact on OS or DFS in patients with stage I-III CRC. In the latter study, patients with marked positive AQP1 expression typically had an improved DFS (17). The results of the present study are similar to the latter study, as a non-significant trend of improved DFS was observed in patients with stage II-III and AQP1-positive CRC compared with that of patients that were AQP1-negative. As there is inconsistency between previous studies and the current study, the prognostic efficacy of AQP1 expression in stage II-III CRC remains to be conclusively demonstrated.

In other types of malignancy, there has been variability in the significance of AQP1 expression levels associated with survival outcomes and tumor phenotype (15,16,24-27). The association between high AQP1 expression and poor prognosis

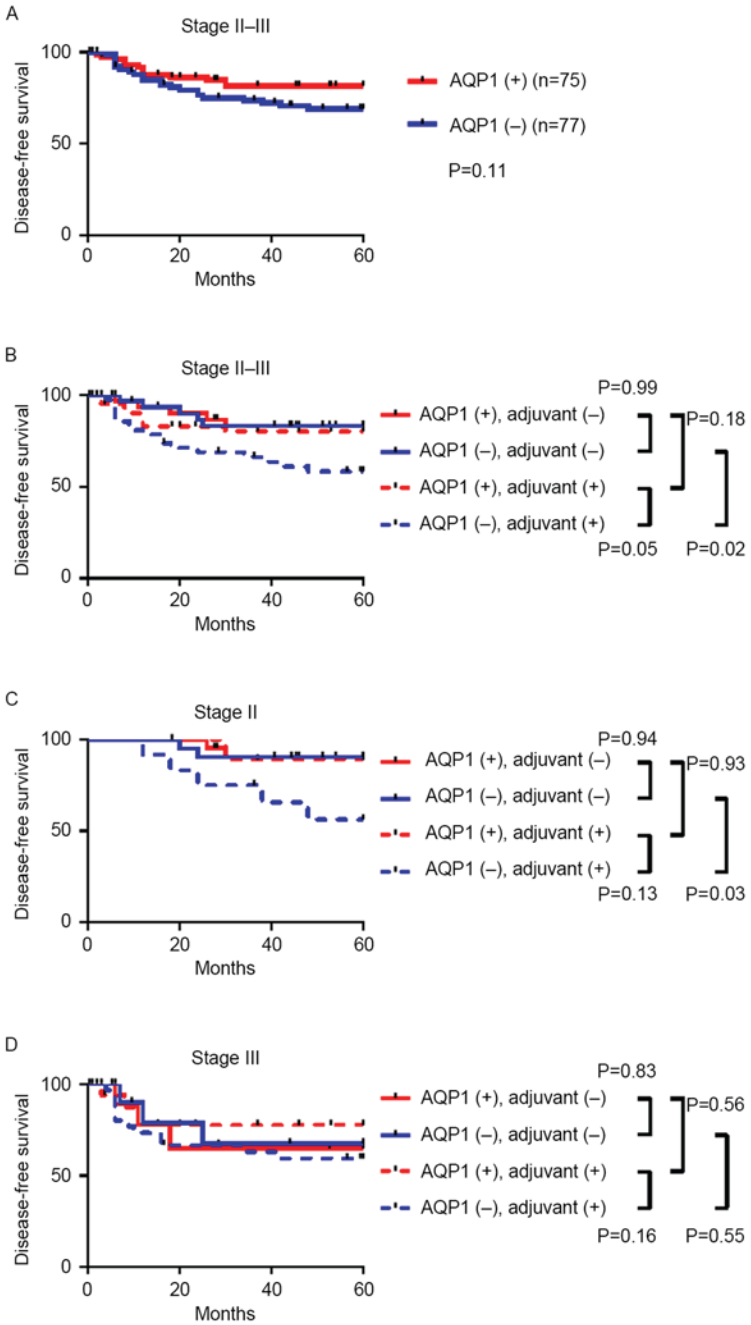

Figure 2. Association between AQP1 expression and DFS following curative surgery. (A) Kaplan-Meier curves for DFS according to the expression of AQP1 in 152 patients with stage II and III colorectal cancer. Kaplan-Meier curves for DFS stratified by receipt of adjuvant chemotherapy and AQP1 expression in (B) stage II and III, (C) stage II or (D) stage III patients. AQP1, aquaporin 1; DFS, disease-free survival.

was demonstrated in numerous types of solid cancer, including lung (15,24), breast (16,25), ovarian (26) and cutaneous melanoma cancer (27). In accordance with this, the upregulation of AQP1 was associated with aggressive subtypes of brain tumors (28,29) and cervical cancer (30). By contrast, in renal cancer (31), mesothelioma (32) and biliary tract cancer (33), AQP1 expression was demonstrated to be associated with improved survival rates. A potential explanation for this discrepancy in clinical impact of AQP1 between cancer types may be due to the multifaceted roles of AQP1 across various organs and tumor cells.

Although a large number of previous studies have analyzed the prognostic values of AQP1 in human cancer, only a small number of *in vitro* studies have investigated

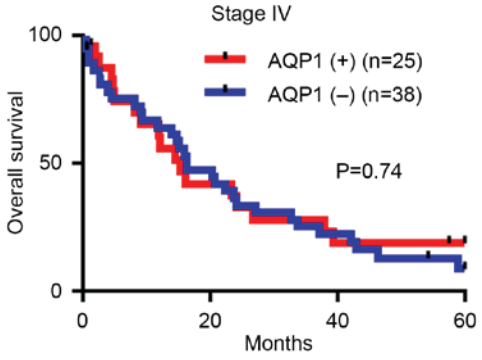

Figure 3. Association between AQP1 expression and overall survival in patients with stage IV colorectal cancer. AQP1, aquaporin 1.

the association between AQP1 expression and response to chemotherapy (18,19,34). In an ovarian cancer cell line, AQP1 expression was demonstrated to be associated with chemosensitivity to cisplatin (19). Pan *et al* (34) utilized a prostate cancer cell line and reported the association between AQP1 expression and the response to ginsenoside, which exerts antitumor effects. Liu *et al* (18) indicated that AQP1 expression was downregulated by combination therapy of celecoxib and afatinib in a lung cancer cell line. In view of these results, it is hypothesized that AQP1 may potentially modulate the sensitivity to anticancer drugs. Therefore, the present study focused on evaluating the prognostic value and also the potential contribution of AQP1 expression to chemotherapeutic outcomes. In patients with stage II-III CRC that were administered 5-FU-based adjuvant chemotherapy, patients with AQP1-positive CRC had improved therapeutic outcomes. By contrast, in AQP1-negative patients, the chemotherapy-treated group demonstrated reduced DFS times compared with the untreated group (treated with surgery alone). This may suggest that AQP1-positive CRC may have a greater sensitivity to 5-FU-based adjuvant chemotherapy compared with AQP1-negative CRC. Also, patients with AQP1-negative CRC may exhibit a poorer response to chemotherapy and may even be harmed by 5-FU-based treatment as demonstrated by the reduction in DFS in the AQP1negative CRC chemotherapy treated group. However, there are no previous studies that have addressed the biological roles of AQP1 in modulating the sensitivity to 5-FU in any type of cancer. Also, there is currently no sufficient explanation as to why AQP1-negative patients have a poorer outcome following treatment with adjuvant chemotherapy, suggesting the requirement for underlying mechanistic investigation in future studies.

The present study demonstrated that AQP1 expression was frequently identified in left-sided CRC, consistent with the results of Yoshida *et al* (20). However, CRC with high-level microsatellite instability (MSI), which has a hypermutable phenotype due to the loss of DNA mismatch repair activity, occurs predominantly in the right-sided colon (3). Also, it has been demonstrated that patients with stage II-III MSI-CRC may not benefit from 5-FU-based adjuvant chemotherapy (3). This suggests that combining AQP1 expression with MSI status may provide improved predictive ability compared with using single biomarkers in stage II-III CRC, although MSI data was not available in the present study.

The present study had several limitations, which included its retrospective nature and the small number of patients that were examined. Although all regimens of adjuvant therapy used in this study included 5-FU-based drugs, ~10% of patients were treated with the mFOLFOX6 regimen, as oxaliplatin-containing regimens have been established as the standard of care for patients with stage III CRC in the adjuvant setting (3,35). We were not able to address the beneficial effect of adding oxaliplatin compared to the conventional 5-FU-based therapy alone, due to the small sample size of patients who were treated with oxaliplatin. Also, in patients with stage II CRC, the survival impact of high risk characteristics of relapse was not examined due to the limited clinical information and the lack of standardized criteria for the identification of high-risk stage II patients, particularly in patients with rectal cancer (36). Therefore, the results from the present study are not definitive and future studies are required to elucidate the clinical implications of AQP1 expression in patients with stage II and III CRC, by addressing the aforementioned limitations.

In conclusion, AQP1 expression was detected in ~40% of patients with CRC and was significantly associated with aggressive clinicopathological characteristics, including lymph node metastasis, lymphatic invasion and venous invasion. In stage II and III patients, AQP1 expression had no significant impact on DFS. By contrast, in patients who received 5-FU-based adjuvant chemotherapy following surgery, AQP1-positive CRC exhibited improved therapeutic outcomes. Adjuvant chemotherapy may not be advantageous for patients with AQP1-negative tumors. Therefore, the results of the current study suggested that AQP1 expression may be a potential predictive biomarker candidate for responsiveness to 5-FU-based adjuvant chemotherapy in stage II-III CRC. However, further investigation using a large number of CRC cases as well as functional studies are required to determine the clinical and biological role of AQP1 expression in modulating the chemosensitivity.

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