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*Original Article*

# Aquaporin 5 expression correlates with tumor multiplicity and vascular invasion in hepatocellular carcinoma

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**Abstract:** Aquaporins (AQPs) are a family of water channel transmembrane proteins that play a key role in transcellular water movement and transport. Recent studies have reported that AQPs are involved in cancer biology and can be a novel biomarker for predicting prognosis. The aim of this study was to identify clinical significance and prognostic impact of AQP5 in surgically resected hepatocellular carcinomas (HCCs). We analyzed the association between the expression of AQP5, Ki-67, and E-cadherin. Immunohistochemical stains for AQP5, KI-67, and E-cadherin were performed on 72 surgically resected HCCs. As a result, 46 patients (63.9%) showed AQP5 expression, 46 patients (63.9%) revealed high expression of Ki-67, and E-cadherin loss was identified in 8 patients (11.1%). No significant relationship among the three markers was found (all $P > 0.05$). AQP5 expression was associated with tumor multiplicity ($P = 0.039$), microvascular invasion ($P = 0.040$), and major vessel invasion ($P = 0.044$). High expression of Ki-67 was related to high serum AFP level ($P = 0.006$), tumor grade ($P = 0.002$), and microvascular invasion ($P = 0.040$). AQP5 expression tended to be associated with worse overall survival (OS) ($P = 0.093$) in the univariate analysis, but no significance was found in the multivariate survival analysis. High expression of Ki-67 was associated with shorter recurrence-free survival (RFS) in both univariate ($P = 0.012$) and multivariate analysis ($P = 0.020$). In conclusion, AQP5 might be a prognostic marker in HCC based on its association with tumor multiplicity, microvascular invasion, and major vessel invasion; and Ki-67 is an independent prognostic factor in HCC.

**Keywords:** Aquaporin 5, Ki-67, hepatocellular carcinoma, prognosis

## Introduction

Liver cancer is one of the leading causes of cancer mortality worldwide, and hepatocellular carcinoma (HCC) represents over 90% of essential liver cancer [1]. Unfortunately, 25-70% of patients with HCC are found with advanced stage disease at diagnosis [2-4]. Despite the improvement of modern therapeutic and diagnostic technology of HCC, long term survival of HCC remains poor. A considerable number of patients with HCC ultimately experience recurrence after curative resection, and HCC has a high resistance rate to chemotherapy [5, 6]. Thus, the need for biomarkers for predicting recurrence has been strongly suggested. Aquaporins (AQPs) are a family of water channel transmembrane proteins in human tissues and there are reported 13 mammalian of AQPs (AQP0-13) [7, 8]. AQPs play a key role in transcellular water movement, fluid transport, glycerol transport, and cell migration [7-9]. Interestingly, AQPs are also involved in tumor biology including tumor-associated edema, tumor proliferation, tumor cell migration, and tumor angiogenesis [10]. Among the AQPs, AQP5 has also been evaluated in carcinogenesis. Recently, various studies have been reported that AQP5 expression is different and has prognostic impact in several tumors, such as esophageal cancer [11], stomach cancer [12], lung cancer [13, 14], colon cancer [15], breast cancer [16], brain tumor [17], prostate cancer [18], ovarian cancer [19], cervical cancer [20], and

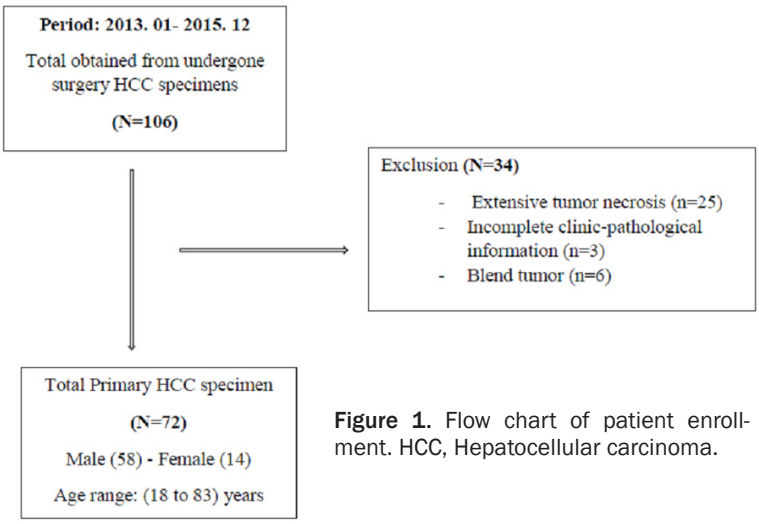


**Figure 1.** Flow chart of patient enrollment. HCC, Hepatocellular carcinoma.

pancreatic cancer [21], compared to normal tissue. In addition, they reported that overexpression of AQP5 was associated with aggressive tumor progression and poor prognosis in these various malignancies [11-21]. Mechanisms and effects of AQP5 on HCC are still unknown, and only a few studies have been reported.

The Ki-67 protein exists during the dynamic stage of the cell cycle (G1, S, G2, and mitosis) but is absent in resting cells (G0) [22]. Ki-67 is an excellent marker to determine the growth fraction of a given cell population [22], thus, Ki-67 index may have the correlation with the clinical course of cancer. The clinical significance of Ki-67 in HCC has not been fully elucidated, and relationship between AQP5 and Ki-67 in HCC is unknown.

Epithelial-mesenchymal transition (EMT) is a course in which epithelial cells lose their cell polarity and cell to cell adhesion, and gain migratory and invasive properties to become mesenchymal cells. Loss of E-cadherin is regarded to be a fundamental event in EMT [23], and some studies reported that loss of E-cadherin is associated with a poor prognosis, intrahepatic metastasis, vascular invasion, advanced stage, and higher tumor grade in HCC [24, 25]. However, very few studies have been reported the clinical significance and prognostic impact of AQP5, Ki-67, and E-cadherin as well as the relationship among these markers so far.

In this study, we aimed to identify clinical significance and prognostic impact of AQP5 in surgically resected HCCs. Furthermore, we assessed the association between the expression of AQP5, Ki-67, and E-cadherin.

## Materials and methods

### *Patient selection*

This retrospective study was based on 106 patients with primary HCC who underwent curative surgery at Kyungpook National University Hospital between January 2013 and December 2015. Of the 106 patients, 34 patients were excluded the following criteria: (1) no viable tumor due to extensive tumor necrosis, (2) blended tumor, (3) incomplete clinico-pathological information, and (4) unavailability of tissue sections during an experiment. Thus, a total of 72 patients were included in the present study (**Figure 1**), and their formalin-fixed, paraffin-embedded (FFPE) tissues were archived. All patients had not received prior radiation therapy or chemotherapy and were treated by a standard strategy after surgery.

The baseline clinicopathological data were obtained though reviewing medical records and pathologic reports as follows: age, gender, presence of hepatitis, serum alpha-fetoprotein (AFP), prothrombin induced by the absence of vitamin K or antagonist-II (PIVKA-II), tumor size, multiplicity of nodules, tumor grade, micro-vascular invasion, major-vascular invasion, presence of liver cirrhosis, and pathologic TNM stage. All patients were diagnosed according to the 2010 World Health Organization (WHO) criteria, and slides were reviewed by two pathologists. The tumor grade was defined according to the Edmondson grading system. The tumor stage was evaluated according to the 8th edition of the Union for International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC) Staging Manual for HCC.

### *Immunohistochemistry*

Immunohistochemistry (IHC) for AQP5, Ki-67, and E-cadherin was performed on whole sections using an automatic immunostainer (BenchMark XT, Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's proto-

**Table 1.** Clinicopathologic characteristics of 72 cases of hepatocellular carcinoma

| Characteristic | n | % |
|---|---|---|
| Age (yr) | 60.5 (18-83) | |
| Sex | | |
| Male | 58 | 80.6 |
| Female | 14 | 19.4 |
| Etiology | | |
| Hepatitis B virus-associated | 59 | 81.9 |
| Hepatitis C virus-associated | 5 | 6.9 |
| Alcohol | 8 | 11.1 |
| Serum alpha-fetoprotein (IU/mL) | 6.8 (0-51935) | |
| Serum PIVKA-II (AU/mL) | 56.5 (0-17661) | |
| Tumor size (cm) | 3.2 (1.0-15.0) | |
| Edmondson-Steiner grade | | |
| I | 9 | 12.5 |
| II | 17 | 23.6 |
| III | 27 | 37.5 |
| IV | 19 | 26.4 |
| Multiplicity | | |
| Absent | 57 | 79.2 |
| Present | 15 | 20.8 |
| Microvascular invasion | | |
| Absent | 46 | 63.9 |
| Present | 26 | 36.1 |
| Major vessel invasion | | |
| Absent | 67 | 93.1 |
| Present | 5 | 6.9 |
| Macro and micronodular cirrhosis | | |
| Absent | 35 | 48.6 |
| Present | 37 | 51.4 |
| Pathologic T stage | | |
| T1 | 36 | 50 |
| T2 | 15 | 20.8 |
| T3 | 18 | 25 |
| T4 | 3 | 4.2 |
| Pathologic Stage | | |
| I | 36 | 50 |
| II | 15 | 20.8 |
| III | 21 | 29.2 |

col. Briefly, 4-μm whole tissue sections were affixed to glass slides coated with poly-L-lysine and dried overnight at 65°C. Tissue sections were deparaffinized in xylene for a total of 15 min and subsequently rehydrated in three graded alcohol chambers, and treated with 3% hydrogen peroxide in methanol. Next, a primary rabbit monoclonal antibody against anti-AQP5 (Clone E3747; diluted 1:100; Abcam, USA), a primary mouse monoclonal antibody against anti-E-cadherin (Clone NCH-38; diluted 1:100; Dako, Denmark) were used and incubated for 32 min. A primary rabbit monoclonal antibody against anti-Ki-67 (Clone 30-9; predilution; Ventana Medical System) was used and incubated for 16 min. The ultraView Universal DAB detection kit (Ventana Medical Systems, Tucson, AZ, USA) was used as the chromogen at 37°C for 8 min, and the sections were counterstained with Harris hematoxylin for 2 min.

*Interpretation of immunohistochemistry*

Two pathologists independently evaluated the IHC results for each marker with no prior knowledge of all clinicopathologic data. AQP5 was expressed in the cytoplasm and/or membrane of tumor cells with various intensities. E-cadherin was shown in membranous staining, while Ki-67 was expressed in the nucleus of tumor with various intensities. Scoring for AQP5 and Ki67 was evaluated by the percentage of expression regardless of intensity (0-100%). Scoring for E-cadherin was assessed the percentage for complete loss of membranous stain (0-100%). AQP-5 expression was considered positive if there were at least 1% positive tumor cells staining. Ki-67 expression was considered as low expression (≤ 20%) and high expression (> 20%). The loss of E-cadherin was defined as loss of expression over 10% of the tumor cells.

*Statistical analysis*

All statistical analyses were conducted using Statistical Package for the Social Sciences software (version 23.0, SPSS Inc, Chicago, IL, USA). The association between clinicopathologic parameters and each marker was assessed using the chi-square or Fisher's exact test. The relationship among the three markers was evaluated using the Pearson correlation (*R*) test. The survival rates for recurrence-free survival (RFS) and overall survival (OS) were estimated using the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test. A multivariate analysis for survival was performed using the Cox proportional hazards regression model. The recurrence-free survival (RFS) was defined as the interval from the time of the first surgery to the time of any relapse of disease, and overall survival (OS) was estimated as the interval from

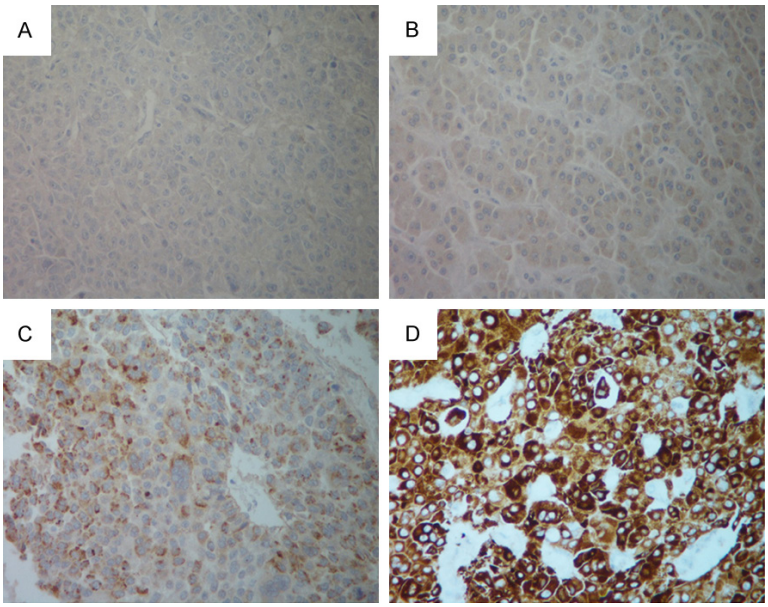


**Figure 2.** Aquaporin 5 expression in hepatocellular carcinoma. The immunohistochemical stain for aquaporin-5 shows various intensities: (A) No staining, (B) Weak staining, (C) Moderate staining, (D) Strong staining.

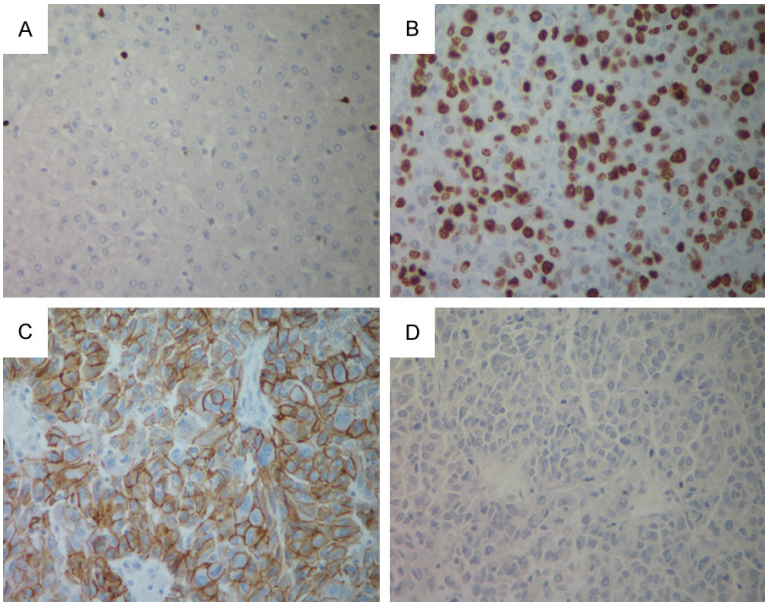


**Figure 3.** Ki-67 and E-cadherin expression in hepatocellular carcinoma. Ki-67 is expressed in the nucleus: (A) Low expression, (B) High expression, and E-cadherin is expressed in the membrane of tumor cells: (C) Absence of E-cadherin loss, (D) Presence of E-cadherin loss.

the time of the first surgery to the time of death or the time of last contact. *P* value of < 0.05 and *p* value of < 0.1 were considered as statistical significance and a trend towards significance, respectively.

## Results

### *Patient characteristics*

The baseline characteristics of the 72 eligible patients are summarized in **Table 1**. The median age was 60.5 years (range, 18-83 years), and 58 patients (80.6%) were male. The majority of patients had a history of HBV-related hepatitis (n = 59, 81.9%). The median levels of serum AFP and PIVKA-II were 6.8 IU/mL (range, 0-51935 IU/mL) and 56.5 AU/mL (range, 0-17661 AU/mL), respectively. Tumor sizes ranged from 1.0 to 15.0 cm (median, 3.2 cm). Tumor grade was revealed as I in 9 patients (12.5%), II in 17 patients (23.6%), III in 27 patients (37.5%), and IV in 19 patients (26.4%). Tumor multiplicity showed in 15 patients (20.8%). Microvascular invasion, major vessel invasion, and macro or micronodular cirrhosis were identified in 26 patients (36.1%), 5 patients (6.9%), and 37 patients (51.4%). After surgical resection, 36 (50.0%) patients were determined to have pathologic T1 (pT1) category, 15 (20.8%) were pT2, 18 (25.0%) were pT3, and 3 (4.2%) were pT4. All patients received standard treatment after surgery, with evidence of pathologic stage I in 50.0% (n = 36), stage II in 20.8% (n = 15), and stage III in 29.2% (n = 21).

### *Immunoexpression of AQP5, Ki-67, and E-cadherin*

AQP-5 was expressed in the cytoplasm and/or membrane of the tumor with various intensities of weak, moderate, and strong intensities (**Figure 2**). Ki-67 was expressed in the nucleus of the tumor cells (**Figure 3A**, **3B**), and

**Table 2.** Expression rate of Aquaporin 5, Ki-67, and E-cadherin

| Expression | Total (n = 72) | |
|---|---|---|
| | n | % |
| AQP5 | | |
| Negative (0%) | 26 | 36.1 |
| Positive (≥ 1%) | 46 | 63.9 |
| Ki-67 | | |
| Low expression (≤ 20%) | 26 | 36.1 |
| High expression (> 20%) | 46 | 63.9 |
| E-cadherin loss | | |
| Absent (≤ 10%) | 64 | 88.9 |
| Present (> 10%) | 8 | 11.1 |

**Table 3.** Correlation among Aquaporin 5, Ki-67, and E-cadherin expression

| Markers | AQP-5 | Ki67 | E-cadherin loss |
|---|---|---|---|
| | *R* (*p* value) | *R* (*p* value) | *R* (*p* value) |
| AQP-5 | - | 0.149 (0.210) | -0.068 (0.571) |
| Ki67 | 0.149 (0.210) | - | -0.114 (0.342) |
| E-cadherin loss | -0.068 (0.571) | -0.114 (0.342) | - |

E-cadherin expression was membranous (**Figure 3C**, **3D**). The percentages of expression of AQP5, Ki-67 and E-cadherin were variable. The results of expression of AQP5, Ki-67, and E-cadherin are shown in **Table 2**. AQP5 was positive in 63.9% (n = 46), Ki-67 showed high expression in 63.9% (n = 46), and E-cadherin loss was identified in 11.1% (n = 8). **Table 3** showed the correlation among expressions of three markers. There was no statistical significance for the relationship among the three markers (all $P > 0.05$).

*Association between clinicopathological features and AQP5, Ki-67, and E-cadherin*

The relationship between clinicopathological features and the expression level of AQP5, Ki-67, and E-cadherin is shown in **Table 4**. The expression of AQP5 was significantly associated with tumor multiplicity ($P = 0.039$), microvascular invasion ($P = 0.040$), and major vessel invasion ($P = 0.044$). High expression of Ki-67 was significantly related to serum AFP level ($P = 0.006$), tumor grade ($P = 0.002$), and microvascular invasion ($P = 0.040$). The loss of E-cadherin showed no association with any clinicopathologic variables. The clinicopathologic features of age, gender, serum PIVKA-II level, tumor size, presence of liver cirrhosis, and pathologic stage had no significant association with expression of all three markers.

*Survival outcomes according to expression of AQP-5, Ki-67, and E-cadherin*

The median follow-up period was 30.6 months (range: 0.23-56.2 months). In this period, 19 patients (26.4%) experienced recurrence and 5 (6.9%) died. In the univariate analysis, the patients with AQP5 positivity showed a trend of worse OS compared to the patients with AQP5-negative tumors ($P = 0.093$) (**Figure 4A**), although the expression of AQP5 was not associated with RFS ($P = 0.926$) (**Figure 4B**). On the other hand, high expression of Ki-67 was associated with shorter RFS ($P = 0.012$) (**Figure 4D**), but no relation with OS was found ($P = 0.873$) (**Figure 4C**). E-cadherin loss did not have a significant association with either OS or RFS in the survival analysis (**Figure 4E**, **4F**). In the multivariate analysis, only high expression of Ki-67 showed a significant association with poor RFS ($P = 0.020$) (**Table 5**). The expression of AQP5 showed no prognostic significance for OS and RFS in the multivariate analysis.

## Discussion

In the present study, we investigated the clinical significance and prognostic impact of AQP5 expression in 72 surgically resected HCCs, and association of AQP5 with Ki-67 and E-cadherin was also assessed. As a result, AQP5 expression was associated with tumor multiplicity, microvascular invasion, and major vessel invasion. High expression of Ki-67 was related to high serum AFP level, higher tumor grade, and microvascular invasion. No significant association among expressions of AQP5, Ki-67, and E-cadherin was found. The expression of AQP5 tended to be associated with worse OS, but no significance was found. High expression of Ki-67 was associated with shorter RFS.

AQPs are a family of water channel transmembrane proteins outflow various in human cancers which is associated with edema in tumor,

**Table 4.** Association between clinicopathologic parameters and Aquaporin 5, Ki-67, and E-cadherin expression

| Characteristic | AQP5 expression | | *P* value | Ki-67 index | | *P* value | E-cadherin loss | | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| | Negative | Positive | | Low | High | | Absent | Present | |
| Age (yr) | | | | | | | | | |
| < 60 | 12 (46.2) | 21 (45.7) | 1 | 10 (38.5) | 23 (50.0) | 0.461 | 29 (45.3) | 4 (50.0) | 1 |
| ≥ 60 | 14 (53.8) | 25 (54.3) | | 16 (61.5) | 23 (50.0) | | 35 (54.7) | 4 (50.0) | |
| Sex | | | | | | | | | |
| Male | 22 (84.6) | 36 (78.3) | 0.757 | 19 (73.1) | 39 (84.8) | 0.353 | 52 (81.3) | 6 (75.0) | 0.648 |
| Female | 4 (15.4) | 10 (21.7) | | 7 (26.9) | 7 (15.2) | | 12 (18.8) | 2 (25.0) | |
| Etiology | | | | | | | | | |
| HBV-associated | 18 (69.2) | 41 (89.1) | 0.054† | 23 (88.5) | 36 (78.3) | 0.352 | 52 (81.3) | 7 (87.5) | 1.000 |
| Non-HBV-associated | 8 (30.8) | 5 (10.9) | | 3 (11.5) | 10 (21.7) | | 12 (18.8) | 1 (12.5) | |
| Serum AFP (IU/mL) | | | | | | | | | |
| ≤ 7 | 15 (57.7) | 21 (45.7) | 0.462 | 19 (73.1) | 17 (37.0) | 0.006* | 31 (48.4) | 5 (62.5) | 0.71 |
| > 7 | 11 (42.3) | 25 (54.3) | | 7 (26.9) | 29 (63.0) | | 33 (51.6) | 3 (37.5) | |
| Serum PIVKA-II (AU/mL) | | | | | | | | | |
| ≤ 40 | 12 (46.2) | 20 (43.5) | 1.000 | 13 (50.0) | 19 (41.3) | 0.622 | 27 (42.2) | 5 (62.5) | 0.453 |
| > 40 | 14 (53.8) | 26 (56.5) | | 13 (50.0) | 27 (58.7) | | 37 (57.8) | 3 (37.5) | |
| Tumor size (cm) | | | | | | | | | |
| < 3.0 | 10 (38.5) | 21 (45.7) | 0.625 | 10 (38.5) | 21 (45.7) | 0.625 | 26 (40.6) | 5 (62.5) | 0.278 |
| ≥ 3.0 | 16 (61.5) | 25 (54.3) | | 16 (61.5) | 25 (54.3) | | 38 (59.4) | 3 (37.5) | |
| Multiplicity | | | | | | | | | |
| Absent | 17 (65.4) | 40 (87.0) | 0.039* | 23 (88.5) | 34 (73.9) | 0.227 | 51 (79.7) | 6 (75.0) | 0.669 |
| Present | 9 (34.6) | 6 (13.0) | | 3 (11.5) | 12 (26.1) | | 13 (20.3) | 2 (25.0) | |
| Edmondson-Steiner grade | | | | | | | | | |
| I & II | 10 (38.5) | 16 (34.8) | 0.802 | 16 (61.5) | 10 (21.7) | 0.002* | 22 (34.4) | 4 (50.0) | 0.448 |
| III & IV | 16 (61.5) | 30 (65.2) | | 10 (38.5) | 36 (78.3) | | 42 (65.6) | 4 (50.0) | |
| Microvascular invasion | | | | | | | | | |
| Absent | 21 (80.8) | 25 (54.3) | 0.040* | 21 (80.8) | 25 (54.3) | 0.040* | 41 (64.1) | 5 (62.5) | 1.000 |
| Present | 5 (19.2) | 21 (45.7) | | 5 (19.2) | 21 (45.7) | | 23 (35.9) | 3 (37.5) | |
| Major vessel invasion | | | | | | | | | |
| Absent | 26 (100.0) | 39 (84.8) | 0.044* | 25 (96.2) | 40 (87.0) | 0.410 | 57 (89.1) | 8 (100.0) | 1.000 |
| Present | 0 (0.0) | 7 (15.2) | | 1 (3.8) | 6 (13.0) | | 7 (10.9) | 0 (0) | |
| Macro and micronodular cirrhosis | | | | | | | | | |
| Absent | 10 (38.5) | 25 (54.3) | 0.227 | 15 (57.7) | 20 (43.5) | 0.327 | 29 (45.3) | 6 (75.0) | 0.146 |
| Present | 16 (61.5) | 21 (45.7) | | 11 (42.3) | 26 (56.5) | | 35 (54.7) | 2 (25.0) | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pathologic T stage | | | | | | | | | |
| T1 & T2 | 18 (69.2) | 33 (71.7) | 1.000 | 18 (69.2) | 33 (71.7) | 1.000 | 46 (71.9) | 5 (62.5) | 0.684 |
| T3 & T4 | 8 (30.8) | 13 (28.3) | | 8 (30.8) | 13 (28.3) | | 18 (28.1) | 3 (37.5) | |
| Pathologic Stage | | | | | | | | | |
| I & II | 18 (69.2) | 33 (71.7) | 1.000 | 18 (69.2) | 33 (71.7) | 1.000 | 46 (71.9) | 5 (62.5) | 0.684 |
| III | 8 (30.8) | 13 (28.3) | | 8 (30.8) | 13 (28.3) | | 18 (28.1) | 3 (37.5) | |

HBV, Hepatitis B virus; AFP, alpha-fetoprotein; PIVKA-II, prothrombin induced by the absence of vitamin K or antagonist-II. *Statistical significance. †Trend towards significance.

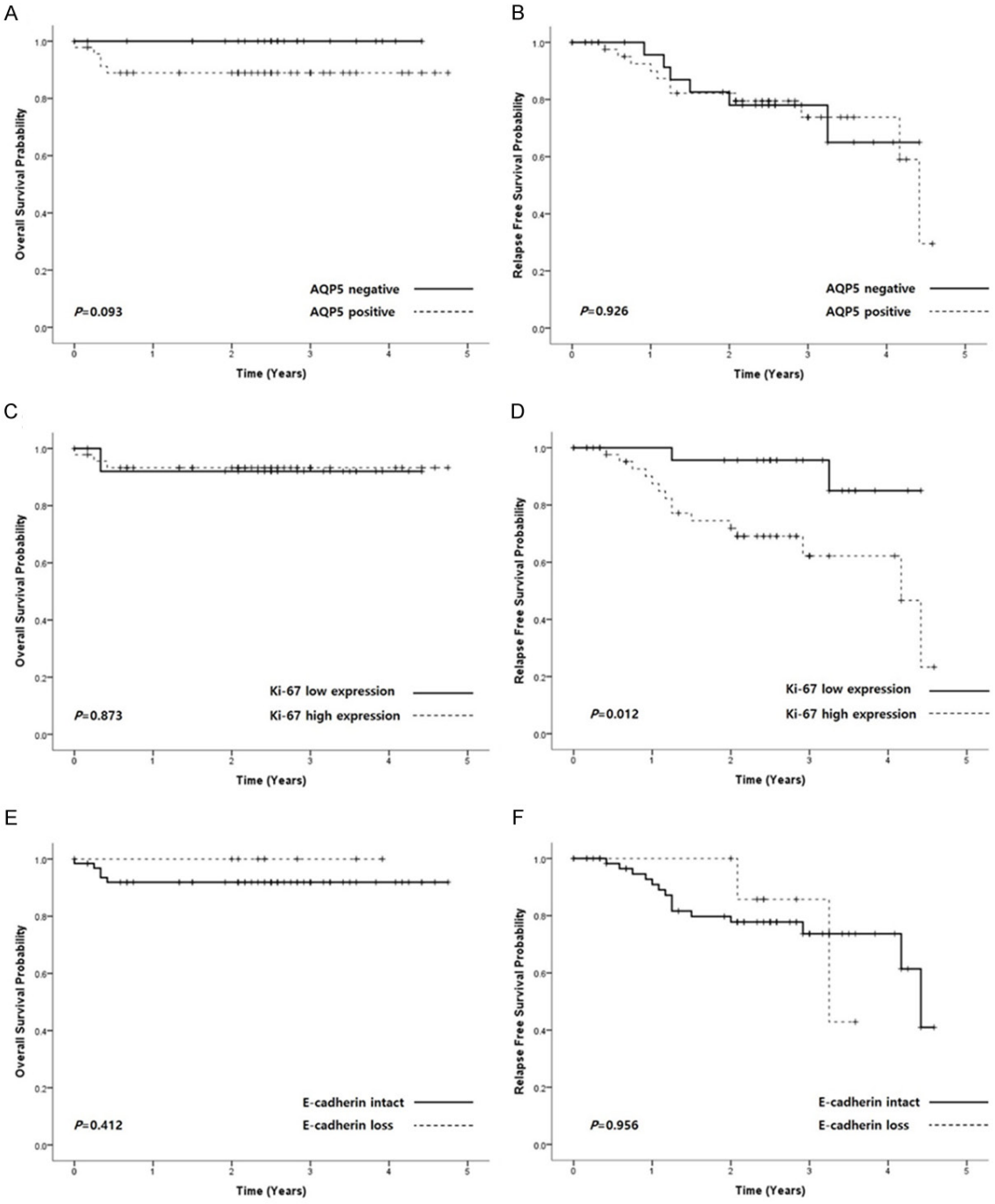


**Figure 4.** Kaplan-Meier survival curves for OS and RFS. Survival curves for OS (A) and RFS (B) according to the expression of AQP5. Survival curves for OS (C) and RFS (D) according to the expression of Ki-67. Survival curves for OS (E) and RFS (F) according to the expression of E-cadherin.

tumor proliferation, tumor cell migration, and angiogenesis [10, 26]. According to their permeability, AQPs are categorized as 3 groups: (1) water-selective transporters for example (AQP0, 1, 2, 4, 5, 6, 8); (2) aquaglyceroporins (AQP3, 7, 9, 10); (3) superaquaporins (AQP11, 12) [27]. Recently, some studies have been reported that AQP5 overexpression is associated with certain clinical features or poor prognosis in various cancers. For instance, Zhang et

**Table 5.** Multivariate analysis for overall survival and relapse free survival

| Variables | OS | | | RFS | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* value | HR | 95% CI | *p* value |
| Age ≥ 60 years | 3.435 | 0.363-32.523 | 0.282 | 0.363 | 0.118-1.112 | 0.363 |
| Tumor size ≥ 3.0 cm | 3.862 | 0.343-43.437 | 0.274 | 1.242 | 0.283-5.442 | 0.774 |
| High Edmondson-Steiner grade (III & IV) | 1.129 | 0.127-10.050 | 0.914 | 0.999 | 0.331-3.018 | 0.998 |
| Advanced stage (III) | 0.886 | 0.122-6.467 | 0,905 | 3.023 | 0.808-11.315 | 0.100 |
| AQP5 expression (≥ 1%) | 445122.097 | 0.000-3.433 | 0.969 | 0.483 | 0.152-1.534 | 0.217 |
| High expression of Ki-67 (> 20%) | 0.698 | 0.078-6.281 | 0.749 | 6.442 | 1.333-31.136 | 0.020* |

OS, overall survival; RFS, recurrence-free survival; HR, hazard ratio; CI, confidence interval. *Statistical significance.

al. demonstrated that outflow of AQP5 protein over intestinal adenocarcinoma might have been significantly connected with lymph node metastasis [28]; Kang et al. and Wang et al. reported that overexpression of AQP5 in colon cancer is related to lymph node metastasis and TNM stage [15, 29]; Huang et al. and Shen et al. uncovered that expression of AQP5 in gastric cancer is also related to lymphovascular invasion and lymph node metastasis [12, 30]; Sekine et al. found that the survival of biliary tract carcinoma patients for secondary AQP5 outflow might have been longer contrasted with those showing low AQP-5 statement [31]; Chae et al. and Song et al. revealed that overexpression of AQP5 in lung cancer is associated with lymph node metastasis, histological type, TNM stage, tumor repetition and poor survival [32, 33]; Jung et al., Shi et al., and Lee et al. reported that high expression of AQP5 was associated with tumor repetition, grade, metastasis, and poor outcome in breast cancer [16, 34, 35]; Yang et al. showed that positive expression of AQP5 was found related to lymph node metastasis and ascites in ovarian cancer patients [36]; Zhang et al. revealed that high expression AQP5 in cervical cancer might have been associated with Ki-67 expression, lymph node metastasis, and poorer prognosis [20]; Li et al. and Pust et al. demonstrated that overexpression of AQP-5 is related with Ki67 positivity, gene deletions, gene amplification, tumor grade, high Gleason score, and lymph node metastasis in prostate cancer [18, 37].

Few studies have been reported regarding effects of AQP5 on HCC. He et al. found that AQP5 was highly expressed in HCC cell lines and down-regulation of AQP5 interrupted invasion and metastasis in vitro and in vivo [7], which suggested that AQP5 might be associated with poor prognosis. But they did not examine the survival outcome association, and validation in clinical setting was not conducted. Guo et al. reported that co-expression of AQP3 and AQP5 was independently associated with poor prognosis in HCC [38]. In the current study, although the expression of AQP5 was not significantly associated with survival in patients with surgically resected HCC, we found AQP5 expression was related to tumor multiplicity, microvascular invasion, and major vessel invasion. Our results suggest AQP5 might be associated with poor prognosis through the mechanisms of vascular invasion and tumor multiplicity. Multiplicity of tumor, microvascular invasion, and major vessel invasion might have an effect on lymph node metastasis, distant metastasis, or recurrence. However, although no patient with distant metastasis was included in the present study, only 6 out of 72 patients were possible to be evaluated pathologic N stages. So our results are not thought to be enough to evaluate effects and mechanisms of AQP5 on lymph node metastasis or distant metastasis. Such a hypothesis should be investigated in further studies to determine the precise prognostic significance and mechanisms of AQP5 in HCC. In addition, AQP5 expression is marginally associated with OS of HCC patients in the univariate analysis in the current study, so further evaluation including a larger scale of patients is worth doing.

Interestingly, in cervical cancer and prostate cancer, overexpression of AQP5 showed a positive correlation with Ki67 index [18, 20]. But the relationship between AQP5 and Ki-67 in HCC was unknown. In the present study, we did not find any significant association among expression of AQP5, Ki-67, and E-cadherin. However, high expression of Ki-67 over 20%

was associated with shorter RFS in our study both in univariate analysis (P = 0.012) and multivariate analysis (P = 0.020). Moreover, we found that high expression of Ki-67 is associated with some clinicopathological variables including higher serum AFP level, Edmondson-Steiner grade, microvascular invasion. Thus, according to our results, Ki-67 is considered as poor prognostic factor for HCC patients. Cao et al. [39] showed that high K-i67 index was an independent poor prognostic factor along with high expression of TopoIIα in patients with HCC also.

He et al. reported that down-regulation of AQP5 inhibited the EMT process in HCC cells by modulating EMT-related molecules such as E-cadherin, α-catenin, N-cadherin, and Vimentin [12]. Although we found no direct association between expression of AQP5 and E-cadherin in the present study, our results that AQP5 is associated with multiplicity of tumor, microvascular invasion, and major vessel invasion suggests the possibility of relationship of AQP5 and EMT. Chen et al. demonstrated that diminished E-cadherin in HCC patients indicated a poor prognosis, and tendency to be associated with intrahepatic metastasis, vascular invasion, advanced TMN stage and tumor grade [24, 25]. But in the present study, the expression of E-cadherin did not have any significant association with any clinicopathological variable or survival.

In conclusion, expression of AQP5 is associated with tumor multiplicity, microvascular invasion, and major vessel invasion, and high expression of Ki-67 is associated with serum AFP level, tumor grade, and microvascular invasion in surgically resected HCCs. Ki-67 is an independent prognostic marker for RFS in HCC. Although further investigation is needed to clarify the distinct biologic significance of AQP5, our results suggest the possibility of AQP5 and Ki-67 as prognostic biomarkers in HCC.

## Disclosure of conflict of interest

None.

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