Aquaporin 3 Expression Predicts Survival in Patients with HER2-positive Early Breast Cancer

SUNHEE KANG[1*], YEE SOO CHAE[2], SOO JUNG LEE[2], BYUNG WOOG KANG[2], JONG GWANG KIM[2], WAN WOOK KIM[3*], JIN HYANG JUNG[3], HO YONG PARK[3], JAE-HWAN JEONG[3], JI YUN JEONG[4] and JI-YOUNG PARK[4]

[1]Department of Surgery, Dongsan Medical Center, Keimyung University School of Medicine, Daegu, Republic of Korea; Departments of [2]Oncology/Hematology, [3]Surgery and [4]Pathology, Kyungpook National University Medical Center, Kyungpook National University School of Medicine, Daegu, Republic of Korea

Abstract. *Background/Aim: Recent studies have revealed aquaporins (AQPs) as targets for novel anti-tumor therapy since they are likely to play a role in carcinogenesis, tumor progression and invasion. Accordingly, we analyzed the prognostic impact of AQP3 expression and polymorphisms in a number of patients with early breast cancer (EBC). Materials and Methods: AQP3 expression was investigated on the basis of the immunohistochemistry of tissue microarray specimens from 447 EBC patients who underwent surgery between 2003 and 2008. We scored the staining intensity (0 through 3) and percentage of positive tumor cells (0 through 4); the staining score was defined as sum of these scores used to categorize the AQP3 expression as negative (0 through 2), weak (3 through 5) or strong (6 or more). For AQP3 polymorphisms, seven single nucleotide polymorphisms (SNPs) (rs10813981, rs34391490, rs2228332, rs2227285, rs591810, rs17553719 and rs3860987) were selected using in silico analysis and genotyped using the Sequenom MassARRAY. Results: A total of 180 (40.3%) patients were identified as AQP3-positive (staining score >2), including 86 (19.2%) cases of strong expression (stating score >5). In a univariate analysis, AQP3 expression was significantly associated with survival for the patients with HER2-over-expressing EBC. Moreover, a multivariate survival analysis revealed that AQP3 expression was an independent prognostic marker of disease-free survival (DFS): hazard ratio (HR)=3.137, 95% confidence interval (CI)=1.079-9.125, p=0.036; distant DFS (DDFS): HR=2.784, 95%CI=0.921-8.414, p=0.070, for the HER2-over-expressing EBC patients. Meanwhile, none of selected AQP3 polymorphisms were related to AQP3 expression in tumor tissue or survival in the current study. Conclusion: AQP3 expression in tumor tissue may be considered as a potential prognostic marker in patients with HER2-over-expressing EBC after curative surgery.*

Breast cancer is a common malignant tumor affecting women with an increasing rate of incidence in many countries and mostly diagnosed at an early stage by widespread use of screening. Although several prognostic criteria have already been introduced to assist management after curative surgery for early breast cancer (EBC), the need for molecular markers has always been strongly suggested to discriminate individual variability and, thus, predict relapse or survival in patients with a similar clinical status, especially when considering that adjuvant regimens containing more toxic chemotherapeutic agents, such as anthracylines, are acknowledged for their efficacy over survival in patients with EBC (1).

Aquaporins (AQPs), a family of transmembrane water channel proteins that are widely distributed in various tissues throughout the body, play a key role in water homeostasis by regulating cellular water transport (2, 3). AQPs are also involved in the transport of other molecules, such as glycerol and urea, and, in addition, mediate transmembrane signaling by transporting signal molecules or coupling with other molecules as membrane proteins (4). Importantly, recent studies have revealed certain AQP subtypes as targets for novel anti-tumor therapy since they likely play a role in carcinogenesis, tumor progression and invasion (5-10). However, the prognostic role of

[*]These authors have contributed equally to this study and are both considered as first authors.

Correspondence to: Yee Soo Chae, MD, Ph.D., Department of Oncology, Kyungpook National University Medical Center, Kyungpook National University School of Medicine, 807 Hogukno, Buk-Gu, Daegu 702-210, Korea. Tel: +82 532002627, Fax: +82 532002029, e-mail: yschae@knu.ac.kr and Ho Yong Park, MD, Ph.D., Department of Surgery, Kyungpook National University Medical Center, Kyungpook National University School of Medicine, 807 Hogukno, Buk-Gu, Daegu 702-210, Korea. Tel: +82 532002702, Fax: +82 532002027, e-mail: phy123@knu.ac.kr

Key Words: Early breast cancer, aquaporin 3, polymorphism, prognosis.

Table I. *Seven selected AQP3 variants and information from dbSNP (www.ncbi.nlm.nih.gov/projects/SNP).*

AQP3 variants	Region	Ancestral allele	Chromosome	Chromosome position	Contig	Contig position	Minor allele	Minor allele frequency			
								European	African	Asian	KNUH*
rs2228332	cds-synon	T	9	33442954	NT_008413.19	33432954	C	0.381	0.199	0.326	0.312
rs2227285	Intron	C	9	33444002	NT_008413.19	33434002	G	0.261	0.307	0.556	0.594
rs10813981	Intron	G	9	33444760	NT_008413.19	33434760	A	0.381	0.433	-	0.260
rs34391490	Intron	G	9	33444863	NT_008413.19	33434863	A	0.238	0.467	-	0.189
rs591810	cds-synon	C	9	33447426	NT_008413.19	33437426	G	0.283	0.183	0.256	0.250
rs17553719	UTR-5	G	9	33447581	NT_008413.19	33437581	G	0.348	0.438	-	0.045
rs3860987	nearGene-5	G	9	33448961	NT_008413.19	33438961	A	0.239	0.042	0.300	0.305

AQP3, Aquaporin 3; KNUH, Kyungpook National University Hospital; cds-synon, coding region variant - synonymous mutation; UTR, untranslated region; SNP, single nucleotide polymorphisms. *Minor allele frequencies of the current study.

AQPs is still unknown and, thus, there is a need for further research. Among them, AQP5 over-expression in tumor tissue is found to be a potential prognostic factor in patients with estrogen receptor/ progesterone receptor (ER/PgR)-positive EBC after complete surgery, regardless of the clinical or pathologic characteristics in previous studies by the current authors (11, 12).

AQP3 is also known to play an important role in cellular homeostasis and water/substrate transport across cell membrane. Furthermore, it is widely expressed in a variety of cancers and identified to be associated with tumor progression and prognosis of squamous cancer in esophagus, cervix and head and neck; however, there is no study of breast cancer yet. It is also suggested that the AQP3 expression or its alteration, possibly caused by AQP3 variants, may affect outcomes in patients with breast cancer. In particular, AQP3 was over-expressed after therapy, while its inhibition by small interfering RNA (siRNA) was associated with a decreased cancer cell survival to cryotherapy suggesting its cytoprotective role (13). It is, thus, possible that the AQP3 expression or its alteration, possibly caused by *AQP3* variants, may affect outcomes in patients with breast cancer. Accordingly, the current study evaluated the prognostic role and association of AQP3 expression or its variants in a number of patients with EBC who underwent curative surgery.

Patients and Methods

Patients' characteristics. Four hundred and forty seven female patients who underwent surgery for EBC at Kyungpook National University Hospital (KNUH) between June 2003 and August 2008 were enrolled for evaluation in the current study. Patients with ductal carcinoma *in situ*, lobular carcinoma of the breast or who underwent any type of neoadjuvant treatment prior to surgery were excluded. The patient data was obtained from the KNUH breast cancer registry and patient files. The tumors were classified and staged according to the WHO classification and TNM staging system. The present study was approved by the local Ethics Committee of KNUH (No. 08-1008).

Tissue microarray. Tissue microarrays (TMAs), 2 mm in diameter, were constructed using formalin-fixed, paraffin-embedded cancer tissue blocks from 447 patients with EBC. The original hematoxylin and eosin (H&E) stained slides were reviewed and marked for tissue cores by two study pathologists. Representative areas from each tumor were arrayed to the triplicate blocks to minimize tissue loss and overcome tumor heterogeneity.

AQP3 immunohistochemistry. Immunohistochemistry was performed on a 4-μm-thick section from each TMA block using an automated immunostainer according to the manufacturer's instructions (Ventana Medical Systems Inc., Tucson, AZ, USA) The sections were labeled with anti-AQP3 antibody (1:200; Abcam, Cambridge, UK) at 4˚C overnight and, then, with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:200, P448; DAKO, Carpinteria, CA, USA) for 90 min at room temperature, as previously described (14). The AQP3 immunolabeling of the TMAs was reviewed and graded semiquantitatively considering both the staining intensity and the percentage of positive tumor cells by study pathologists blinded to the clinicopathological variables.

Scoring of AQP3 immunohistochemistry. The sections were scored on the basis of the staining intensity and percentage of stained cells relative to the background. The staining intensity (IS) was scored as 0 (no staining), 1 (faint/barely perceptible membrane staining), 2 (weak to moderate) and 3 (strong), relative to the internal positive control, while the percentage of positive cells (PC) was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%) for positive tumor cells. The AQP3 expression in the cancer tissue was defined as the staining score based on the sum of IS and PC. A staining score of 0 through 2 was considered AQP3-negative, 3 through 5 as weak AQP3-positive and 6 through 7 as strong AQP3-positive (Figure 1). The scoring was performed blindly towards the clinicopathological data.

Genotyping of AQP3 polymorphisms. Genomic DNA of fresh frozen breast tissue taken at the time of surgery was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The seven selected single nucleotide polymorphisms (SNPs) (rs2228332, rs2227285, rs10813981, rs34391490, rs591810, rs17553719 and rs3860987; Table I) were determined using the Sequenom MassARRAY (Sequenom Inc., San Diego, CA, USA) as

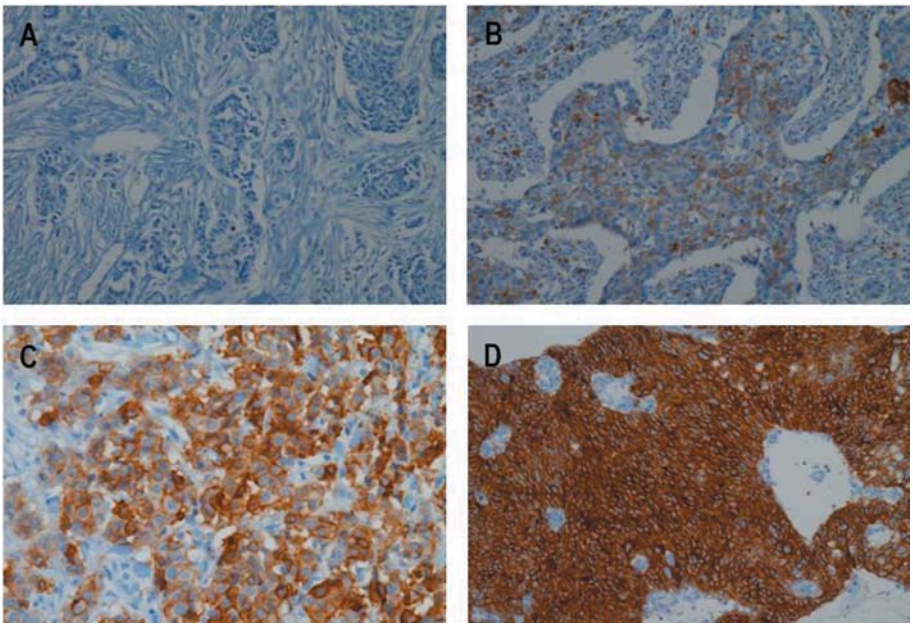

Figure 1. *AQP3 expression and intensity scoring: score 0 (A), 1 (B), 2 (C) and 3 (D).*

described in detail in our previous publication (15) and the genotyping analysis was performed blindly as regards the subjects. The selected polymerase chain reaction (PCR)-amplified DNA samples (n=2, for each genotype) were also examined using DNA sequencing to confirm the genotyping results.

Statistics. Relapse was confirmed by biopsy, when possible, and categorized as local, regional or distant; however, contralateral breast cancer during the follow-up period was not considered a relapse in this study. Disease-free survival (DFS), distant DFS (DDFS) and overall survival (OS) were defined as the time from the date of surgery to the date of any relapse, distant metastasis or death from any cause or the date of the last follow-up, respectively. The SNP genotype was analyzed as a three-group categorial variable (referent model) and grouped according to a dominant and recessive model. The Hardy-Weinberg equilibrium for each polymorphism was analyzed using a χ^2-square test. The cumulative incidence of relapse was defined as the time from the date of surgery to the date of the first event, where the curves were constructed based on the Kaplan-Meier method and analyzed using a log-rank test according to possible clinical (age, menopausal status and use of adjuvant therapies), histopathological risk factors (tumor size, number of involved lymph nodes, histological grade and immunohistochemical expression of ER, PgR and human epidermal growth factor receptor 2 (HER2)), the AQP3 expression score and the genotype of *AQP3* variants. The hazard ratio (HR) and 95% confidence interval (95% CI) for the genotypes of selected variants were calculated from a Cox regression analysis adjusted to age, stage, histological grade and ER/PgR, as well as HER2 status. In the multivariate analysis, the possible clinical and pathologic risk factors and AQP3 expression or variants significantly associated with survival in the adjusted univariate analysis were then analyzed as prognostic factors of relapse or survival for operated invasive ductal breast cancer. The differences in the continuous variables were compared using the Student's *t*-test or an ANOVA test, while a χ^2-test was used for the categorical variables. The statistical analyses were all performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics and clinical outcomes. For the 447 patients, the median age was 49 years (range=23-79) at the time of diagnosis, where 38.7% and 71.8% were node-positive and ER/PgR-expressing, respectively. The other basic clinical and pathological characteristics of the patients are listed in Table II. After curative surgery, 90.8% and 32.2% received adjuvant chemotherapy and radiotherapy, respectively, plus adjuvant hormonal treatment with tamoxifen or aromatase inhibitor that was given if required except for 1 patient. The median time for patients alive at the last follow-up was 7.2 (4.8-10.3) years. Sixty-nine (15.4 %) patients had experienced relapses, including 17 loco-regional and 59 distant relapses; 7 distant relapses were identified after a local relapse. In addition, 46 (10.3%) patients had died from breast cancer among 51 deaths. The estimated 5- and 10-year DFS, DDFS and OS were 87.4, 89.9 and 94.4% and 86.3, 88.4 and 92.4%, respectively.

AQP3 expression in tumor samples. Positive expression of AQP3 (score >2) was observed in 180 (40.3%) of the breast cancer TMA samples, where 21.0% showed weak (score 2-5) and 19.2% strong expression (score 6-7). Although the

Table II. *Comparison of patient characteristics according to the expression of AQP3.*

Characteristics	Total (%)	IS+PC (%)∬		*p*-Value
		Negative	Positive	
Number	447	266	180	
Age, median, range (years)	49.0, 23-79	48.0, 23-79	49.0 32-77	
mean±SD	50.38±10.71	49.6±10.9	51.5±10.3	0.075
≤50 years	252 (56.4)	153 (57.5)	99 (55.0)	
>50 years	195 (43.6)	114 (42.9)	81 (45.0)	0.630
≤40	78 (17.4)	55 (20.7)	23 (12.8)	
40-55	241 (53.9)	142 (53.4)	99 (55)	0.152
56-70	100 (22.4)	54 (20.3)	46 (25.6)	
>70	28 (6.3)	16 (6)	12 (6.7)	
Menopausal status				
Pre-menopause	194 (43.4)	117 (44)	77 (42.8)	0.846
Post-menopause	253 (56.6)	150 (56.4)	103 (57.2)	
Histological grade†				
1	45 (10.1)	29 (10.9)	16 (8.9)	
2	334 (74.0)	193 (72.6)	138 (76.7)	0.695
3	67 (15.0)	41 (15.4)	26 (14.4)	
Not assessed	4 (0.9)			
Site				
Left	200 (44.7)	123 (46.2)	77 (42.8)	
Right	237 (53.0)	138 (51.9)	99 (55.0)	0.785
Both	10 (2.2)	6 (2.3)	4 (2.2)	
Tumor size (T), pathologic‡				
1	226 (50.6)	135 (50.8)	91 (50.6)	
2	199 (44.5)	113 (42.5)	86 (47.8)	0.027
3	22 (4.9)	19 (7.1)	3 (1.7)	
Nodal involvement. (N), pathologic‡				
0	274 (61.3)	164 (61.7)	110 (61.1)	
1	122 (27.3)	70 (26.3)	52 (28.9)	0.668
2	51 (11.4)	33 (12.4)	18 (10.0)	
Stage (AJCC), pathologic				
I	149 (33.3)	87 (32.7)	62 (34.4)	
IIA	181 (40.5)	110 (41.4)	71 (39.4)	0.307
IIB	60 (13.4)	31 (11.7)	29 (16.1)	
IIIA	57 (12.8)	39 (14.7)	18 (10.0)	
ER				
Negative	125 (28.0)	75 (28.2)	50 (27.8)	
Weak	38 (8.5)	24 (9)	14 (7.8)	0.883
Strong	283 (63.3)	167 (62.8)	116 (64.4)	
Not assessed	1 (0.2)			
PR				
Negative	149 (33.3)	87 (32.7)	62 (34.4)	
Weak	76 (17.0)	47 (17.7)	29 (16.1)	0.882
Strong	197 (44.1)	117 (44)	80 (44.4)	
Not assessed	25 (5.6)			
HER2				
Positive§	64 (14.3)	38 (14.3)	26 (14.4)	
Negative	368 (82.3)	214 (80.5)	154 (85.6)	0.855
Unknown	15 (3.4)			
Tumor type¶				
Hormone-responsive	298 (66.7)	176 (66.2)	122 (67.8)	
HER2-overexpressed	64 (14.3)	38 (14.3)	26 (14.4)	0.250
Triple negative	79 (17.7)	47 (17.7)	32 (17.8)	
Unknown	6 (1.3)	6 (2.3)	0 (0)	
Surgery				
Mastectomy	367 (82.1)	217 (81.6)	150 (83.3)	
Breast conserving	80 (17.9)	50 (18.8)	30 (16.7)	0.557
Adjuvant chemotherapy				
None	41 (9.2)	25 (9.4)	16 (8.9)	
CMF	229 (51.2)	132 (49.6)	97 (53.9)	
Anthracycline without taxane	45 (10.1)	32 (12)	13 (7.2)	0.409
Anthracycline with taxane	132 (29.5)	78 (29.3)	54 (30.0)	
Adjuvant hormonal therapy				
None	112 (25.1)	64 (24.1)	47 (26.1)	
Tamoxifen	173 (38.6)	105 (39.5)	68 (37.8)	0.964
AI	120 (26.9)	72 (27.1)	48 (26.7)	
Tamoxifen followed by AIs	43 (9.4)	26 (9.8)	17 (9.4)	

AQP3, Aquaporin 3; IS, intensity score; PC, positive tumor cell; SD, standard deviation; AJCC, American Joint Committee on Cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CMF, cyclophosphamide/methotrexate/5 fluorouracil; AI, aromatase inhibitor. †modified Scarff-Bloom-Richardson grading system. §IHC (3+) or FISH (+). ¶according to immunohistochemical (IHC) stain of ER, PR and HER2. The staining intensity (IS) was scored as 0 (no staining), 1 (faint/barely perceptible membrane staining), 2 (weak to moderate) and 3 (strong), relative to the internal positive control. ∫The proportion of positive tumor cells (PC) was scored as following: 0, 0%; 1, 1% to 9%; 2, 10 to 33%; 3, 34 to 66%; 4, 67% or more. ∬AQP3 expression in the cancer tissue was defined by the staining score calculated as the sum of IS and PC.

distribution of tumor size (T) was statistically different based on AQP3 expression, the proportion of tumor equal to 2 cm or less in size (T1) was almost the same in both groups (50.8 *vs*. 50.6%). Otherwise, no statistical associations between the AQP3 expression and clinicopathological characteristics were observed in the current study (Table II).

AQP3 over-expression associated with worse prognosis for HER2-over-expressing EBC. There was no statistical correlation between AQP3 expression and survival in terms of DFS, DDFS and OS (hazard ratio (HR)=1.013, 0.886 and 1.179; p=0.951, 0.668 and 0.602, respectively). However, only for the patients with HER2-over-expressing EBC, AQP3 expression (IS+PC ≥3)

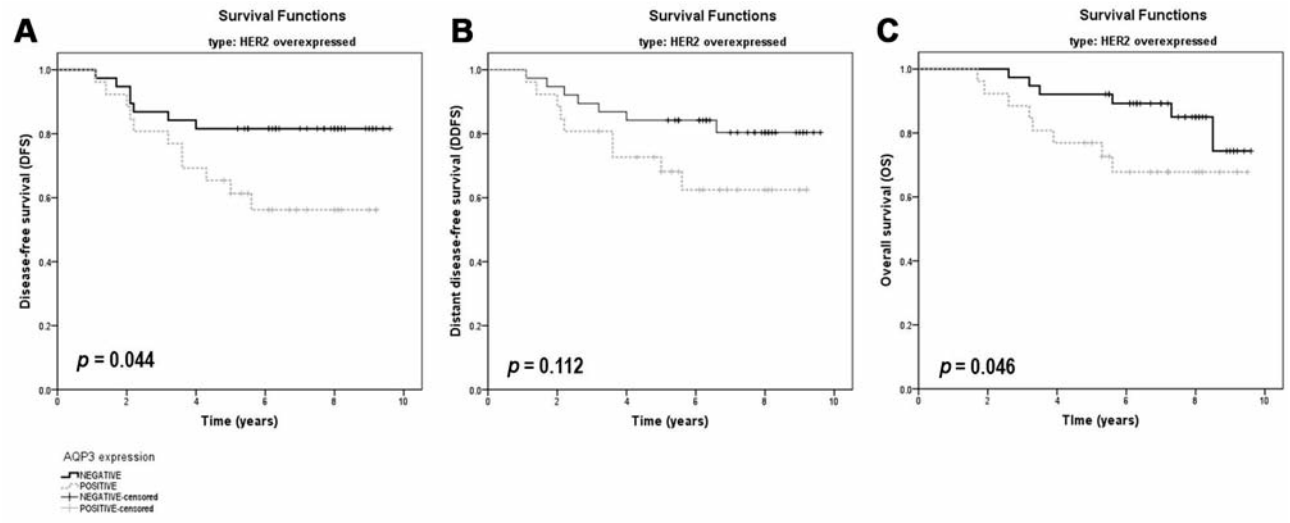

Figure 2. *Survival according to the AQP expression in patients with HER2 over-expressing breast cancer: DFS (A), DDFS (B) and OS (C).*

was significantly associated with a poor survival when compared to negative expression in a univariate survival analysis (Figure 2). Since no difference in survival was found between the patients with weak and strong AQP3 expression, only two categories (negative *vs*. positive expression) were used for the multivariate survival analysis. As a result of the multivariate survival analysis, AQP3 over-expression was identified as an independent prognostic factor for DFS (HR=3.137; 95%CI=1.079-9.125; p=0.036) but showed a trend for poor DDFS and OS (HR=2.784 and 2.439; p=0.070 and 0.179, respectively) regardless of the clinicopathological parameters including the stage and use of adjuvant chemotherapy (Table III).

Association between AQP3 variants and tumor expression of AQP3 or survival. Genotyping for *AQP3* variants was available for 374 out of total 447 patients. There was no statistical difference between genotype of each variant and AQP3 expression in tumor tissue or survival. Furthermore, for the patients with HER2-over-expressing breast cancer, whom AQP3 expression was statistically associated with survival, no association was observed between genotypes of each variant and tumor expression of AQP3 (Table IV).

Discussion

AQP3 is well-known to be over-expressed in breast cancer together with AQP1 and AQP5; however, its clinical impact has not yet been identified. Therefore, the current study analyzed the association of AQP3 expression in tumor with survival based on a significant cohort of patients with EBC and an extended follow-up of about 10 years after curative surgery suggesting AQP3 expression as a potential prognostic marker for patients with HER2-positive EBC.

Aquaporins (AQPs) are a family of water-transporting transmembrane proteins. Yet, in addition to osmotic water transport, several studies have provided evidence that certain AQP subtypes perform unexpected functions in cell migration, angiogenesis and tumor development and progression (16-18). The expression of several AQPs in breast tissue has already been identified and their role in breast cancer also investigated, although still poorly characterized. Among these AQPs, the current authors previously demonstrated a correlation between the expression of AQP5 in breast cancer cells and survival after curative surgery in patients with EBC suggesting AQP5 as a potential biomarker (12).

AQP3 is a well-known aquaglyceroporin transporting water, glycerol and urea in normal tissue; however, the role of AQP3 in breast cancer has not yet been elucidated. Similar to AQP5, AQP3 is expressed in breast cancer and also stomach, esophageal, head and neck, as well as cervical cancers (19-22). In addition, the expression of AQP3 detected by RT-PCR has been correlated with advanced stage, large tumor size, lymphatic spreading and vascular invasiveness indicating that AQP3 may play roles in tumor angiogenesis, progression, invasion and metastasis (20, 21). A recent study of lung cancer cell lines also demonstrated that AQP3 knock-down by short hairpin RNA (shRNA) in a xenograft model inhibited tumor proliferation by inducing apoptosis and inhibiting angiogenesis (23). Other studies demonstrated that AQP3 and/or AQP5 promotes epithelial-mesenchymal transition (EMT) *via* the PI3K/AKT/Snail signaling pathway and is clinically or pathologically correlated with lymphovascular invasion and regional or distant metastasis in gastric cancer and hepatocellular carcinoma (24, 25). Accordingly, it was speculated that AQP3 expression could be a possible

Table III. *Multivariate analysis for survival in patients with HER2- over-expressing early breast cancer.*

		DFS			DDFS			OS		
Variables		*p*-Value	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value	HR	95%CI
Age (years), >50	≤50	0.463	1.502	0.506-4.456	0.778	1.176	0.382-3.624	0.627	1.440	0.331-6.266
ER/PR positive	*vs.* negative	0.305	0.573	0.198-1.660	0.289	0.534	0.168-1.703	0.066	0.271	0.068-1.089
Pathologic Stage (AJCC)§		0.514			0.414			0.015		
IIA	*vs.* I	0.411	1.822	0.436-7.606	0.291	2.427	0.468-12.602	0.970	1.035	0.173-6.201
IIB	*vs.* I	0.588	1.609	0.287-9.015	0.558	1.808	0.250-13.098	0.022	14.715	1.479-146.384
IIIA	*vs.* I	0.186	3.987	0.513-30.982	0.163	4.933	0.523-46.569	0.002	272.258	7.196-10301.008
Adjuvant chemotherapy		0.697			0.697			0.095		
classic CMF	*vs.* none	0.928			0.945			0.966		
Adriamycin-based	*vs.* none	0.920			0.939			0.976		
AC4→T4	*vs.* none	0.922			0.941			0.982		
AQP3 expression¶	*vs.* negative	0.036	3.137	1.079-9.125	0.070	2.784	0.921-8.414	0.179	2.439	0.665-8.943

DFS, Disease-free survival; DDFS, distant disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CMF, cyclophosphamide/methotrexate/5-fluorouracil; AC, adriamycin/cyclophosphamide; T, taxane; AIs, aromatase inhibitors; AQP3, aquaporin 3; AJCC, American Joint Committee on Cancer. †modified Scarff-Bloom-Richardson grading system. ‡according to immunohistochemical stain of ER, PR and HER2. ¶AQP3 expression in the cancer tissue was defined by the staining score of more than 2 calculated as the sum of IS (0 through 3) and PC (0 through 4) as follows: negative, 0 to 2; weak, 3 to 5; strong, 6 to 7.

Table IV. *Association between genotypes of selected AQP3 variants and AQP3 expression.*

AQP3 expression		rs2228332			rs2227285			rs10813981			rs34391490			rs591810			rs17553719			rs3860987		
		TT	TC	CC	GG	GC	CC	GG	GA	AA	GG	GA	AA	CC	CG	GG	AA	AG	GG	GG	GA	AA
Total	Negative	109	80	27	93	84	51	112	71	20	132	48	13	133	62	22	178	8	5	111	93	18
	positive	69	50	18	53	67	25	78	42	10	91	30	9	80	46	13	115	6	2	66	69	14
	p-Value			0.983			0.176			0.638			0.983			0.983			0.814			0.555
HER2-over-expressing	Negative	13	15	2	13	15	3	17	15	0	23	7	1	18	9	3	26	2	1	12	17	3
	positive	6	13	2	10	8	3	7	10	0	10	4	2	12	4	3	14	2	1	13	8	1
	p-Value			0.557			0.733			0.551			0.437			0.709			0.777			0.284

AQP3, Aquaporin 3; HER2, human epidermal growth factor receptor 2.

biomarker for survival after curative resection in early breast cancer (20). However, no correlation between AQP3 expression and survival was identified in the subjects enrolled in this study as it was also demonstrated for AQP5 in our previous study based on the same patients (12). Considering that the clinical or pathological features and survival outcomes vary with the pathologic or molecular subtypes of breast cancer, it is possible that AQP3 expression may differ according to the breast cancer subtype. Thus, a sub-group analysis revealed that AQP3 expression was associated with a poor recurrence-free survival in the patients with the HER-over-expressing subtype. To further investigate how AQP3 may affect prognosis in EBC, several clinicopathological factors influencing prognosis in EBC were analyzed. Yet, no statistical association has been found between those prognostic factors and the positive rates of AQP3 expression in the current study, thus suggesting that AQP3 expression is an independent prognostic factor for HER2-over-expressing breast cancer regardless of the disease status or pathological characteristics, such as the pathological stage and histological grade, which is inconsistent with the results for gastric and hepatocellular cancer (24, 25). Nevertheless, since the current result is the first cohort study for AQP3 expression in breast cancer, further studies are warranted for a definitive conclusion.

As polymorphisms in tumor-associated genes are rapidly being identified and investigated in human cancers as a novel class of variation, this study also investigated whether seven target variants of *AQP3*, selected using web-based data, were associated with the expression of AQP3 expression in tumor cells and prognosis for Korean ECB patients who underwent curative surgery. None of the selected variants was, nonetheless, found to be associated with AQP3 expression or the prognosis of EBC in the current study. However, while SNPs are thought to be attractive biomarkers as they are stably inherited, highly abundant and show diversity within and among populations, the application of individual SNPs is limited due to their penetrance and the difficulty involved in identifying their effects. Furthermore, since the selected variants were mostly deviated from the Hardy-Weinberg equilibrium in the current study, which can be partially explained by the small sample size for each variant genotype, caution is warranted in terms of drawing definite conclusion from the current study until the present results are further confirmed.

Recently, although ongoing clinical trials have not validated their results, several biological markers, such as Ki-67 (26, 27) and gene signatures (28-30), have been identified and introduced as important prognostic and predictive markers for EBC. Therefore, one of our future goals is to evaluate the association between AQP3 expression and these recently-discovered biological markers. In addition, as anti-HER2 therapy is currently a standard in combination with or following chemotherapy after complete surgery, the exact role of AQP3 expression needs to be clarified in an era of trastuzumab therapy, as none of the patients enrolled in the present study underwent adjuvant trastuzumab treatment. Moreover, due to the absence of a concrete scoring system or positivity guidelines for AQP expression, different scores have been used in different studies (24, 25) resulting in quite different AQP3 expression rates, thus emphasizing the need for validated IHC staining interpretation and more accurate scoring methods. Finally, despite the previous suggestion of EMT by molecular transduction using the EGFR/Ras/ERK signaling pathway and NF-kappaB pathway (24, 31), the specific intracellular mechanism related to AQP3 needs to be clarified in order to understand the exact role of AQP3 based on the correlation between AQP3 over-expression and pathological parameters, such as lymphovascular invasion and tumor stage, including regional and distant metastases.

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References

1 Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet *365*: 1687-1717, 2005.

2 Parisi M, Amodeo G, Capurro C, Dorr R, Ford P and Toriano R: Biophysical properties of epithelial water channels. Biophys Chem *68*: 255-263, 1997.

3 Agre P: The aquaporin water channels. Proc Am Thorac Soc *3*: 5-13, 2006.

4 Rojek AM, Skowronski MT, Fuchtbauer EM, Fuchtbauer AC, Fenton RA, Agre P, Frokiaer J and Nielsen S: Defective glycerol metabolism in aquaporin 9 (AQP9) knockout mice. Proc Natl Acad Sci USA *104*: 3609-3614, 2007.

5 Hoque MO, Soria JC, Woo J, Lee T, Lee J, Jang SJ, Upadhyay S, Trink B, Monitto C, Desmaze C, Mao L, Sidransky D and Moon C: Aquaporin 1 is overexpressed in lung cancer and stimulates NIH-3T3 cell proliferation and anchorage-independent growth. Am J Pathol *168*: 1345-1353, 2006.

6 Liu W, Wang K, Gong K, Li X and Luo K: Epidermal growth factor enhances MPC-83 pancreatic cancer cell migration through the upregulation of aquaporin 3. Mol Med Report *6*: 607-610, 2012.

7 Nico B, Annese T, Tamma R, Longo V, Ruggieri S, Senetta R, Cassoni P, Specchia G, Vacca A and Ribatti D: Aquaporin-4 expression in primary human central nervous system lymphomas correlates with tumour cell proliferation and phenotypic heterogeneity of the vessel wall. Eur J Cancer *48*: 772-781, 2012.

8 Chae YK, Kang SK, Kim MS, Woo J, Lee J, Chang S, Kim DW, Kim M, Park S, Kim I, Keam B, Rhee J, Koo NH, Park G, Kim SH, Jang SE, Kweon IY, Sidransky D and Moon C: Human AQP5 plays a role in the progression of chronic myelogenous leukemia (CML). PLoS One *3*: e2594, 2008.

9 Chae YK, Woo J, Kim MJ, Kang SK, Kim MS, Lee J, Lee SK, Gong G, Kim YH, Soria JC, Jang SJ, Sidransky D and Moon C: Expression of aquaporin 5 (AQP5) promotes tumor invasion in human non small cell lung cancer. PLoS One *3*: e2162, 2008.

10 Kang SK, Chae YK, Woo J, Kim MS, Park JC, Lee J, Soria JC, Jang SJ, Sidransky D and Moon C: Role of human aquaporin 5 in colorectal carcinogenesis. Am J Pathol *173*: 518-525, 2008.

11 Jung HJ, Park JY, Jeon HS and Kwon TH: Aquaporin-5: a marker protein for proliferation and migration of human breast cancer cells. PLoS One *6*: e28492, 2011.

12 Lee SJ, Chae YS, Kim JG, Kim WW, Jung JH, Park HY, Jeong JY, Park JY, Jung HJ and Kwon TH: AQP5 expression predicts survival in patients with early breast cancer. Ann Surg Oncol *21*: 375-383, 2014.

13 Ismail M, Bokaee S, Morgan R, Davies J, Harrington KJ and Pandha H: Inhibition of the aquaporin 3 water channel increases the sensitivity of prostate cancer cells to cryotherapy. Br J Cancer *100*: 1889-1895, 2009.

14 Nejsum LN, Kwon TH, Jensen UB, Fumagalli O, Frokiaer J, Krane CM, Menon AG, King LS, Agre PC and Nielsen S: Functional requirement of aquaporin-5 in plasma membranes of sweat glands. Proc Natl Acad Sci USA *99*: 511-516, 2002.

15 Jung JH, Chae YS, Moon JH, Kang BW, Kim JG, Sohn SK, Park JY, Lee MH and Park HY: TNF superfamily gene polymorphism as prognostic factor in early breast cancer. J Cancer Res Clin Oncol *136*: 685-694, 2010.

16 Papadopoulos MC, Saadoun S and Verkman AS: Aquaporins and cell migration. Pflugers Arch 456: 693-700, 2008.

17 Zhang ZQ, Zhu ZX and Bai CX: Lung fluorescence imaging to evaluate tumor metastasis induced by AQP5 expression in murine model. Med Oncol *29*: 205-211, 2012.
18 Saadoun S, Papadopoulos MC, Hara-Chikuma M and Verkman AS: Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption. Nature *434*: 786-792, 2005.
19 Shen L, Zhu Z, Huang Y, Shu Y, Sun M, Xu H, Zhang G, Guo R, Wei W and Wu W: Expression profile of multiple aquaporins in human gastric carcinoma and its clinical significance. Biomed Pharmacother *64*: 313-318,
20 Chen R, Shi Y, Amiduo R, Tuokan T and Suzuk L: Expression and prognostic value of aquaporin 1, 3 in cervical carcinoma in women of Uygur ethnicity from Xinjiang, China. PLoS One *9*: e98576, 2014.
21 Kusayama M, Wada K, Nagata M, Ishimoto S, Takahashi H, Yoneda M, Nakajima A, Okura M, Kogo M and Kamisaki Y: Critical role of aquaporin 3 on growth of human esophageal and oral squamous cell carcinoma. Cancer Sci *102*: 1128-1136, 2011.
22 Ishimoto S, Wada K, Usami Y, Tanaka N, Aikawa T, Okura M, Nakajima A, Kogo M and Kamisaki Y: Differential expression of aquaporin 5 and aquaporin 3 in squamous cell carcinoma and adenoid cystic carcinoma. Int J Oncol 41: 67-75, 2012.
23 Xia H, Ma YF, Yu CH, Li YJ, Tang J, Li JB, Zhao YN and Liu Y: Aquaporin 3 knockdown suppresses tumour growth and angiogenesis in experimental non-small cell lung cancer. Exp Physiol *99*: 974-984, 2014.
24 Chen J, Wang T, Zhou YC, Gao F, Zhang ZH, Xu H, Wang SL and Shen LZ: Aquaporin 3 promotes epithelial-mesenchymal transition in gastric cancer. J Exp Clin Cancer Res *33*: 38, 2014.
25 Guo X, Sun T, Yang M, Li Z, Li Z and Gao Y: Prognostic value of combined aquaporin 3 and aquaporin 5 overexpression in hepatocellular carcinoma. Biomed Res Int *2013*: 206525, 2013.
26 Urruticoechea A, Smith IE and Dowsett M: Proliferation marker Ki-67 in early breast cancer. J Clin Oncol 23: 7212-7220, 2005.
27 de Azambuja E, Cardoso F, de Castro G, Jr., Colozza M, Mano MS, Durbecq V, Sotiriou C, Larsimont D, Piccart-Gebhart MJ and Paesmans M: Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients. Br J Cancer *96*: 1504-1513, 2007.
28 Paik S: Development and clinical utility of a 21-gene recurrence score prognostic assay in patients with early breast cancer treated with tamoxifen. Oncologist *12*: 631-635, 2007.
29 Lyman GH, Cosler LE, Kuderer NM and Hornberger J: Impact of a 21-gene RT-PCR assay on treatment decisions in early-stage breast cancer: an economic analysis based on prognostic and predictive validation studies. Cancer *109*: 1011-1018, 2007.
30 Mook S, Schmidt MK, Weigelt B, Kreike B, Eekhout I, van de Vijver MJ, Glas AM, Floore A, Rutgers EJ and van 't Veer LJ: The 70-gene prognosis signature predicts early metastasis in breast cancer patients between 55 and 70 years of age. Ann Oncol *21*: 717-722, 2010.
31 Tsubaki M, Komai M, Fujimoto S, Itoh T, Imano M, Sakamoto K, Shimaoka H, Takeda T, Ogawa N, Mashimo K, Fujiwara D, Mukai J, Sakaguchi K, Satou T and Nishida S: Activation of NF-kappaB by the RANKL/RANK system up-regulates snail and twist expressions and induces epithelial-to-mesenchymal transition in mammary tumor cell lines. J Exp Clin Cancer Res *32*: 62, 2013.

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