Prognostic relevance of the *AQP5*-1364C>A polymorphism in primary breast cancer

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Abstract. Besides known prognostic factors in breast cancer, disseminated tumor cells are regarded as a surrogate marker for minimal residual disease in breast cancer. Their prognostic significance is comparable to that of lymph node status. However, the mechanism by which the primary tumor directs cells to disseminate into lymph nodes or into blood vessels is unclear. Aquaporins (AQPs) are small integral membrane proteins that provide a major pathway for water transport throughout several organs, and have been shown to be of potential importance in various types of cancer. Here, we analyzed the single nucleotide polymorphism (SNP) A(-1364)C in AQP5 in 107 patients with early stage breast cancer in order to test the hypothesis that this polymorphism is associated with lymphogenous and hematogenous tumor cell dissemination and overall survival. Paraffin-embedded tumor tissue was dewaxed, and DNA was extracted from tumor tissues using the QIAamp Blood DNA Mini Kit. The quantification and quality of the extracted DNA were determined spectrophotometrically. Genotyping of the -1364A>C polymorphism was performed by Pyrosequencing. Cytokeratin-positive (CK^+) bone marrow (BM) cells were isolated by density gradient centrifugation followed by immunocytochemistry, applying the pan CK antibody A45-B/B3. There was no evidence of an association between the AQP5C>A genotypes and an increased risk of developing breast cancer, nor between the genotypes and tumor size, lymph node involvement, distant metastasis, grade, histopathology, expression of estrogen receptor and HER2, or the dissemination of tumor cells to BM. In contrast, when comparing C-allele carriers with patients carrying the AA genotype, expression of the progesterone receptor differed significantly between these genotype groups (mean AA genotype 51%, mean AC and CC genotype 73%; p=0.039). Additionally, a significant correlation was found between progesterone receptor expression and adjuvant chemotherapy (p=0.021) and adjuvant endocrine therapy (p=0.017), respectively. SNPs in AQP5 are not associated with hematogenous or lymphogenous tumor cell spread. However, we observed for the first time an association between SNPs in AQP5 and progesterone receptor positivity, which might have implications for future adjuvant treatment. Further investigations must include more than one AQP as well as factors promoting angiogenesis to elucidate the different modes of tumor cell dissemination.

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Abbreviations: AQP, aquaporins; BM, bone marrow; CK^+, cytokeratin-positive; DTC, disseminated tumor cells; SNP, single nucleotide polymorphism

Key words: polymorphism, aquaporin 5, disseminated tumor cells, primary breast cancer

Introduction

Current therapy for patients with early stage breast cancer is determined by the size of the primary tumor, the presence or absence of axillary lymph node metastasis, the grade of tumor differentiation, receptor and HER2 status, age and the hormonal status of the patient. Although these patients are considered to be potentially curable, a substantial number develop recurrent carcinoma, including nearly 30% of those with negative axillary lymph nodes (1). This recurrence rate is explained by tumor cell dissemination to distant organs, in particular to bone marrow (BM). This is a common phenomenon, noted in breast cancer at primary diagnosis in up to 40% of patients (2-4). The presence of DTCs in BM is increasingly being regarded as a clinically relevant prognostic factor for breast cancer. A pooled analysis of BM findings in more than 4,700 patients documented that DTC presence is associated with a poor prognosis (5). It has been demonstrated that these cells show a considerable heterogeneity in the expression of carcinoma-associated cell surface molecules, (6) and that they survive even high-dose chemotherapy (7). Their persistence in BM after conventional adjuvant chemotherapy is associated with poor prognosis (8-10). Although it has been demonstrated that node-negative but BM-positive patients

and node-positive but BM-negative patients have the same prognosis (3), no data have been published regarding the mechanisms that promote the lymphogenous or hematogenous dissemination of these cells.

The transport of water and some solutes across biological membranes is mediated by a family of membrane proteins termed aquaporins (AQPs), small integral membrane proteins which provide a major pathway for water transport in the kidneys, brain, secretory epithelia and other organs. AQPs have been documented in the reproductive tract of males and females, and their expression has been related to the formation of seminiferous fluid, a process controlled by steroid hormones (11). To date, eleven mammalian AQPs have been identified. These are distributed ubiquitously throughout the human body, and are believed to play a role in hypertension and heart failure (12,13) and, moreover, in oncology, carcinogenesis and angiogenesis (14-22).

AQP5, a 27-kDa protein, was first cloned from the salivary gland and is known to be an exocrine-type water channel with ubiquitous tissue expression. Rare mutations in AQP5 have been detected in patients with Sjögren's syndrome (23), a disorder with decreased salivary and lacrimal secretions. Very recently, single nucleotide polymorphisms (SNPs) in AQP5, which may be capable of altering gene expression and/or function, were associated with alterations of variables of the renin-angiotensin-aldosterone system in young healthy males and in patients with coronary artery disease (24). The expression of AQP5 has been demonstrated to promote tumor invasion in human non-small cell lung cancer (25), is involved in the progression of chronic myelogenous leukemia (26), and plays an key role in ovarian carcinogenesis (27). However, to date no data have been published concerning AQP5 expression and breast cancer.

Here, we analyzed the SNP A(-1364)C in AQP5 in 107 patients with early stage breast cancer, and tested the hypothesis that this SNP is associated with lymphogenous and hematogenous tumor cell dissemination and overall survival.

Patients and methods

Tissue specimens. Tissue specimens from 107 female Caucasian patients with unilateral invasive breast cancer were retrieved from the Institute of Pathology and Neuropathology, University Hospital of Essen. All patients had undergone surgery with curative intent between 1998 and 2003 at the Clinic of Gynecology and Obstetrics. Informed written consent was obtained from the patients, and the study was approved by the Local Essen Research Ethics Committee. Overall survival data for these patients were obtained from the local municipal registry; the median follow-up time was 74 months (0-119 months). For each of the 107 patients, tumor type, TNM stage and grade were assessed according to the WHO Classification of Tumors of the Breast (28) and the sixth edition of the TNM Classification System (29). Oestrogen and progesterone receptor status as well as the Dako score were determined by immunohistochemistry (30).

Preparation of paraffin-embedded tumor tissue. Tumor tissue specimens were retrieved from the Institute of Pathology and Neuropathology. Tumor pieces (3 mm) were processed from paraffin-embedded tumor blocks and re-embedded in paraffin to form 6 sections of 10-20 μm thickness. The sections were placed in a 1.5-ml microfuge tube and dewaxed in 1 ml xylene on a shaker incubator at 45˚C for 5 min. After centrifugation at full speed for 5 min at room temperature, the supernatant was removed. Pellets were washed in 1 ml of ethanol and again centrifuged at full speed for 5 min. The supernatant was removed and the open microfuge tube was incubated at 45˚C for 2-5 min until the ethanol had evaporated.

Genomic DNA was extracted from tumor tissues using the QIAamp Blood DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantification and quality of the extracted DNA were determined spectrophotometrically.

Control samples. Control samples consisted of 122 female healthy Caucasian individuals who were recruited at the local Department for Transfusion Medicine, University Hospital of Essen.

DNA genotyping for the -1364A>C SNP. DNA was extracted from whole blood or heart tissue using the QIAamp Kit (Qiagen). The -1364A>C polymorphism was genotyped by Pyro-sequencing®. PCR was performed using forward primer AQP5-SE 5'-GAAACTGCAGGATGAGAGAAAT-3', and biotinylated reverse primer AQP5-AS 5'-TCTCTGTTCTCC ACCTCTCCA-3', resulting in a 120-nt fragment. After denaturing at 94˚C, 40 cycles of DNA amplification were carried out using Taq PCR Mastermix (Eppendorf, Hamburg, Germany) at 94˚C for 40 sec, 53˚C for 40 sec and 72˚C for 40 sec. The biotinylated strand was captured on streptavidin sepharose beads and annealed with sequencing primer AQP5-Seq 5'-CAGAGAGACTAAGACAGCA-3'. Pyro-sequencing was performed using PSQ HS 96 Gold SNP Reagents and the PSQ HS 96 (Biotage, Uppsala, Sweden).

Preparation of bone marrow. From each patient, bilateral BM aspirations (5-10 ml) were obtained under general anesthesia from the upper iliac crests by needle aspiration under normal coagulation parameter conditions. BM cells were isolated from heparinized BM (5000 U/ml BM) by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400 x g for 30 min. Interface cells were washed (400 x g for 15 min) and resuspended in phosphate-buffered saline (PBS). Cells ($3x10^6$) ($1x10^6$ per slide per area of 240 mm^2) from each aspiration side were spun directly onto glass slides (400 x g for 5 min) coated with poly-L-lysine (Sigma, Deisenhofen, Germany) using a Hettich cytocentrifuge (Tuttlingen, Germany) for the detection of cytokeratin-positive (CK^+) cells.

Immunocytochemistry. After overnight air drying, staining for CK^+ cells was performed using the Epimet® Kit (Micromet, Martinsried, Germany). The identification of epithelial cells using this kit is based on the reactivity of the murine monoclonal antibody Mab A45-B/B3, directed against a common epitope of CK polypeptides. The kit uses Fab fragments of the pan-Mab complexed with alkaline phosphatase molecules. The method followed includes i) permeabilization of the cells with a detergent (5 min), ii) fixation with

Table I. Correlation of the AQP5 polymorphism with clinicopathological data in patients with breast cancer.

Parameters	No. patients (%) (n=107)	No. patients by AQP5 genotype (%)			P-value
		AA (n=74; 69%)	AC (n=27; 25%)	CC (n=6; 6%)	
Mean age at first diagnosis (range)	54 (25-86)	52 (25-86)	57 (31-80)	58 (48-69)	0.132
T					
1	50 (47)	33 (45)	13 (48)	4 (67)	0.499
2	50 (47)	35 (47)	14 (52)	1 (17)	
3	1 (1)	1 (1)	0	0	
4	6 (6)	5 (7)	0	1 (17)	
N					
0	53 (50)	33 (45)	16 (59)	4 (67)	0.631
1	50 (47)	38 (52)	10 (37)	2 (33)	
2	3 (3)	2 (7)	1 (4)	0	
M1	3 (3)	2 (3)	1 (4)	0	0.880
Grade					
1	10 (10)	6 (8)	3 (12)	1 (17)	0.968
2	51 (49)	35 (48)	13 (52)	3 (50)	
3	42 (40)	31 (43)	9 (36)	2 (33)	
4	1 (1)	1 (1)	0	0	
Histopathology					
Ductal	88 (87)	58 (83)	24 (96)	6 (100)	0.151
Lobular	8 (8)	8 (11)	0	0	0.146
Medullar	1 (1)	0	1 (4)	0	0.215
Other	4 (4)	4 (6)	0	0	0.398
ER-positive	73 (68)	47 (64)	22 (82)	4 (67)	0.228
PR-positive	62 (58)	38 (51)	19 (70)	5 (83)	0.099
	62 (58)	38 (51)	24 (73)		**0.039**
Her2Neu					
0	63 (65)	42 (63)	18 (75)	3 (50)	0.315
2	13 (13)	10 (15)	3 (13)	0	
3	21 (22)	15 (22)	3 (13)	3 (50)	
CK^+ cells in BM at first diagnosis	43 (40)	32 (43)	9 (33)	2 (33)	0.627

ER, estrogen receptor; PR, progesteron receptor; CK^+, cytokeratine-positive; BM, bone marrow.

a formaldehyde-based solution (10 min), iii) binding of the conjugate Mab A45-B/B3-alkaline phosphatase to cytoskeletal CKs (45 min), and iv) formation of an insoluble red reaction product at the site of the binding of the specific conjugate (15 min). Subsequently, the cells were mounted with Kaiser's glycerin/gelatine (Merck, Darmstadt, Germany) in Tris EDTA buffer (Sigma). A negative control antibody (conjugate of Fab-fragment; Micromet) served as the negative control. For each test, a positive control slide with the breast carcinoma cell line MCF-7 (ATTC, Rockville, MD) was treated under identical conditions. A BM analysis of 165 non-carcinoma controls resulted in only two false-positive results, indicating that A45B/B-3 gives reliable results for the detection of single DTCs (31).

Evaluation of CK^+ cells. Microscopic evaluation was carried out using the Ariol SL-50 (Applied Imaging), an automated scanning microscope and image analysis system with a slide loader, camera, computer and software for the detection and classification of cells of interest based on a particular color, intensity, size, pattern and shape. To use the system, immuno-stained slides are loaded onto the SL-50 slide loader. The system loads each slide in turn onto the automated microscope stage and scans each frame of the cytospin through red, green and blue filters to recognize candidate objects. These objects are automatically scrutinized by color subtraction, then analyzed and classified by 23 different morphometric parameters prior to final color ratio analysis. Quantitative data

Table II. Correlation of the AQP5C>A polymporphism with breast cancer-related therapies, disease-free survival and overall survival.

Parameter	No. patients (%) (n=107)	No. patients by AQP5 genotype (%) AA (n=74; 69%)	AC (n=27; 25%)	CC (n=6; 6%)	P-value
Type of surgery					
Breast-sparing	70 (67)	48 (66)	18 (67)	4 (80)	0.808
Mastectomy	35 (33)	25 (34)	9 (33)	1 (20)	
Adjuvant chemotherapy	66 (62)	51 (69)	12 (44)	3 (50)	0.068
	66 (62)	51 (69)	15 (46)		**0.021**
Adjuvant radiotherapy	77 (72)	51 (69)	20 (74)	6 (100)	0.254
Adjuvant endocrine treatment	72 (68)	45 (61)	21 (81)	6 (100)	**0.038**
	72 (68)	45 (61)	27 (84)		**0.017**
Recurrent disease	17 (16)	12 (16)	4 (15)	1 (17)	0.948
DFS (months)	96	98	92	72	0.972
OS (months)	102	102	100	79	0.824

Disease-free survival (DFS) and overall survival (OS) were estimated according to the Kaplan-Meier method.

and high-quality images of analyzed objects were presented for review and classification.

Statistical analysis. Patients were classified as tumor cell positive when at least one CK^+ cell was detected by immunocytochemistry. Data from reporter assays were analyzed using the Wilcoxon signed rank test. Continuous parametric variables were compared by the unpaired or paired t-test, as indicated. Nonparametric variables were compared using the Mann Whitney U test and Kruskal-Wallis test, as indicated. All genotype distributions were tested for accordance with the Hardy-Weinberg equilibrium using a goodness-of-fit χ^2 test. Differences were regarded as significant at $p<0.05$. All statistical analyses were conducted using SPSS 15.0 (SPSS, Chicago, IL, USA). Continuous variables are presented as the means ± SEM.

Results

AQP5 -1364C>A genotype distribution in cases and controls. In the control group consisting of unrelated healthy Caucasian blood donors, the genotype distribution of patients was 74 AA (69%), 43 AC (36%) and 2 CC (2%). In patients with breast cancer, the genotype distribution was 74 AA (62%), 27 AC (25%) and 6 CC (6%). These genotype distributions were in accordance with/within Hardy Weinberg equilibrium. As both genotype distributions and allele frequencies were not significantly different in patients (A-allele frequency 0.82) and controls (A-allele frequency 0.80), there was no evidence for an association between *AQP5* -1364C>A genotype and an increased risk of developing breast cancer.

Correlation of the AQP5 polymorphism with clinicopathological data in patients with breast cancer. Clinicopathological data of all patients and data upon stratification by AQP5 genotypes are displayed in Table I. We found no association between the *AQP5* -1364C>A genotype and tumor size, lymph node involvement, distant metastasis, grade, histopathology, expression of estrogen receptor and HER2. In contrast, comparing the C-allele carriers with patients carrying the AA genotype, expression of the progesterone receptor was significantly different between the genotype groups (mean AA genotype 51%, mean AC and CC genotype 73%; p=0.039). No association could be drawn between the presence of DTCs in BM and the AQP5 polymorphism.

Correlation of the AQP5C>A polymporphism with breast cancer-related therapy, disease-free and overall survival. Data regarding breast cancer-related therapy, disease-free and overall survival in correlation with the AQP5 genotype are documented in Table II. Comparing the C-allele carriers with patients carrying the AA genotype, significantly more progesterone receptor-positive tumors (p=0.039) were registered, resulting in significant differences in adjuvant chemotherapy (p=0.021) and adjuvant endocrine therapy (p=0.017). No correlations were found between the *AQP5* -1364C>A polymorphism and the type of surgery performed (breast sparing vs. mastectomy), adjuvant radiotherapy, recurrent disease or disease-free survival. The median survival time of the entire group was 102 months.

Discussion

Tumor cell dissemination is a common phenomenon that is noted in breast cancer patients – even at primary diagnosis – in up to 40% of cases. The prognostic significance of DTCs has been clearly demonstrated, and has a value comparable to that of lymph node status, which has for years been the single most

reliable predictor of final outcome in breast cancer and the primary determinant for the use of systemic therapy (3). However, the mechanism by which the primary tumor directs cells to disseminate into lymph nodes or blood vessels is unclear.

Concomitant changes in water and sodium homeostasis could contribute to or modulate the development of pathologies known to be associated with disturbed water and sodium handling. Using RT-PCR, immunohistochemistry and Western blotting, the potential importance of AQPs in oncology and carcinogenesis has been demonstrated in various types of cancer. AQP1 water channels have been identified in multiple myeloma (32), pancreatic adenocarcinoma (33), renal cell carcinoma (34), erythroleukemia cells (35), mammary carcinoma (36), glioblastomas and astrocytomas (37), in which other AQPs including AQP4 are also known to be involved (38-40). The expression of AQP1 and AQP5 is induced in the early stages of colorectal carcinogenesis (22,41), promotes tumor invasion in human non-small cell lung cancer (25), is involved in the progression of chronic myelogenous leukemia (26), and plays an important role in ovarian carcinogenesis (27,42). However, concerning AQPs in breast cancer, the published data are limited. AQP1 could not be detected in a series of liver metastases of metastatic breast cancer patients (15), and Mobasheri *et al* demonstrated that the expression of AQP1 was differentially altered in tumors of the breast (16), showing that AQP1 expression was upregulated in the microvessel endothelia and neoplastic cells of selected ductal adenocarcinomas. This was in agreement with data previously published by Endo *et al* (36).

It is well established that increased microvessels (i.e., increased angiogenesis) are a feature of breast cancer. Consequently, it seems likely that AQPs are conditionally expressed in the neoplastic cells of mammary tumors, and that they may promote tumor cell dissemination into distant organs.

We did not find any association between the *AQP5* -1364C>A genotype and tumor size, distant metastasis, grade, histopathology, or the expression of estrogen receptor and HER2. Furthermore, no relationship between SNPs in AQP5 and tumor cell dissemination to the lymph nodes or BM was found. Different hypotheses need to be discussed with regard to our findings. Tumor cell extravasation into surrounding tissue involves the active migration of tumor cells across the endothelial barrier and their penetration through the underlying basement membrane. In this regard, Hu and Verkman found that AQP1-expressing tumor cells have increased metastatic potential and local invasiveness compared to control cells, but similar adherence to various basement membrane substrates and similar growth rates (18). Furthermore, it is well known that tumor microvessels exhibit abnormalities, including fragmented basement membranes and the absence of pericytes, leading to increased permeability for plasma proteins, cytokines and other macromolecules. The resulting increase in hydrostatic interstitial pressure may induce factors such as vascular endothelial growth factor (VEGF), which may in turn promote either lymphangiogenesis or hemangiogenesis.

On the other hand, the expression of several types of AQPs in tumor cells may be advantageous compared to normal cells with limited AQP expression, and it is possible that tumor cells may require several AQPs for high metabolic turnover or the tumor-specific metabolic pathways needed for survival. Thus, the coexpression of AQP5 with other AQPs might be of interest in breast cancer. This assumption also holds true for ovarian cancer, where a positive correlation between lymph node metastasis and the expression of AQP5 protein and mRNA was demonstrated, although without statistical significance (27).

One significant finding in our study was the fact that, in comparing the C-allele carriers with patients carrying the AA genotype, the parameters of the expression of progesterone receptor, adjuvant chemotherapy and adjuvant endocrine therapy differed significantly between the genotype groups. However, this observation did not result in a significant difference in survival, though the endocrine response of tumors is a prognostic parameter. The association between the *AQP5* -1364C>A genotype and progesterone receptor status cannot be explained functionally. A relationship between AQPs and hormones has been studied in the rat uterus. Branes *et al* demonstrated that the expression of AQP5, 8 and 9 was differentially regulated by ovarian hormones, and provided evidence that different levels of AQP9 corresponded to 17ß-estradiol and P4 at the mRNA and translation levels, respectively (43). In addition, an increase in apical AQP5 protein expression in luminal epithelial cells was shown to be controlled by progesterone (44). However, the reciprocal interaction of AQPs with hormone receptor expression has not been described.

In conclusion, although several expression profiles have been provided along with preliminary functional evidence, confirmatory data on the role of AQPs as one of the key elements directly involved in human carcinogenesis is lacking. Particularly in gynaecological cancer, further investigations must include more than one aquaporin as well as factors promoting angiogenesis to explain the different modes of tumor cell dissemination.

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