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Prognostic Value of Aquaporin-3, Vimentin and E-cadherin Expressions in Invasive Breast Carcinoma: An Immunohistochemical Study

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Abstract

Background: The preponderance of breast cancer-related deaths are the result of local invasion and distant metastasis; therefore, it is necessary to identify the factors underlying invasion and metastasis in order to develop novel treatment strategies and improve the survival of patients. In this regard, this study aimed to investigate the immunohistochemical expression and prognostic impact of aquaporin-3 (AQP3) and certain markers associated with epithelial-mesenchymal transition concerning invasive breast carcinoma of no special type.

Method: Immunohistochemical expressions of AQP3, vimentin and E-cadherin were performed in 50 paraffin embedded specimens of such cases. We also assessed the relationship of their expressions with the clinicopathological variables and patients' disease-free survival and overall survival.

Results: There were significant associations between positive AQP3 and positive vimentin expressions and high tumor grade, large tumor size, lymph node metastasis, and advanced tumor stage. On other hand, negative E-cadherin expression had a significant correlation with high tumor grade, large tumor size, lymph node metastasis, distant metastasis, and advanced tumor stage. A significant association also existed between positive AQP3, positive vimentin and negative E-cadherin expressions and high tumor recurrence, short 'three-year' disease-free survival and overall survival.

Conclusion: Positive AQP3, positive vimentin, and negative E-cadherin expressions are known as adverse prognostic markers and may predict survival in invasive breast carcinoma of no special type. It is proposed that AQP3 might play a role in breast cancer progression, invasion, and metastasis through induction of epithelial-mesenchymal transition.

Keywords: Aquaporin -3, Vimentin, E-Cadherin, Breast, Invasive carcinoma, Epithelial mesenchymal transition

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Introduction

Breast cancer is one of the most prevalent causes of cancer-related mortality among women in developing countries such as Egypt.[1] Approximately 90% of breast cancer deaths are caused by local invasion and distant metastasis of tumor cells.[2] Although the mechanisms of breast cancer invasion and metastasis are not completely fathomed, there exists a general agreement that the metastatic cascade encompasses changes in phenotypic features. These changes transform mammary epithelial cells to mesenchymal cells; therefore, the epithelial cells have the capacity to invade other tissues and establish metastatic tumors. This phenomenon is known as epithelial-mesenchymal transition (EMT).[3] Understanding the complex steps of EMT and metastasis could contribute to the development of enhanced antimetastatic drug strategies against the circulating metastatic cells and therapy-resistant cancer cells.[4]

EMT is a primary developmental process occurring in the early stages of embryogenesis. In these stages certain epithelial cells change to form a third layer of embryonic disc, called mesenchyme.[5] This process occurs in some tumors where the epithelial cells lose their polarity and acquire the invasive properties of cancers. Therefore, EMT plays a pivotal role in the invasive characteristics and tumor metastases of cells.[6] Studying this process requires investigating the expression of markers regarding 'epithelial and mesenchymal' components. Epithelial markers include the E-cadherin, while the vimentin is a mesenchymal one. Moreover, aquaporin-3 (AQP3) induces the suppression of E-cadherin and promotes EMT in gastric cancer.[7]

AQP3 is a small, integral transmembrane protein playing an important role in cellular homeostasis and water/glycerol transport across cell membrane. In addition to its physiological functions, evidence points to its role in carcinogenesis, tumor progression, and invasion of tumor cells. Different studies have reported the overexpression of AQP3 in various types of human cancer, aggravating the EMT of cancer cells; however, its mechanism is yet to be fully elucidated.[8, 9] Also, AQP3 has received much scientific attention over the past years as a potential novel targeted antitumor therapy reducing the invasion and metastasis of cancer cells.[10] AQP3 was associated with tumor progression and prognosis in many cancers such as squamous cell carcinoma of esophagus and cervix, head and neck cancer, and cancer ovary.[9,11,12] However, very few studies have investigated its role and prognostic values in breast cancer.[5]

Vimentin and E-cadherin are EMT-related molecules.[6] Vimentin is an intermediate filament protein normally expressed in cells of mesenchymal origin. However, it can also be expressed in epithelial cells undergoing EMT.

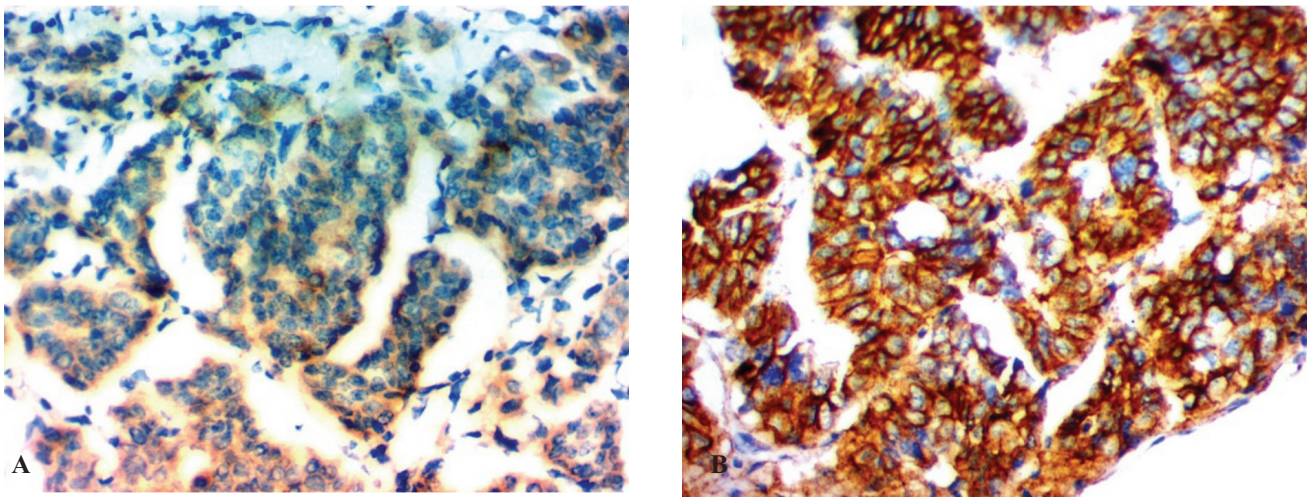

Figure 1. AQP-3 immunohistochemical expression: (A) Invasive carcinoma NST showing negative AQP-3 expression, (B) Invasive carcinoma NST showing strong positive membranous AQP-3 expression (Original magnification, ×400).

Vimentin is expressed aberrantly in epithelial cancers of prostate, gastrointestinal tract, central nervous system, lung, and melanomas.[13] Being a mesenchymal marker, this molecule is overexpressed in EMT in intrahepatic cholangiocarcinoma. On the other hand, E-cadherin, an epithelial marker, decreases in EMT tumors.[6] E-cadherin is a Ca2-dependent transmembrane glycoprotein mediating cellular adhesion in normal epithelial cells. Expression of E-cadherin decreases; then, the epithelial tumor cells lose their adhesion and become migratory and invasive; thus, this glycoprotein has a central role in the cellular motility and invasion during EMT.[14,15] E-cadherin also plays a major role in cell-cell junctions. The loss of E-cadherin expression in epithelial cells is regarded as the most important hallmark of EMT; this loss induces the destruction of intracellular junction; therefore, epithelial cells acquire the ability to migrate.[16]

Accordingly, the present study evaluated the expression of AQP3 with key molecules involved in EMT (E-cadherin and vimentin) regarding invasive breast carcinoma no special type (NST); we also correlated their expressions with different clinicopathological parameters and patients' survival in trial to assess their role in breast cancer progression; ultimately, we assessed their prognostic values in these cases.

Materials and Methods

Patients and tissue specimens

We conducted the current retrospective study in the Departments of Pathology, Clinical Oncology, and General Surgery, Zagazig University, Egypt. In this study, the archive of Pathology Department provided 50 formalin-fixed paraffin-embedded tissue specimens of invasive breast carcinoma NST from November 2013 to November 2015. Inclusion criteria were all invasive breast cancer cases of NST.

Exclusion criteria were as follows:

* Carcinomas of other histological types.
* Patients receiving neoadjuvant chemotherapy.
* Patients refusing to share in the study and those losing follow-up.

A general surgeon and a clinical oncologist contacted all patients. All subjects signed informed consent. The local Ethics Committee of our faculty approved the study (Ethics code: ZU_IRP#5700/17-11-2015).

We retrieved the clinicopathological data from the patients' files, including age, family history, tumor size (T), tumor grade, lymph node status (N), distant metastasis (M), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) status and follow-up data. We cut formalin fixed paraffin-embedded tissue specimens into 4-5µm thick sections and stained them with hematoxylin and eosin for light microscopic examination. We reviewed all cases for histological type,

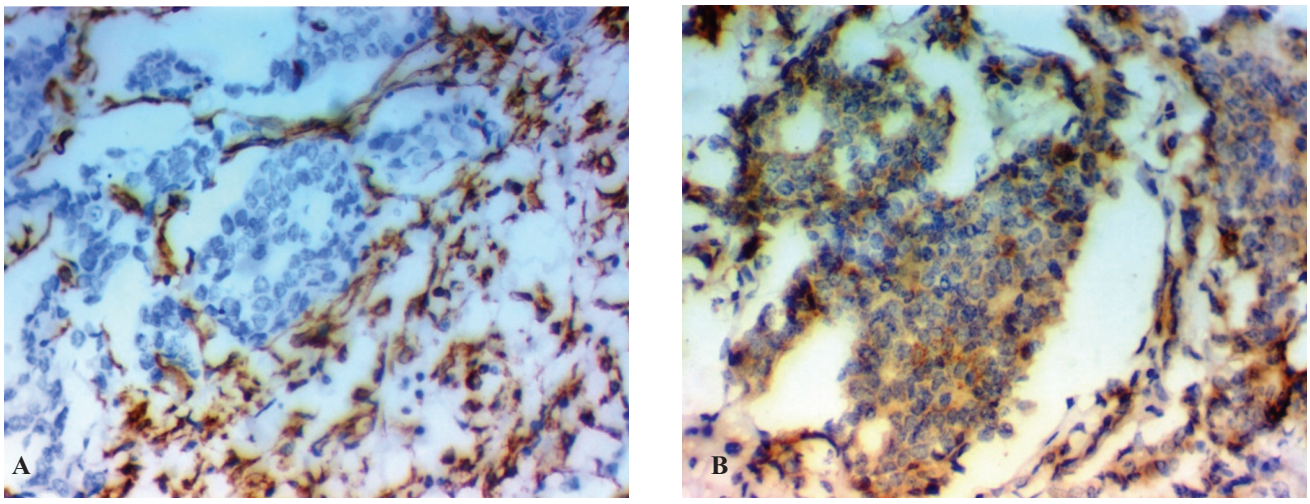

Figure 2. Vimentin immunohistochemical expression: (A) Invasive carcinoma NST showing negative vimentin expression of tumor cells and positive immunoreactivity of stromal cells, (B) Invasive carcinoma NST showing negative vimentin expression of tumor cells (original magnification, ×400).

histological grade, and lymph node status. According to Modified Scarff–Bloom–Richardson grading system, we calculated the grading. The tumors were classified according to the WHO classification[17] and staged based on TNM staging system.[18]

Immunohistochemical procedure

We stained paraffin sections of 4-5 µm using the streptavidin-biotin-peroxidase technique, deparaffinized the tissue sections in xylene, and rehydrated them through graded alcohol. Boiling in citrate buffer (pH 6.0) for 20 min provided the epitope then washed in phosphate buffer saline. We incubated the tissue sections overnight with a rabbit polyclonal anti-AQP3 antibody (dilution 1:200; Abcam, Cambridge, UK), a mouse monoclonal anti-E-cadherin antibody (clone SPM471, dilution 1:100; Thermo Scientific, CA, USA), and a mouse monoclonal antivimentin antibody (Clone V9, dilution 1:100, Thermo Scientific, CA, USA). Via Meyer's hematoxylin, we counterstained, dehydrated, and mounted the slides.

Evaluation of AQP3 immunostaining

We scored AQP3 immunostaining based on the staining intensity and percentage of stained tumor cells showing membranous positivity. The staining intensity scores were 0 (no staining), 1 (faint/barely perceptible membrane staining), 2 (weak to moderate), and 3 (strong).The percentage of positive cells was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (>75%) for positive tumor cells. We calculated the final scores (0-7) as the sum of intensity score and the percentage of positive cells. A staining score of 0-2 was negative, 3-5 was weak positive, and more than 6 was strong positive.[8]

Evaluation of vimentin immunostaining

We analyzed vimentin expression according to immunoscore calculated as follows: Immunoscore = % of positive cells × staining intensity [no staining (0), weak (1+), moderate (2+), strong (3+)]. A score of more than 10 was considered as positive.[13]

Evaluation of E-cadherin immunostaining

E-cadherin expression was semiquantitatively analyzed according to the percentage of cells showing membrane positivity into negative immunostaining as 0 (0-10%), and positive immunostaining as +1 (11-30%), +2 (31-70%), + 3 (>70%).[14]

Follow-up

We performed the follow-up of the patients at clinical oncology and general surgery departments; the objective was the early detection of local recurrence, distant metastasis, treatment complication management, and assignment of clinical outcome in the form of disease-free survival (DFS) and overall survival (OS). We

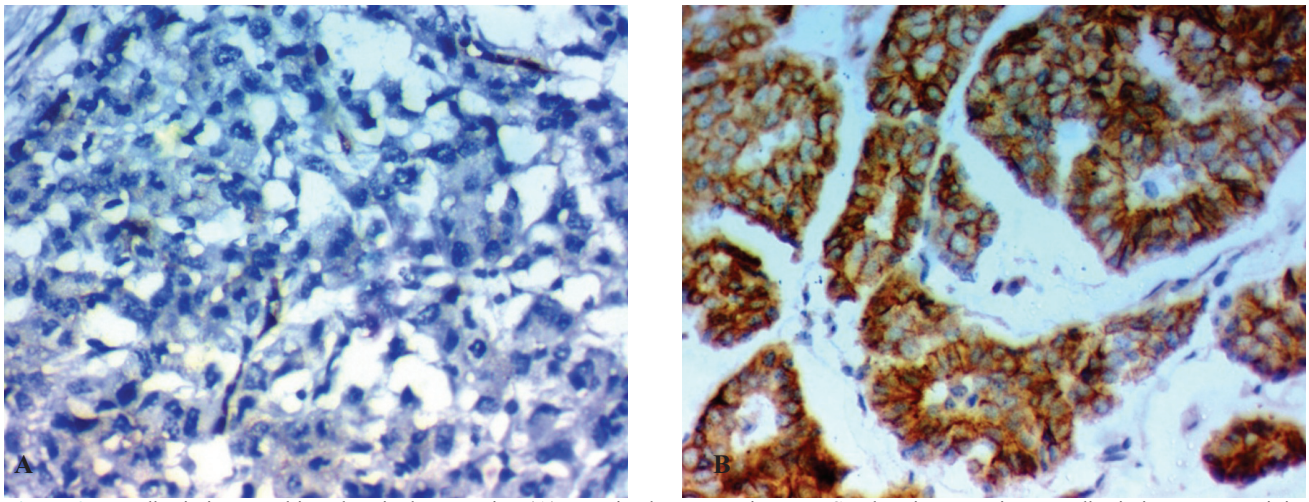

Figure 3. E-cadherin immunohistochemical expression (A) Invasive breast carcinoma NST showing negative E-cadherin immunoreactivity (B) Invasive carcinoma NST showing positive membranous E-cadherin immunoreactivity (original magnification, ×400).

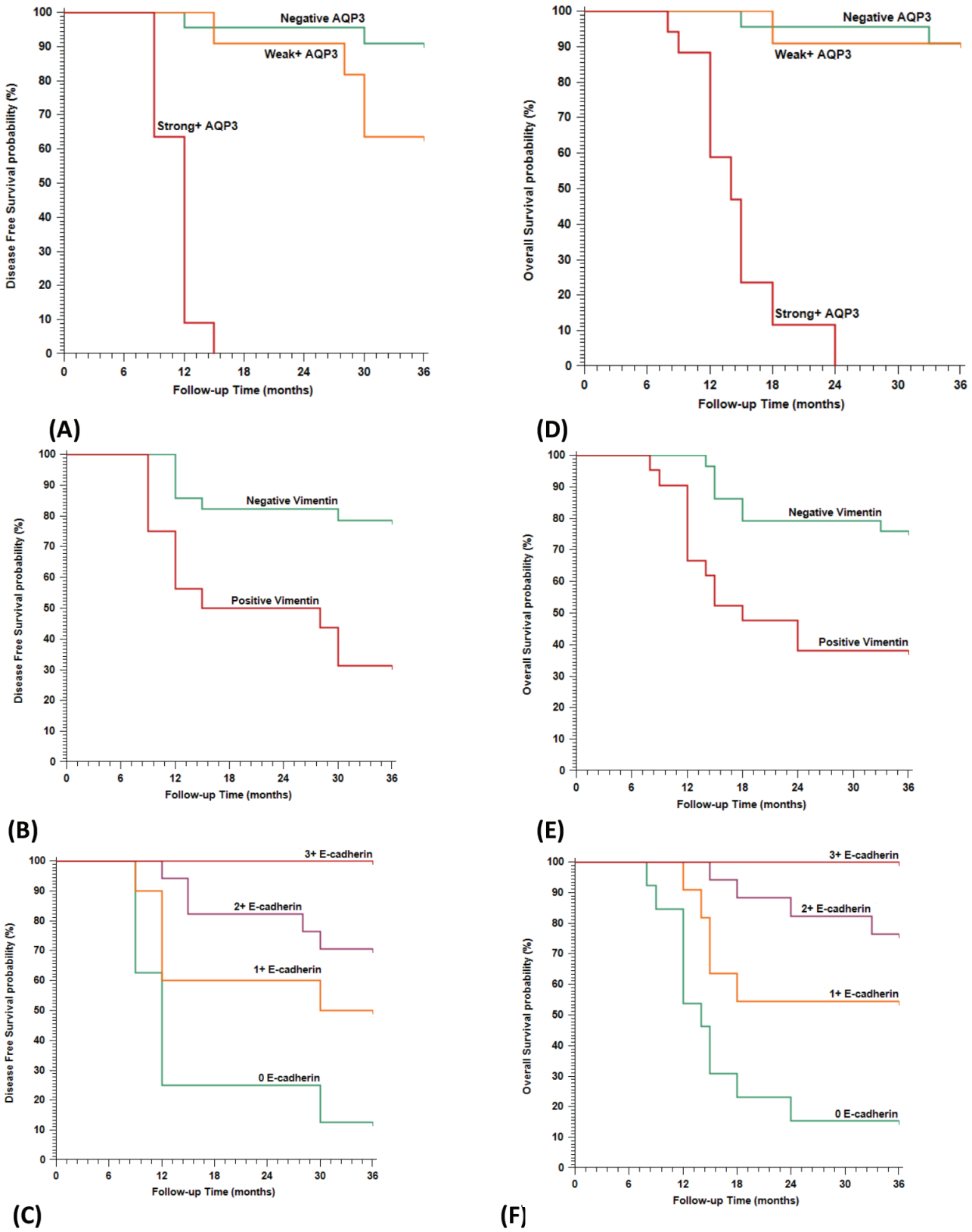

Figure 4. Kaplan Meier plot, Left panel: Disease-free survival, Right panel: Overall survival; (A & D) stratified by AQP3, (B & E) stratified by vimentin & (C & F) stratified by E-cadherin.

Table 1. Clinicopathological features, immunohistochemical markers and outcome of 50 patients with invasive breast carcinoma of no special type (NST).

Characteristics	All patients (N=50) No.	%
Age		
≤50 years	19	38%
>50 years	31	62%
Menstrual status		
Premenopausal	20	40%
Postmenopausal	30	60%
Family history		
Negative	43	86%
Positive	7	14%
Grade		
Grade I	6	12%
Grade II	27	54%
Grade III	17	34%
LVI		
Absent	32	64%
Present	18	36%
LN metastasis		
Absent	11	22%
Present	39	78%
pT		
T1	6	12%
T2	21	42%
T3	18	36%
T4	5	10%
pN		
N0	11	22%
N1	10	20%
N2	19	38%
N3	10	20%
M		
M0	44	88%
M1	6	12%
TNM stage		
Stage I	6	12%
Stage IIA	5	10%
Stage IIB	9	18%
Stage IIIA	16	32%
Stage IIIB	4	8%
Stage IIIC	4	8%
Stage IV	6	12%

Characteristics	All patients (N=50) No.	%
ER		
Negative	16	32%
Positive	34	68%
PR		
Negative	18	36%
Positive	32	64%
Her2/neu		
Negative	39	78%
Positive	11	22%
Molecular subtype		
Luminal A	20	40%
Luminal B	14	28%
Her2 enriched	6	12%
Triple negative	10	20%
AQP3		
Negative	22	44%
Weak+	11	22%
Strong+	17	34%
Vimentin		
Negative	29	58%
Positive	21	42%
E-cadherin		
0	13	26%
1+	11	22%
2+	17	34%
3+	9	18%
Follow-up duration	(months)	
Mean±SD	27.90±10.64	
Median (Range)	36 (8 – 36)	
Relapse	(N=44)	
Absent	27	61.4%
Present	17	38.6%
Death	(N=50)	
Alive	30	60%
Died	20	40%

Categorical variables were expressed as number (percentage). Continuous variables were expressed as mean ± SD & median (range).
ER: Estrogen; PR: Progesterone; Her2/neu: Human epidermal growth factor receptor 2; AQP3: Aquaporin-3; LVI: Lymph vascular invasion; LN: Lymph node; T: Tumor size; N: Lymph node; M: metastasis.

asked the patients for regular visits every three months in the first two years, every six months in the third year of follow-up, and annually.

Statistical analysis

Continuous variables were expressed as the mean ± SD & median (range), and the categorical variables were expressed as a number

Table 2. Relation between clinicopathological features and immunohistochemical staining for AQP3 and vimentin in IC NST patients (N=50). (continued)

	All	AQP3				Vimentin		
	(N=50)	Negative (N=22)	Weak+ (N=11)	Strong+ (N=17)	*P*-value	Negative (N=29)	Positive (N=21)	*P*-value
Characteristics	**No. %**	**No. %**	**No. %.**	**No. %**		**No. %**	**No. %**	
Age								
≤50 years	19(38%)	10(52.6%)	1(5.3%)	8(42.1%)	0.082‡	11(57.9%)	8(42.1%)	0.991‡
>50 years	31(62%)	12(38.7%)	10(32.3%).	9(29%)		18(58.1%)	13(41.9%)	
Menstrual status								
Premenopausal	20(40%)	9(45%)	1(5%)	10(50%)	0.032‡	10(50%)	10(50%)	0.349‡
Postmenopausal	30(60%)	13(43.3%)	10(33.3%)	7(23.3%)		19(63.3%)	11(36.7%)	
Family history								
Negative	43(86%)	18(41.9%)	10(23.3%)	15(34.9%)	0.737‡	25(58.1%)	18(41.9%)	1.000‡
Positive	7(14%)	4(57.1%)	1(14.3%).	2(28.6%)		4(57.1%)	3(42.9%)	
Grade								
Grade I	6(12%)	6(100%)	0(0%)	0(0%)	<0.001§	4(66.7%)	2(33.3%)	0.005§
Grade II	27(54%)	16(59.3%)	9(33.3%)	2(7.4%)		21(77.8%)	6(22.2%)	
Grade III	17(34%)	0(0%)	2(11.8%)	15(88.2%)	4(23.5%)	13(76.5%)		
LVI								
Absent	32(64%)	22(68.8%)	5(15.6%)	5(15.6%)	<0.001‡	24(75%)	8(25%).	0.001‡
Present	18(36%)	0(0%)	6(33.3%)	12(66.7%)		5(27.8%)	13(72.2%)	
LN metastasis								
Absent	11(22%)	10(90.9%)	0(0%)	1(9.1%)	0.002‡	8(72.7%)	3(27.3%)	0.319‡
Present	39(78%)	12(30.8%)	11(28.2%)	16(41%)		21(53.8%)	18(46.2%)	
pT								
T1	6(12%)	6(100%)	0(0%)	0(0%)	<0.001§	4(66.7%)	2(33.3%).	0.008§
T2	21(42%).	13(61.9%).	5(23.8%).	3(14.3%)		17(81%)	4(19%)	
T3	18(36%).	3(16.7%)	4(22.2%).	11(61.1%)		7(38.9%)	11(61.1%)	
T4	5(10%).	0(0%)	2(40%)	3(60%)		1(20%)	4(80%)	
pN								
N0	11(22%)	10(90.9%)	0(0%)	1(9.1%)	<0.001§	8(72.7%)	3(27.3%).	0.025§
N1	10(20%)	4(40%)	3(30%)	3(30%)		7(70%)	3(30%)	
N2	19(38%)	8(42.1%)	7(36.8%)	4(21.1%)		12(63.2%)	7(36.8%)	
N3	10(20%).	0(0%)	1(10%)	9(90%)		2(20%)	8(80%)	
M								
M0	44(88%)	22(50%)	11(25%)	11(25%)	0.001‡	28(63.6%)	16(36.4%).	0.070‡
M1	6(12%)	0(0%)	0(0%)	6(100%)		1(16.7%)	5(83.3%)	
TNM stage								
Stage I	6(12%)	6(100%)	0(0%)	0(0%)	<0.001§	4(66.7%)	2(33.3%).	0.007§
Stage IIA	5(10%).	4(80%)	0(0%)	1(20%)		4(80%)	1(20%)	
Stage IIB	9(18%)	4(44.4%)	3(33.3%)	2(22.2%)		7(77.8%)	2(22.2%)	
Stage IIIA	16(32%)	8(50%)	5(31.2%)	3(18.8%)		11(68.8%)	5(31.2%)	
Stage IIIB	4(8%)	0(0%)	2(50%)	2(50%)		1(25%)	3(75%)	
Stage IIIC	4(8%)	0(0%)	1(25%)	3(75%)		1(25%)	3(75%)	
Stage IV	6(12%)	0(0%)	0(0%)	6(100%)		1(16.7%)	5(83.3%)	
ER								
Negative	16(32%)	0(0%)	4(25%)	12(75%)	<0.001‡	3(18.8%)	13(81.2%).	<0.001‡
Positive	34(68%)	22(64.7%)	7(20.6%).	5(14.7%)		26(76.5%)	8(23.5%)	
PR								
Negative	18(36%)	0(0%)	6(33.3%)	12(66.7%)	<0.001‡	3(16.7%)	15(83.3%).	<0.001‡
Positive	32(64%)	22(68.8%)	5(15.6%)	5(15.6%)		26(81.2%)	6(18.8%)	
Her2/neu								
Negative	39(78%)	20(51.3%)	8(20.5%)	11(28.2%)	0.131‡	25(64.1%)	14(35.9%).	0.166‡
Positive	11(22%)	2(18.2%0	3(27.3%)	6(54.5%)		4(36.4%)	7(63.6%)	
Molecular subtype								
Luminal A	20(40%)	17(85%)	3(15%)	0(0%)	<0.001‡	18(90%)	2(10%).	<0.001‡
Luminal B	14(28%)	5(35.7%)	4(28.6%)	5(35.7%)		8(57.1%)	6(42.9%)	
Her2 enriched	6(12%)	0(0%)	2(33.3%)	4(66.7%)		2(33.3%)	4(66.7%)	
Triple negative	10(20%)	0(0%)	2(20%)	8(80%)		1(10%)	9(90%)	

Table 2. Relation between clinicopathological features and immunohistochemical staining for AQP3 and vimentin in IC NST patients (N=50). (continued)

AQP3								
Negative	22(44%)					20(90.9%)	2(9.1%)	<0.001§
Weak+	11(22%)					5(45.5%)	6(54.5%)	
Strong+	17(34%)					4(23.5%)	13(76.5%)	
Vimentin								
Negative	29(58%)	20(69%)	5(17.2%).	4(13.8%)	<0.001‡			
Positive	21(42%)	2(9.5%)	6(28.6%).	13(61.9%)				
E-cadherin								
0	13(26%)	1(7.7%)	1(7.7%)	11(84.6%)	<0.001§	2(15.4%)	11(84.6%).	<0.001§
+1	11(22%)	2(18.2%)	4(36.4%).	5(45.5%)		5(45.5%)	6(54.5%)	
+	17(34%)	10(58.8%)	6(35.3%).	1(5.9%)		14(82.4%)	3(17.6%)	
+	9(18%)	9(100%)	0(0%)	0(0%)		8(88.9%)	1(11.1%)	

Categorical variables were expressed as number (percentage); ‡ Chi-square test; § Chi-square test for trend; P<0.05 is significant.; AQP3: Aquaporin-3; IC NST: Invasive

(percentage). Shapiro-Wilk test checked the continuous variables for normality. When appropriate, Pearson's chi-squared or Fisher's exact tests compared the percentage of categorical variables t. Chi-squared test for trend compared the trend of change in the distribution of relative frequencies between ordinal data. OS was calculated as the time from diagnosis to death or the most recent follow-up contact (censored). We also calculated DFS as the period stretching from the start of the treatment to the date of relapse or the most recent follow-up contact that patient was known as relapse-free. Stratification of OS and DFS was done according to the markers. We estimated time-to-event distributions using Kaplan-Meier plot and compared them via two-sided exact log-rank test. All tests were two sided. A *P*-value <0.05 was considered significant. SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium) performed all the statistics.

Results

Clinicopathological features

The present study included 50 female patients aged 31-68 years with a mean value of 51.8 and SD of 8.42. The majority of cases were older than 50 years (62%). Out of the 50 studied subjects, six (12%) had grade I, 27 (54%) had grade II, and 17 cases (34%) had grade III. Regarding tumor stage, six cases (12%) had stage I, 14 (28%) had stage II, 24 (48%) had stage III, and 12 (12%) had stage IV. The preponderance of cases had positive lymph node metastasis (78%) and only 12% had distant metastasis. Concerning molecular subtypes, we classified the tumors as 40% luminal A, 28% luminal B, 12% HER2 enriched, and 20% triple negative. Table 1 summarizes other clinicopathological data of the studied cases.

AQP3 expression

AQP3 staining was mainly expressed in the cell membrane and /or cytoplasm. Only membranous staining was considered positive and cytoplasmic staining was considered non-specific. Out of the 50 studied patients, 22 (44%) showed negative expression (Figure 1A). Positive AQP3 expression occurred in 28 cases (56%), of which 11 cases (22%) showed weak positive staining, and 17 cases (34%) showed strong positive staining (Figure 1B). Based on the correlations between different AQP3 expression categories and clinicopathological variables, there was a significant association between positive AQP3 expression and high tumor grades (*P*<0.001). Furthermore, positive AQP3 expression had a significant relationship with the tumors with more aggressive characteristics such as large tumor size (*P*<0.001), positive lymph node (LN) metastasis (*P*<0.002), distant metastasis (*P* =0.001), advanced tumor stage (*P*<0.001), and lymphovascular invasion LVI (*P*<0.001). AQP3 expression level was higher in the premenopausal patients compared with the postmenopausal patients (*P*=0.032). In addition, the correlation between hormonal receptor status and molecular subtypes showed that AQP3 was significantly overexpressed in ER-negative and

Table 3. Relation between clinicopathological features and immunohistochemical staining for E-cadherin in breast cancer patients (N=50). (continued)

		E-cadherin				
Characteristics	**All (N=50) No. (%)**	**0 (N=13) No. (%)**	**1+ (N=11) No. (%)**	**2+ (N=17) No. (%)**	**3+ (N=9) No. (%)**	***P*-value**
Age						
≤50 years	19(38%)	7(36.8%)	4(21.1%)	2(10.5%)	6(31.6%)	0.023‡
>50 years	31(62%)	6(19.4%)	7(22.6%)	15(48.4%)	3(9.7%)	
Menstrual status						
Premenopausal	20(40%)	7(35%)	6(30%)	2(10%)	5(25%)	0.036‡
Postmenopausal	30(60%)	6(20%)	5(16.7%)	15(50%)	4(13.3%)	
Family history						
Negative	43(86%)	11(25.6%)	10(23.3%)	15(34.9%)	7(16.3%).	0.845‡
Positive	7(14%)	2(28.6%)	1(14.3%)	2(28.6%)	2(28.6%)	
Grade						
Grade I	6(12%)	1(16.7%)	1(16.7%)	0(0%)	4(66.7%)	
<0.001§						
Grade II	27(54%)	2(7.4%)	5(18.5%)	15(55.6%)	5(18.5%)	
Grade II	17(34%)	10(58.8%)	5(29.4%)	2(11.8%)	0(0%)	
LVI						
Absent	32(64%)	4(12.5%)	4(12.5%)	15(46.9%)	9(28.1%).	<0.001‡
Present	18(36%)	9(50%)	7(38.9%)	2(11.1%)	0(0%)	
LN metastasis						
Absent	11(22%)	2(18.2%)	1(9.1%)	1(9.1%)	7(63.6%).	<0.001‡
Present	39(78%)	11(28.2%)	10(25.6%)	16(41%)	2(5.1%)	
pT						
T1	6(12%)	1(16.7%)	1(16.7%)	0(0%)	4(66.7%)	<0.001§
T2	21(42%)	2(9.5%)	0(0%)	14(66.7%)	5(23.8%)	
T3	18(36%)	8(44.4%)	7(38.9%)	3(16.7%)	0(0%)	
T	5(10%)	2(40%)	3(60%)	0(0%)	0(0%)	
pN						
N0	11(22%)	2(18.2%)	1(9.1%)	1(9.1%)	7(63.6%).	<0.001§
N1	10(20%)	2(20%)	0(0%)	6(60%)	2(20%)	
N2	19(38%)	1(5.3%)	9(47.4%)	9(47.4%)	0(0%)	
N3	10(20%)	8(80%)	1(10%)	1(10%)	0(0%)	
M						
M0	44(88%)	8(18.2%)	10(22.7%)	17(38.6%)	9(20.5%).	0.007‡
M1	6(12%)	5(83.3%)	1(16.7%)	0(0%)	0(0%)	
TNM stage						
Stage I	6(12%)	1(16.7%)	1(16.7%)	0(0%)	4(66.7%).	<0.001§
Stage IIA	5(10%)	1(20%)	0(0%)	1(20%)	3(60%)	
Stage II	9(18%)	1(11.1%)	0(0%)	6(66.7%)	2(22.2%)	
Stage IIIA	16(32%)	1(6.2%)	6(37.5%)	9(56.1%)	0(0%)	
Stage IIIB	4(8%)	1(25%)	3(75%)	0(0%)	0(0%)	
Stage IIIC	4(8%)	3(75%)	0(0%)	1(25%)	0(0%)	
Stage IV	6(12%)	5(83.3%)	1(16.7%)	0(0%)	0(0%)	
ER						
Negative	16(32%)	11(68.8%)	4(25%)	1(6.2%)	0(0%).	<0.001‡
Positive	34(68%)	2(5.9%)	7(20.6%)	16(47.1%)	9(26.5%)	
PR						
Negative	18(36%)	11(61.1%)	6(33.3%)	1(5.6%)	0(0%).	<0.001‡
Positive	32(64%)	2(6.2%)	5(15.6%)	16(50%)	9(28.1%)	
Her2/neu						
Negative	39(78%)	9(23.1%)	8(20.5%)	13(33.3%)	9(23.1%).	0.345‡
Positive	11(22%)	4(36.4%)	3(27.3%)	4(36.4%)	0(0%)	
Molecular subtype						
Luminal A	20(40%)	1(5%)	1(5%)	9(45%)	9(100%).	<0.001‡
Luminal B	14(28%)	1(7.1%)	6(42.9%)	7(50%)	0(0%)	
Her2 enriched	6(12%)	3(50%)	3(50%)	0(0%)	0(0%)	
Triple negative	10(20%)	8(80%)	1(10%)	1(10%)	0(0%)	

Table 3. Relation between clinicopathological features and immunohistochemical staining for E-cadherin in breast cancer patients (N=50). (continued)

AQP3						
Negative	22(44%)	1(4.5%)	2(9.1%)	10(45.5%)	9(40.9%)	<0.001§
Weak+	11(22%)	1(9.1%)	4(36.4%)	6(54.5%)	0(0%)	
Strong+	17(34%)	11(64.7%)	5(29.4%)	1(5.9%)	0(0%)	
Vimentin						
Negative	29(58%)	2(6.9%)	5(17.2%)	14(48.3%)	8(27.6%).	<0.001‡
Positive	21(42%)	11(52.4%)	6(28.6%)	3(14.3%)	1(4.8%)	

Categorical variables were expressed as number (percentage); ‡ Chi-square test; § Chi-square test for trend; $P<0.05$ is significant.; E-cadherin: cadherin of type E.

triple-negative groups.

Vimentin expression

We detected vimentin staining as cytoplasmic staining. Stromal fibroblasts and endothelial lining of blood vessels showed positive vimentin expression. Vimentin expression was negative in 29 cases (58%) (Figure 2A) and positive in 21 subjects (42%) (Figure 2B). Positive vimentin expression had a correlation with higher tumor grade (P=0.005), larger tumor size (P=0.008), pathological LN lymph node stage (P=0.025), advanced tumor stage (P=0.007), and LVI (P=0.001). In addition, there was a significant association between vimentin expression and different molecular subtypes (P<0.001). No significant relationship existed between vimentin expression and distant metastasis (P=0.07).

E-cadherin expression

We detected E-cadherin staining in the cellular membrane of tumor cells. E-cadherin immunoreactivity was negative in 13 cases (26%) (Figure 3C), positive E-cadherin expression existed in 37 subjects (74%) (Figure 3D), of which 11 (22%) were +1, 17 (34%) were +2, and 9 (18%) were +3 immunostaning. Our results showed that increased tumor grade and positive LVI significantly downregulated E-cadherin immunoreactivity (P<0.001). Negative E-cadherin expression was more common in advanced stages in comparison with the early stages (P<0.001); this expression was also more prevalent in cases with large tumor size (P<0.001), lymph node involvement, and distant metastasis in contrast to those with negative lymph node and no distant metastasis (P<0.001and P<0.007, respectively). Negative E-cadherin was significantly associated with ER-negative and triple-negative cases.

Association among AQP3, vimentin, and E-cadherin expressions

The correlation analysis of our marker expression among the studied cases revealed a significant positive correlation between AQP3 and vimentin expression (P<0.001); however, E-cadherin expression had a negative relationship with both markers (P<0.001) (Tables 2, 3). These two tables show the association of AQP3, E-cadherin, and E-cadherin expressions with the clinicopathological parameters and the correlation between the expressions of the markers.

Association of AQP3, E-Cadherin, and vimentin expressions with tumor recurrence and patients' survival

Negative expression of AQP3 had a significant relationship with the decrease in the incidence of tumor recurrence (P<0.001). Patients with breast cancer with negative expression of AQP3 had significant better 3 years' DFS and OS (P<0.001). However, the positive expression of AQP3 was significantly correlated with the increase in the incidence of tumor recurrence (P<0.001) and poor survival outcomes. Negative expression of vimentin had a significant association with the reduction in the incidence of tumor recurrence (P=0.002) and significant better 3 years' DFS and OS (P=0.001, 0.003, respectively). The positive expression of vimentin, on the other hand, was significantly related to the increase in the incidence of tumor recurrence and poor survival outcomes. Positive expression of E-cadherin had a significant association with the decrease in the incidence of tumor recurrence and significant better 3 years' DFS and OS (P<0.001) (Figures 4A-4F). Tables 4 and 5 show

Table 4. Relation between immunohistochemical staining for AQP3 and vimentin and outcome in invasive carcinoma NST patients (N=50).

		AQP3				Vimentin		
Outcome	**All. No.(%)**	**Negative No.(%)**	**Weak+ No.(%)**	**Strong+ No.(%)**	***P*-value**	**Negative No.(%)**	**Positive No.(%)**	***P*-value**
Relapse	**(N=44)**	**(N=22)**	**(N=11)**	**(N=11)**		**(N=28)**	**(N=16)**	
Absent	27(61.4%)	20(90.9%).	7(63.6%)	0(0%)	<0.001‡	22(78.6%)	5(31.2%)	0.002‡
Present	17(38.6%)	2(9.1%)	4(36.4%).	11(100%)		6(21.4%)	11(68.8%)	
Disease Free Survival								
Mean (months)	28.18 months.	34.63 months.	32.27 month.	11.18months.	<0.001†.	31.60.	22.18	
(95%CI)	(24.93-31.42).	(32.50-36.76).	(28.60-35.94).	(10.03-12.32)		(28.29-34.92).	(16.48-27.89).	0.001†
1-year DFS	75%	95.5%	100%	9.1%		85.7%	56.3%	
2-year DFS	70.5%.	95.5%	90.9%	0%		82.1%	50%	
3-year DFS	61.4%	90.9%	63.6%	0%		78.6%	31.3%	
Death	(N=50)	(N=22)	(N=11)	(N=17)		(N=29)	(N=21)	
Alive	30(60%)	20(90.9%).	10(90.9%)	0(0%)	<0.001‡	22(75.9%).	8(38.1%)	0.007‡
Died	20(40%)	2(9.1%)	1(9.1%)	17(100%)		7(24.1%).	13(61.9%)	
Overall Survival								
Mean (months)	27.90months	34.90months.	34.36months.	14.64months.	<0.001†.	31.72months.	22.61months.	0.003†
(95%CI)	(24.97-30.82)	(33.07-36.74)	(31.30-37.42).	(12.55-16.73)		(28.75-34.69).	(17.85-27.38)	
1-year OS	86%	100%	100%	58.8%		100%	66.7%	
2-year OS	62%	100%	90.9%	0%		79.3%	38.1%	
3-year OS	60%	90.9%	90.9%	0%		75.9%	38.1%	

Continuous variables were expressed as mean (95%CI); Categorical variables were expressed as number (percentage); ‡ Chi-square test; † Log rank test; P<0.05 is significant.; AQP3: Aquaporin-3; IC NST: Invasive carcinoma of no special type.

the association of AQP3, E-cadherin, and vimentin expressions with three-year DFS and OS.

Discussion

According to our results, AQP3 expression was positive in 56% of invasive breast cancer cases. Additionally, the expression of AQP3 was significantly correlated with the tumors of aggressive nature (high tumor grade, large tumor size, positive LN and distant metastasis, advanced tumor stage, and tumors with LVI). These results point to the role of AQP3 in the invasion and metastasis of tumor cells. AQP3 expression level was higher in the premenopausal patients compared with postmenopausal patients. In addition, the correlation between hormonal receptor status and molecular subtypes showed that AQP3 was significantly overexpressed in ER-negative and triple-negative groups. These findings are similar to Kang et al. who reported that AQP3 overexpression in the early cases of breast cancer was associated with worse prognosis in patients with HER2-overexpression following curative surgical operations.[8] Its expression was correlated with advanced stage, large tumor size, and LVI. Their study concluded that AQP3 expression might be considered as a prognostic marker in these cases. Additionally, Huang et al. showed the increased expression of AQP3 protein in ER positive invasive breast carcinoma of premenopausal compared to postmenopausal cases; also, AQP3 protein had a relationship with higher histological grade and positive lymph nodes metastasis.[19]

Involvement of AQP3 in increased cellular motility and invasiveness corroborates its role in EMT. Moreover, a few studies have directly implicated AQP3 in EMT progression in cancer cells. In breast cancer cells, AQP3 overexpression reduced the protein levels of E-cadherin.[19] Similarly, in a study using gastric cancer cell lines, AQP3 overexpression down-regulated E-cadherin expression while up-regulating vimentin and fibronectin expression.[7] Therefore, the mechanisms underlying the regulation of EMT by AQP3 overexpression can be important future research topics for unraveling the role of AQP3 in cancer. In the present work, a significant positive correlation existed between AQP3 and mesenchymal marker vimentin expressions. Additionally, we found an inverse association between AQP3 and E-cadherin expressions. Accordingly, we suggested that the overexpression of AQP3 might aggravate EMT of cancer cells through converting epithelial cells to a more mesenchymal morphology with weakened cell–

cell adhesions.

EMT was considered as transient, occurring during progression towards metastases in several types of solid tumors.[20] Our findings proposed that AQP3 was associated with EMT induction in breast invasive carcinoma cases. Thus, further research is required to study the potential effectiveness of AQP-based therapy for inhibiting cancer cells from metastasizing. These results are similar to the observations of Huang et al. They reported that the up-regulation of AQP3 could influence the expression of molecules related to epithelial-mesenchymal transition. This resulted in the enhancement of cell migration and invasion in ER-positive breast cancer cells.[19] The authors concluded that the overexpression of AQP3 in invasive breast carcinoma significantly enhanced cell migration and invasion.

Vimentin is a widely employed marker of the mesenchymal tissues. Positive vimentin expression was considered as a feature of EMT, indicating the acquisition of a mesenchymal phenotype of tumor cells;[20, 21] this expression was further observed in undifferentiated tumors and tumors bound to form distant metastases to tissues such as lung and brain, hence with unfavorable prognosis.[20,22,23] Several studies have addressed the correlation between vimentin expression and cell migration. Inhibition of vimentin filament integrity caused mesenchymal cells to adopt epithelial shape and inhibit migration.[24] Many aggressive breast cancer cell lines express vimentin.[25] Furthermore, the overexpression of vimentin in a vimentin-negative, non-invasive MCF7 breast cancer cell line increased integrin traffic, migration, and invasiveness.[26] In particular, vimentin expression was identified as a marker of basal-like breast cancer cells possibly representing the clinical 'triple-negative' tumor-type associated with a poor prognosis.[25, 27] In the present work, positive vimentin expression occurred in 42% of the cases. Moreover, positive vimentin expression had a significant correlation with tumor grade, tumor staging, tumor size, lymph node involvement, and LVI; however, it was not correlated with distant metastasis. These data are comparable with Karihtala et al. They noticed positive staining for vimentin in 51% of cases; they also observed positive vimentin expression in invasive cancer cells, particularly in highly proliferating and poorly differentiated cancers.[28] On the other hand, Calf et al. observed positive staining for vimentin in 21% of the subjects; they proposed that positive vimentin expression was an indicator of breast cancer progression.[29] Therefore, vimentin expression seems to predict survival in ductal breast carcinoma patients. Hemalatha et al. documented the lower percentage of vimentin positivity (only 18%); also, it was associated with high grade tumors and had no correlation with tumor size, nodal metastasis, and survival status.[13] This might be because they considered all subtypes of breast carcinoma and not just the invasive breast carcinoma NST. In addition, there was a significant correlation between vimentin expression and triple-negative carcinoma. This result is in agreement with findings of Yamashita et al. They reported a significant association between positive vimentin protein expression and poor prognosis and reduced survival in triple-negative breast cancer cases.[30] Also, vimentin expression and its association with ER-negative phenotype in breast cancer was reported several years ago.

In the present study, we observed negative E-cadherin expression in 26% of cases; a significant correlation also existed between the down-expression of E-cadherin and large tumor size, positive lymph node metastasis, advanced TNM stage, and higher histological grade. These findings are in concordance with the study performed by Shibata et al. They showed negative E-cadherin expression in 29% of invasive carcinoma NST cases.[31] On the other hand, a higher percentage of negative E-cadherin expression was reported by Ricciardi et al. who observed negativity in 47% of the subjects.[14] Such difference might be attributed to the various cut-off points of positivity and the fact that all their cases were of triple-negative type. The current results are also in line with Li et al.[32] who suggested that reduced E-cadherin expression had a significant association with poorer DFS and OS and clinicopathological characteristics such as tumor size, lymph node

Table 5. Relation between immunohistochemical staining for E-cadherin and outcome in invasive carcinoma NST patients (N=50)

Outcome		E-cadherin				
	All No. (%) (N=44)	0 No. (%) (N=8)	1+ No. (%) (N=10)	2+ No. (%) (N=17)	3+ No. (%) (N=9)	*P*-value
Relapse						
Absent	27(61.4%)	1(12.5%)	5(50%)	12(70.6%)	9(100%)	<0.001‡
Present	17(38.6%)	7(87.5%)	5(50%)	5(29.4%)	0(0%)	
Disease-free survival						
Mean (months)	28.18 months	16.12 months	25.50 months	31.29 months	36 months	<0.001†
(95%CI)	(24.93-31.42)	(7.23-25.01)	(16.60-34.39)	(26.87-35.71)		
1-year DFS	75%	62.5%	60%	94.1%	100%	
2-year DFS	70.5%	25%	60%	82.4%	100%	
3-year DFS	61.4%	12.5%	50%	70.6%	100%	
Death	(N=50)	(N=13)	(N=11)	(N=17)	(N=9)	
Alive	30(60%)	2(15.4%)	6(54.5%)	13(76.5%)	9(100%).	<0.001‡
Died	20(40%)	11(84.6%)	5(45.5%)	4(23.5%)	0(0%)	
Overall Survival						
Mean (months)	27.90 months	17.15 months	26.36 months	32.82 months	36 months	<0.001†
(95%CI)	(24.97-30.82)	(11.54-22.76)	(18.86-33.85)	(29.31-36.33)		
1-year OS	86%	53.8%	90.9%	100%	100%	
2-year OS	62%	23.1%	54.5%	82.4%	100%	
3-year OS	60%	15.4%	54.5%	76.5%	100%	

Continuous variables were expressed as mean (95%CI); Categorical variables were expressed as numbers. (percentage); ‡ Chi-square test; † Log rank test; P<0.05 is significant.; E-cadherin: cadherin of type E.

status, TNM stage, and histological tumor grade.[32] However, Wang et al. found no relationship between E-cadherin expression and prognosis.[33] This difference might stem from the various primary antibody sources and antibody dilution ratios, leading to differences in immunohistochemistry sensitivity. There were no uniform scoring criteria to define E-cadherin positive expression. Furthermore, the cut-off values defining reduced E-cadherin expression varied from 5 to 70% without an optimal threshold.

During EMT, the expression of E-cadherin adhesion molecule decreases; whereas, vimentin expression increases. These molecular changes possibly ensue dysfunctional cell-cell adhesions and loss of cell-cell junctions. The involvement of EMT varies among different types of cancer; although there has been research on breast cancer, much remains to be elucidated.[35] In the present study, we found a negative correlation between vimentin and E-cadherin expression. These results suggest the role of these markers concerning invasion and metastasis in breast cancer patients. In agreement with Kachroo et al. who reported the loss of E-cadherin as a key step of EMT, Tsubaki et al. observed vimentin as a marker of mesenchymal differentiation.[34, 35]

Repression of E-cadherin with increased vimentin expression predicted poor prognosis for invasive breast carcinoma of breast. These results are consistent with those reported by Hemalatha et al. and Kachroo et al.[13, 34] Therefore, these proteins are involved in the processes associated with malignant progression. In the present study, Kaplan-Meier curve analysis revealed a significant association of positive AQP3, positive vimentin, and negative E-cadherin expression with shorter three-year DFS and shorter OS, hence the poor patient outcomes. Kang et al. showed that AQP3 expression might predict the survival of breast cancer patients.[8] Similarly, other researchers reported that positive vimentin expression conferred a poor survival and a worse prognosis.[13, 29, 30] Moreover, Shibata et al. and Li et al. demonstrated that negative E-cadherin expression had a correlation with worse prognosis and reduced survival.[31,32]

It is concluded that AQP3, vimentin, and E-cadherin could predict prognosis in patients with invasive breast carcinoma (NST); also, AQP3 might promote EMT development in breast cancer cases. The use of AQP3 in breast cancer is a potential diagnostic and prognostic biomarker and might provide an important target for

therapeutic intervention. Therefore, it is necessary that future studies involve large sample sizes and long-term follow-ups.

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Conflict of Interest

None declared.

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