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Original Article

Expression of alpha B crystallin and BCL2 in patients with infiltrating ductal carcinoma

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Abstract: Alpha B crystallin was characterized as a negative prognostic factor in breast cancer. BCL2 has an antiapoptotic role and sustains cell survival in vitro, ironically BCL2 expression has been associated with a good prognosis of breast cancer patients. To investigate the significance of alpha B crystallin and BCL2 expression in breast cancer and the relationship between these proteins, we performed immunohistochemical staining for both proteins in human breast cancer tissues. In the present study, overexpression of alpha B crystallin was observed more frequently in triple negative cancer (9/20, 45%) than in luminal type cancer (8/53, 15.1%, *P*=0.02161). BCL2 tended to be more highly expressed in luminal type cancer than in HER2 and triple negative cancer types (luminal: 36/53, 68%, HER2: 2/9, 22%, triple negative: 7/20 35%, *P*=0.008652). In multivariate analysis using ANCOVA, alpha B crystallin was related to short overall survival (*P*=0.017173). These findings suggest that alpha B crystallin is an independent prognostic factor of infiltrating ductal carcinoma. BCL2 was not associated with survival in multivariate analysis using ANCOVA. Thus, in our study BCL2 was not an independent prognostic indicator.

Keywords: Alpha B crystallin, BCL2, breast cancer, overall survival

Introduction

Breast cancer is a heterogeneous disease. One of the primary challenges in breast cancer management is to determine the proper treatment for each patient. In particular, it is important to be able to select patients who should undergo adjuvant therapy and predict their prognosis. Although there are a number of known independent prognostic factors, such as the Nottingham Prognostic Index, we cannot predict prognosis of all patients. Alpha B crystallin was characterized as a negative prognostic factor in breast cancer [1-5]. In tumor pathogenesis, alpha B crystallin functions as an antiapoptotic regulator. In a previous study human alpha A crystallin and alpha B crystallin showed affinity to the proapoptotic agents Bax and Bcl-X_s. Through these interactions, alpha crystallins suppress the mitochondrial translocation of Bax and Bcl-X_s and prevent apoptosis [6]. These findings demonstrate the antiapoptotic function of alpha B crystallin and support the hypothesis that patients with alpha B crystallin overexpression might have a poor prognosis. BCL2 protein is known to be an antiapoptotic regulator in the intrinsic pathway of apoptosis. Although BCL2 has an antiapoptotic role and sustains cell survival *in vitro*, ironically BCL2 expression has been associated with a good prognosis of breast cancer patients [7-9]. In previous experiments using rabbit lens epithelial cells, the human *BCL2* gene provisionally acted as an apoptotic regulator. The human *BCL2* gene downregulated the *CRYAB* gene encoding alpha B crystallin and attenuated resistance against oxidative stress-induced apoptosis [10]. BCL2 had an apoptotic function in rabbit lens epithelial cells and was associated with alpha B crystallin. To investigate the significance of alpha B crystallin and BCL2 expression in breast cancer and the relationship between these proteins, we performed immunohistochemical staining for both proteins in human breast cancer tissues.

Materials and methods

Patients and tissue sampling

A total of 82 formalin-fixed paraffin-embedded breast tissue samples were obtained from

Table 1. Clinical characteristics in 82 breast cancer patients

Clinicopathologic feature	N	%
Gender		
Male	0	0
Female	82	100
Age		
≤ 60	56	68.3
> 60	26	31.7
pT stage		
T1	28	34.1
T2	45	54.9
T3	3	3.7
T4	6	7.3
pN stage		
N0	48	58.5
N1	26	31.7
N2	6	7.3
N3	2	2.4
pM stage		
M0	82	100
M1	0	0
Hormonal therapy		
Yes	71	86.6
No	11	13.4
Chemotherapy		
Yes	61	74.4
No	21	25.6
Radiation therapy		
Yes	23	28
No	59	72
Type		
Luminal A	33	40.2
Luminal B	20	24.4
HER2	9	11
Triple negative	20	24.4
Distant metastasis (follow up)		
Yes	17	20.7
No	65	79.3
Recurrence		
Yes	9	11
No	73	89
αB crystallin		
Strong	18	22
Weak	64	78
BCL2		
Strong	45	54.9
Weak	37	45.1

patients with stage IA to IIIC infiltrating ductal carcinoma. All patients underwent partial or total mastectomy at Samsung Changwon hospital between 2003 and 2009. All clinical information was acquired through medical records. The choice of adjuvant treatment by hormonal therapy, chemotherapy, or radiation therapy was made according to widely used guidelines (hormonal therapy: 71/82 [86.6%], chemotherapy 61/82 [74.4%], radiation therapy 23/82 [28%]). All patients were women because males were excluded from the study, and 56 patients (68.3%) were aged 60 or younger. Patients with suspicion of distant metastasis at the time of mastectomy were excluded. The proportion of T/N stages was as follows: T1: 28 patients (34.1%); T2: 45 (54.9%); T3: 3 (3.7%); T4: 6 (7.3%)/N0: 48 (58.5%); N1: 26 (31.7%); N2: 6 (7.3%); N3: 2 (2.4%). The stage of the tumor was determined according to the TNM system of the American Joint Committee on Cancer (AJCC), 7th edition. Breast cancer subtyping was performed based on previous reports: the luminal A subtype as defined as ER and/or PR positive, and HER2 negative, and Ki67 low (Ki67 < 14%); the luminal B subtype as ER and/or PR positive, and HER2 negative, and Ki67 high (Ki67 ≥ 14%) or as ER and/or PR positive and HER2 positive; the HER2 enriched group as HER2 positive, and ER negative, and PR negative; and the triple negative subgroup was ER negative, PR negative, and HER2 negative [11, 12]. These types were determined by immunohistochemical staining performed at the time of diagnosis. Three cases had discrepancy between previous records of type and the present review; samples from these cases were re-stained to confirm the diagnosis of subtype. The reviews were conducted by two experienced pathologists (E. H. Lee and H. W. Lee). The proportion of tumor subtypes among the patients in this study were categorized as follows: luminal A: 33 patients (40.2%); luminal B: 20 (24.4%); HER2: 9 (11%); triple negative: 20 (24.4%). Seventeen patients (20.7%) showed distant metastasis and 9 patients (11%) underwent recurrence during the follow-up period. The mean follow-up duration was 2,563.3 days. The mean overall survival was 2,563.3 days and disease-free survival was 1,855.7 days. The clinical characteristics of the 82 patients are summarized in **Table 1**.

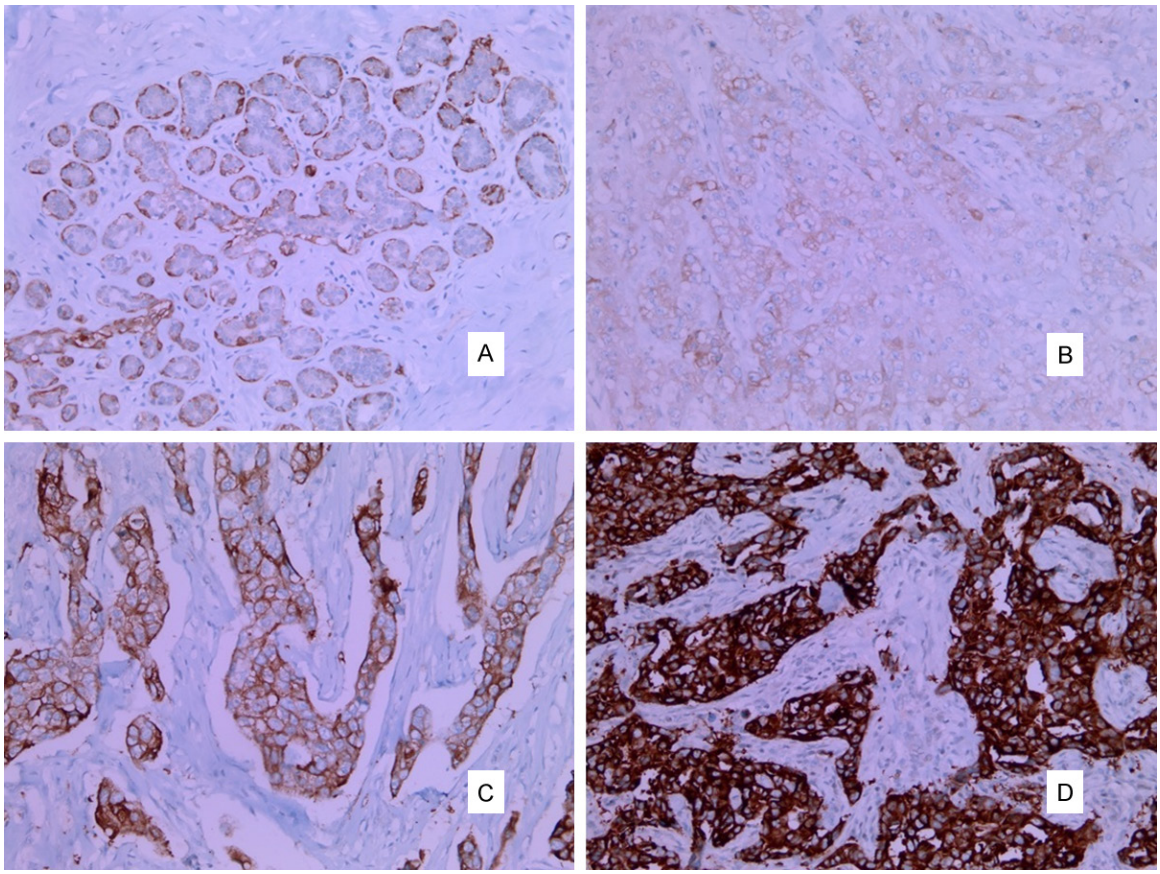

Figure 1. (A) aB-crystallin expression in myoepithelial component of normal breast, no expression in luminal cells (B) Weak (1+) expression of aB-crystallin in infiltrating ductal carcinoma (C) Moderate (2+) expression of aB-crystallin in infiltrating ductal carcinoma (D) Strong (3+) expression of aB-crystallin in infiltrating ductal carcinoma.

Immunohistochemical staining

Formalin-fixed paraffin-embedded (FFPE) tissue samples from patient breast tumors were collected retrospectively. Representative sections from the tissue blocks were cut with a microtome at 4-μm thickness and dried overnight at 37°C on a salinized slide. Immunochemical staining was performed using a Benchmark XT slide stainer (Ventana, Inc.) according to the manufacturer's instructions. The antibodies used for immunohistochemical staining are as follows: mouse IgG1 monoclonal antibody, clone 1B6.1-3G4 (Enzo Life Sciences, Inc.) was used for the detection of alpha B crystallin (1:200 dilution, 1 hour incubation at room temperature); rabbit monoclonal primary antibody, clone SP66 (Ventana Medical Systems, Inc.) was used for the detection of BCL2 (predilution, 1 hour incubation at room temperature). To evaluate the expression of alpha B crystallin and BCL2 protein, the intensity was scored using a scoring system from 0-3 (0: negative; 1: weak; 2: moderate; 3: strong) and multiplied by the percentage of cells with staining. The range of the total score was 0-300. For alpha B crystallin expression, cytoplasmic staining and membranous staining were considered positive and nuclear staining was excluded from the score [1, 13]. BCL2 staining was considered positive in cases with cytoplasmic staining [14]. Human pilocytic astrocytoma tissue was used as an internal control for alpha B crystallin and follicular lymphoma tissue was used as an internal control for BCL2. Immunohistochemical staining was evaluated by experienced pathologists (E. H. Lee and H. W. Lee) who were blinded to patient information.

Statistical analysis

Statistical analysis was conducted using R software (version 3.1.0) with packages named "prettyR" and "survival". To identify the significance of alpha B crystallin or BCL2 expression with respect to clinicopathologic characteris-

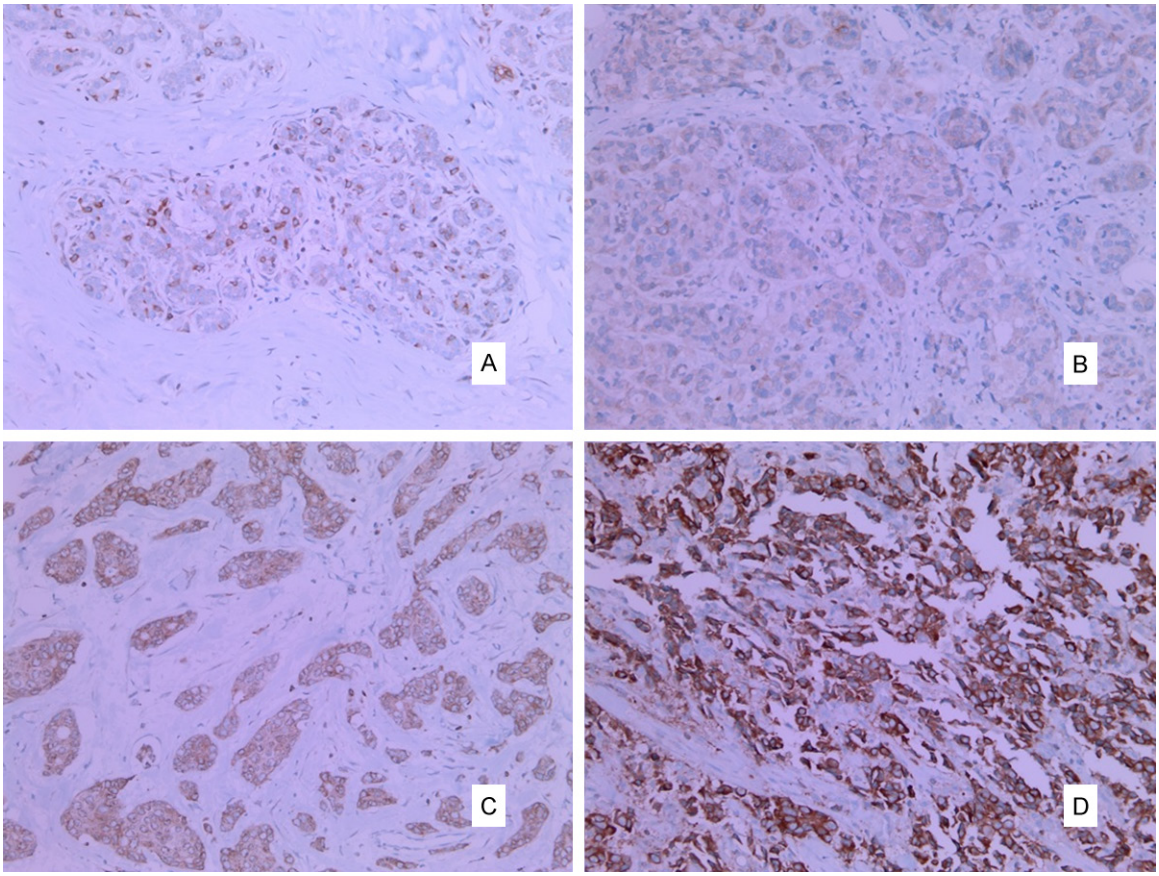

Figure 2. (A) For BCL2 staining, a few strongly positive cells were scattered in tubulo-lobular units of normal breast (B) Weak (1+) expression of BCL2 in infiltrating ductal carcinoma (C) Moderate (2+) expression of BCL2 in infiltrating ductal carcinoma (D) Strong (3+) expression of BCL2 in infiltrating ductal carcinoma.

Table 2. Results of immunohistochemical staining

Alpha B crystallin (n=82)				BCL2 (n=82)			
Score (0: negative 1: weak 2: moderate 3: strong)							
0	1	2	3	0	1	2	3
64	5	5	8	33	17	20	12
Proportion (%)							
0-25%	26-50%	51-75%	76-100%	0-25%	26-50%	51-75%	76-100%
65	7	3	7	37	3	0	42

tics, we used the Fisher's exact test and t-test. To determine the relationship between survival and alpha B crystallin or BCL2 expression according to clinicopathologic characteristics, we used analysis of covariance (ANCOVA). Kaplan-Meier analysis was performed to estimate the overall survival and disease-free survival of the alpha B crystallin or BCL2 positive group and negative group. Log rank test was used to compare the survival distribution of the two groups (positive vs. negative) for alpha B crystallin and BCL2. Correlation analysis was performed to identify relationships between alpha B crystallin and BCL2. A P value < 0.05 was considered statistically significant.

Results

Immunohistochemical staining of αB crystallin and BCL2

Immunohistochemical staining for alpha B crystallin expression was positive in myoepithelial cells of normal breast (**Figure 1A**). For BCL2 staining, a few strongly positive cells were scattered in tubulo-lobular units of normal breast (**Figure 2A**). The intensity of positive staining for alpha B crystallin and BCL2 was weak (**Figures 1B**, **2B**), moderate (**Figures 1C**, **2C**), or strong (**Figures 1D**, **2D**). For alpha B crystallin, when

Table 3. αB crystallin expression and clinicopathologic characteristics

		αBcrystallin	αBcrystallin	95% confidence interval		*P*-value
		Positive	Negative	Lower	Upper	
Age	Mean	55.44444	53.67188	-9.290273	5.745134	0.6402
Type	Luminal A	3 (9%)	30 (91%)			0.02161
	Luminal B	5 (25%)	15 (75%)			
	HER2	1 (11%)	8 (89%)			
	Triple negative	9 (45%)	11 (55%)			
T stage	1	10 (36%)	18 (64%)			0.01807
	2	5 (11%)	40 (89%)			
	3	0 (0%)	3 (100%)			
	4	3 (50%)	3 (50%)			
N stage	0	11 (23%)	37 (77%)			0.04117
	1	3 (12%)	23 (88%)			
	2	4 (67%)	2 (33%)			
	3	0 (0%)	2 (100%)			
Metastasis	Negative	15 (23%)	50 (77%)	0.1167374	3.0921043	0.7519
	Positive	3 (18%)	14 (82%)			
Recurrence	Negative	16 (22%)	57 (78%)	0.09430478	6.09821272	1
	Positive	2 (22%)	7 (78%)			
Overall survival	Mean	2026.167	2714.312	129.2433	1247.0483	0.01646
Disease-free survival	Mean	1484.889	1959.953	-55.66783	1005.79630	0.07865
BCL2	Positive	5 (11%)	40 (89%)	0.05806607	0.81141410	0.01463
	Negative	13 (35%)	24 (65%)			

Table 4. BCL2 expression and clinicopathologic characteristics

		BCL2	BCL2	95% confidence interval		*P*-value
		Positive	Negative	Lower	Upper	
Age	Mean	52.93333	55.43243	-3.737993	8.736191	0.4276
Type	Luminal A	24 (73%)	9 (27%)			0.008652
	Luminal B	12 (60%)	8 (40%)			
	HER2	2 (22%)	7 (78%)			
	Triple negative	7 (35%)	13 (65%)			
T stage	1	19 (68%)	9 (32%)			0.02463
	2	25 (56%)	20 (44%)			
	3	0 (0%)	3 (100%)			
	4	1 (17%)	5 (83%)			
N stage	0	30 (63%)	18 (37%)			0.1777
	1	13 (50%)	13 (50%)			
	2	2 (33%)	4 (67%)			
	3	0 (0%)	2 (100%)			
Metastasis	Negative	36 (55%)	29 (45%)	0.2714743	3.0786270	1
	Positive	9 (53%)	8 (47%)			
Recurrence	Negative	42 (58%)	31 (42%)	0.0560909	1.9124351	0.2871
	Positive	3 (33%)	6 (67%)			
Overall survival	Mean	2751.133	2334.757	-889.41442	56.66127	0.08366
Disease-free survival	Mean	1948.089	1743.270	-652.6390	243.0018	0.3655
αB crystallin	Positive	5 (28%)	13 (72%)	0.05806607	0.8114141	0.01463
	Negative	40 (63%)	24 (37%)			

Table 5. αB crystallin expression and clinicopathologic characteristics, luminal type

		αBcrystallin	αBcrystallin	95% confidence interval		*ph*-value
		Positive	Negative	Lower	Upper	
Age	Mean	57.37500	52.75556	-15.429618	6.190729	0.395
T stage	1	5 (26%)	14 (74%)			0.4002
	2	3 (10%)	28 (90%)			
	3	0 (0%)	1 (100%)			
	4	0 (0%)	2 (100%)			
N stage	0	5 (18%)	23 (82%)			0.6019
	1	2 (10%)	18 (90%)			
	2	1 (33%)	2 (67%)			
	3	0 (0%)	2 (100%)			
Metastasis	Negative	7 (17%)	34 (83%)	0.008996329	4.168344239	0.6652
	Positive	1 (8%)	11 (92%)			
Recurrence	Negative	7 (15%)	40 (85%)	0.02117529	12.79451146	1
	Positive	1 (17%)	5 (83%)			
Overall survival	Mean	1700.250	2730.711	253.5571	1807.3652	0.01034
Disease-free survival	Mean	1372.875	1965.378	-136.0609	1321.0665	0.1087
BCL2	Positive	4 (11%)	32 (89%)	0.06600406	2.57709188	0.413952
	Negative	4 (24%)	13 (76%)			

Table 6. BCL2 expression and clinicopathologic characteristics, luminal type

		Bcl2	Bcl2	95% confidence interval		*P*-value
		Positive	Negative	Lower	Upper	
Age	Mean	52.66667	55.11765	-5.871179	10.773140	0.557
T stage	1	16 (84%)	3 (16%)			0.02251
	2	20 (65%)	11 (35%)			
	3	0 (0%)	1 (100%)			
	4	0 (0%)	2 (100%)			
N stage	0	22 (79%)	6 (21%)			0.04618
	1	13 (65%)	7 (35%)			
	2	1 (33%)	2 (67%)			
	3	0 (0%)	2 (100%)			
Metastasis	Negative	29 (70%)	12 (30%)	0.1282395	2.8309514	0.4901
	Positive	7 (58%)	5 (42%)			
Recurrence	Negative	33 (70%)	14 (30%)	0.05125843	3.62555534	0.3719
	Positive	3 (50%)	3 (50%)			
Overall survival	Mean	2794.000	2111.765	-1288.55698	-75.91361	0.02819
Disease-free survival	Mean	1984.722	1645.588	-904.3497	226.0817	0.2339
αB crystallin	Positive	4 (50%)	4 (50%)	0.06600406	2.57709188	0.413952
	Negative	32 (71%)	13 (29%)			

the intensity score was multiplied by the percentage of positive cells in the tumor, the mean total score was 26 and the median total score was 0. The cut-off value was determined by the median score (0) and the study population was divided into a positive group and negative group on the basis of median score. Thus, all patients with positive staining belonged to the positive group. The percentage of cells with positive staining in the positive group was 15-90%. The mean score for BCL2 expression was 89 and the median score was 20. The group was similarly divided into a positive group (≥ 20) and negative group (< 20) on the basis of median score. The proportion of positive cells in the positive group was 15-100%. Detailed

Table 7. αB crystallin expression and clinicopathologic characteristics, triple negative type

		αBcrystallin	αBcrystallin	95% confidence interval		*P*-value
		Positive	Negative	Lower	Upper	
Age	Mean	51.11111	57.09091	-8.011837	19.971433	0.3811
T stage	1	5 (63%)	3 (37%)			0.3652
	2	2 (25%)	6 (75%)			
	3	0 (0%)	1 (100%)			
	4	2 (67%)	1 (33%)			
N stage	0	6 (40%)	9 (60%)			0.3615
	1	1 (33%)	2 (67%)			
	2	2 (100%)	0 (0%)			
	3	0	0			
Metastasis	Negative	8 (44%)	10 (56%)	0.0141804	107.7022281	1
	Positive	1 (50%)	1 (50%)			
Recurrence	Negative	8 (42%)	11 (58%)	0.03133874	Inf	0.45
	Positive	1 (100%)	0 (0%)			
Overall survival	Mean	2506.222	2695.364	-930.5841	1308.8669	0.7268
Disease-free survival	Mean	1716.222	2052.545	-716.152	1388.799	0.5105
BCL2	Positive	1 (14%)	6 (86%)	0.002025593	1.415072105	0.07028
	Negative	8 (62%)	5 (38%)			

Table 8. BCL2 expression and clinicopathologic characteristics, triple negative type

		BCL2	BCL2	95% confidence interval		*P*-value
		Positive	Negative	Lower	Upper	
Age	Mean	57.71429	52.61538	-19.800601	9.602798	0.4756
T stage	1	2 (25%)	6 (75%)			0.8786
	2	4 (50%)	4 (50%)			
	3	0 (0%)	1 (100%)			
	4	1 (33%)	2 (67%)			
N stage	0	6 (40%)	9 (60%)			0.5739
	1	0 (0%)	3 (100%)			
	2	1 (50%)	1 (50%)			
	3	0	0			
Metastasis	Negative	5 (28%)	13 (72%)	0.3691069	Inf	0.1105
	Positive	2 (100%)	0 (0%)			
Recurrence	Negative	7 (37%)	12 (63%)	0.00000	72.34783	1
	Positive	0 (0%)	1 (100%)			
Overall survival	Mean	2559.857	2637.385	-1093.830	1248.885	0.891
Disease-free survival	Mean	1894.857	1904.615	-1101.654	1121.170	0.9855
αBcrystallin	Positive	1 (11%)	8 (89%)	0.002025593	1.415072105	0.07028
	Negative	6 (55%)	5 (45%)			

staining results for alpha B crystallin and BCL2 are summarized in **Table 2**.

Univariate analysis of alpha B crystallin expression and clinicopathologic characteristics

Results of univariate analysis of alpha B crystallin expression and clinicopathologic characteristics are shown in **Table 3**. Eighteen cases showed positive alpha B crystallin expression. Cancer type, T and N stage, overall survival, and BCL2 expression were related to alpha B crystallin expression. Alpha B crystallin expression was observed more frequently in triple negative carcinoma than in other types (luminal

Table 9. ANCOVA: Overall survival according to αB crystallin expression and clinicopathologic characteristics

Independent Variable	d.f.	Sum Square	Mean Square	F-value	*P*-value
αB crystallin	1	4989663	4989663	5.9550	0.017173*
Age	1	5352244	5352244	6.3877	0.013719*
Cancer type	3	6266195	2088732	2.4928	0.066979
Chemotherapy	1	12735878	12735878	15.1999	0.000217***
Hormonal therapy	1	161	161	0.0002	0.988989
Radiation therapy	1	1120663	1120663	1.3375	0.251356
T stage	1	2477644	2477644	2.9570	0.089863
N stage	1	2867512	2867512	3.4223	0.068483
Residuals	71	59490341	837892	-	-

Significance codes. p<0.1, *p<0.05, **p<0.01, ***p<0.001; 0.1. Cancer type: luminal A, B vs. HER2 vs. triple negative.

Table 10. ANCOVA: Disease-free survival according to αB crystallin expression and clinicopathologic characteristics

Independent Variable	d.f.	Sum Square	Mean Square	F-value	*P*-value
αB crystallin	1	1497172	1497172	2.0268	0.1589258
Age	1	3902170	3902170	5.2826	0.0244879*
Cancer type	3	5551963	1850654	2.5053	0.0659715
Chemotherapy	1	9550841	9550841	12.9295	0.0005936***
Hormonal therapy	1	1917804	1917804	2.5962	0.1115554
Radiation therapy	1	4034815	4034815	5.4621	0.0222578*
T stage	1	92880	92880	0.1257	0.7239459
N stage	1	4112637	4112637	5.5675	0.0210506*
Residuals	71	52446889	738689	-	-

Significance codes. p<0.1, *p<0.05, **p<0.01, ***p<0.001; 0.1. Cancer type: luminal A, B vs. HER2 vs. triple negative.

Table 11. ANCOVA: Overall survival according to BCL2 expression and clinicopathologic characteristics

Independent Variable	d.f.	Sum Square	Mean Square	F-value	*P*-value
BCL2	1	1283772	1283772	1.4423	0.2337589
Age	1	5352244	5352244	6.0132	0.0166602*
Cancer type	3	6266195	2088732	2.3467	0.0799872
Chemotherapy	1	12735878	12735878	14.3086	0.0003207***
Hormonal therapy	1	161	161	0.0002	0.9893170
Radiation therapy	1	1120663	1120663	1.2590	0.2656119
T stage	1	2477644	2477644	2.7836	0.0996387
N stage	1	2867512	2867512	3.2216	0.0769285
Residuals	71	63196231	890088	-	-

Significance codes. p<0.1, *p<0.05, **p<0.01, ***p<0.001; 0.1. Cancer type: luminal A, B vs. HER2 vs. triple negative.

A: 3/33 [9%], luminal B: 5/20 [25%], HER 2: 1/9 [11%], triple negative: 9/20 [45%]) and this finding was statistically significant (P=0.02161). The proportion of cells with alpha B crystallin expression differed significantly according to T stage (T1: 10/28 [36%], T2: 5/45 [11%], T3: 0 [0%], T4: 3/6 [50%]; P=0.01807). Alpha B crystallin expression was also significantly related to N stage (N0: 11/48 [23%], N1: 3/26 [12%], N2: 4/6 [67%], N3: 0 [0%]; *P*=0.04117). The alpha B crystallin positive group showed shorter overall survival (2,026.167 days) than the negative group (2,714.312 days, *P*= 0.01646). Furthermore, alpha B crystallin expression was related to BCL2 expression in univariate analysis; the frequency of alpha B crystallin expression was significantly higher in the BCL2 negative group (13/37 [35%]) than in the positive group (5/45 [11%], *P*=0.01463).

Univariate analysis of BCL2 expression and clinicopathologic characteristics

Results of univariate analysis of BCL2 expression and clinicopathologic characteristics are shown in **Table 4**. Cancer type, T stage, and alpha B crystallin expression were related to BCL2 expression. Positive BCL2 expression was detected in 45 cases. BCL2 overexpression was observed more frequently in luminal type carcinoma than in other types (luminal A: 24/33 [73%], luminal B: 12/20 [60%], HER 2: 2/9 [22%], triple negative: 7/20 [35%];

Table 12. ANCOVA: Disease-free survival according to BCL2 expression and clinicopathologic characteristics

Independent Variable	d.f.	Sum Square	Mean Square	F-value	*P*-value
BCL2	1	19208	19208	0.0253	0.8740983
Age	1	3902170	3902170	5.1378	0.0264587*
Cancer type	3	5551963	1850654	2.4367	0.0717079
Chemotherapy	1	9550841	9550841	12.5751	0.0006969***
Hormonal therapy	1	1917804	1917804	2.5251	0.1164932
Radiation therapy	1	4034815	4034815	5.3124	0.0241012*
T stage	1	92880	92880	0.1223	0.7275994
N stage	1	4112637	4112637	5.4149	0.0228228*
Residuals	71	53924853	759505	-	-

Significance codes. p<0.1, *p<0.05, **p<0.01, ***p<0.001; 0.1. Cancer type: luminal A, B vs. HER2 vs. triple negative.

Table 13. Correlation between αB crystallin and BCL2 protein expression

Method	Correlation coefficient	Remarks
Pearson's product-moment correlation	cor -0.2335857	t=-2.1487, df=80 *P*-value=0.03468
Kendall's rank correlation tau	tau -0.1871347	z=-1.9623 *P*-value=0.04973
Spearman's rank correlation rho	rho -0.2157422	S=111703.6 *P*-value=0.05158

P=0.008652). The proportion of cases with BCL2 expression was significantly different between T stages (T1: 19/28 [68%], T2: 25/45 [56%], T3: 0 [0%], T4: 1/6 [17%]; *P*=0.02463).

Univariate analysis of alpha B crystallin expression and clinicopathologic characteristics in luminal type

Results of univariate analysis of alpha B crystallin expression and clinicopathologic characteristics in luminal type cancer are shown in **Table 5**. Only overall survival was related to alpha B crystallin expression. Out of a total of 53 cases, 8 showed positive alpha B crystallin expression. The alpha B crystallin positive group showed a shorter overall survival (1,700.250 days) than negative (2,730.711 days, *P*=0.01034).

Univariate analysis of BCL2 expression and clinicopathologic characteristics in luminal type

Results of univariate analysis of BCL2 expression and clinicopathologic characteristics in luminal type cancer are shown in **Table 6**. T, N stage and overall survival were related to BCL2 expression. Thirty-six cases showed positive BCL2 expression. The frequency of BCL2 expression differed significantly between T stages (T1: 16/19 [84%], T2: 20/31 [65%], T3: 0 [0%], T4: 0 [0%]; *P*=0.02251) and N stages (N0: 22/28 [79%], N1: 13/20 [65%], N2: 1/3 [33%], N3: 0 [0%], *P*=0.04618). The BCL2 positive group showed a longer overall survival (2,794 days) than the negative group (2,111.765 days, *P*=0.02819).

Univariate analysis of alpha B crystallin expression and clinicopathologic characteristics in triple negative type

Results of univariate analysis of alpha B crystallin expression and clinicopathologic characteristics in the triple negative group are shown in **Table 7**. None of the clinicopathologic characteristics were related to alpha B crystallin expression.

Univariate analysis of BCL2 expression and clinicopathologic characteristics in triple negative type

Results of univariate analysis between BCL2 expression and clinicopathologic characteristics in the triple negative group are shown in **Table 8**. None of the clinicopathologic characteristics were related to BCL2 expression.

ANCOVA analysis of overall survival according to αB crystallin expression and clinicopathologic characteristics

Results of multivariate analysis (ANCOVA) between overall survival and alpha B crystallin expression and clinicopathologic characteristics are shown in **Table 9**. Alpha B crystallin

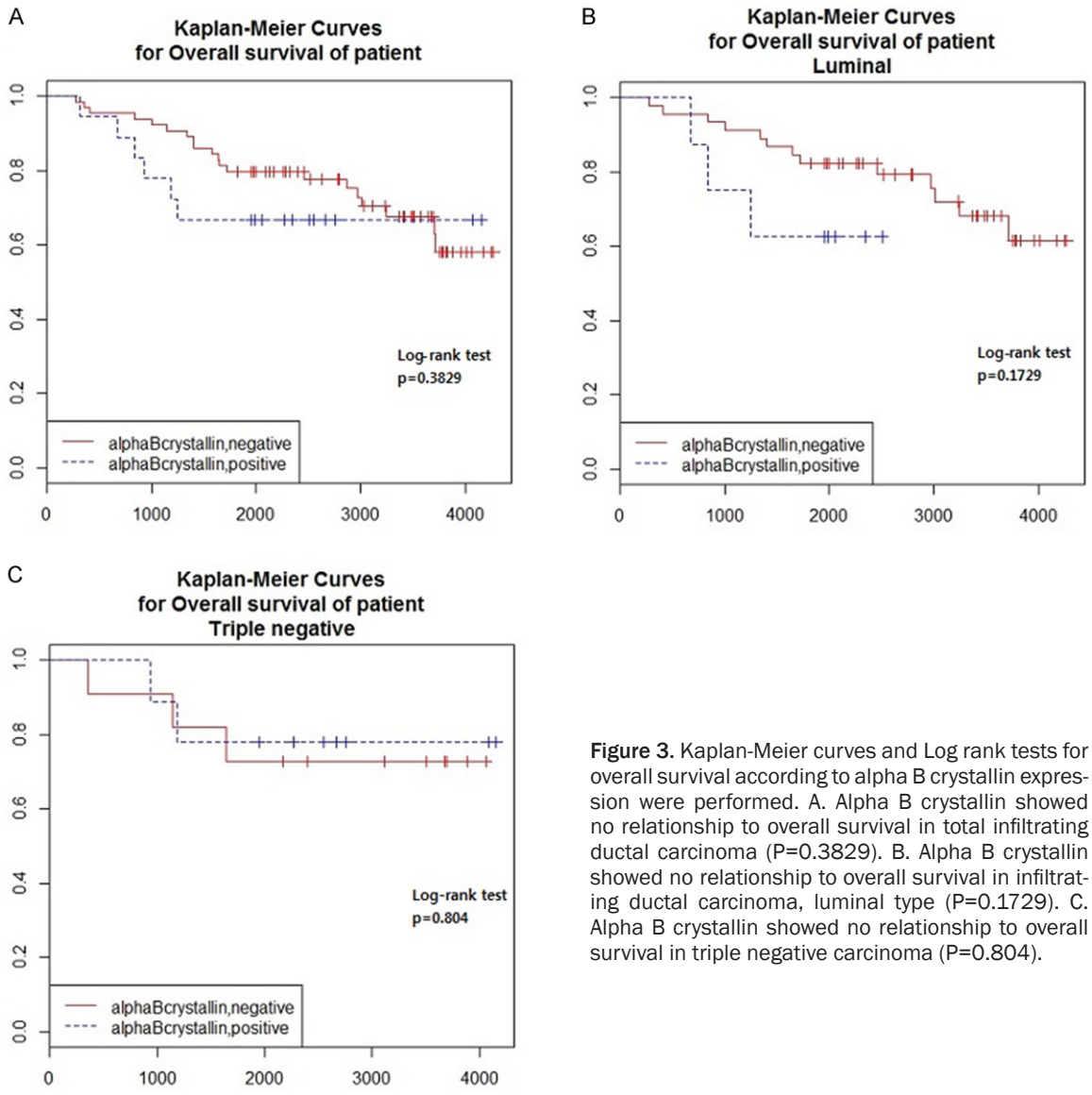

Figure 3. Kaplan-Meier curves and Log rank tests for overall survival according to alpha B crystallin expression were performed. A. Alpha B crystallin showed no relationship to overall survival in total infiltrating ductal carcinoma (P=0.3829). B. Alpha B crystallin showed no relationship to overall survival in infiltrating ductal carcinoma, luminal type (P=0.1729). C. Alpha B crystallin showed no relationship to overall survival in triple negative carcinoma (P=0.804).

expression (*P*=0.017173), age (*P*=0.013719), and chemotherapy (*P*=0.000217) were related to overall survival.

ANCOVA analysis of disease-free survival according to αB crystallin expression and clinicopathologic characteristics

Results of multivariate analysis (ANCOVA) between disease-free survival and alpha B crystallin expression and clinicopathologic characteristics are shown in **Table 10**. Alpha B crystallin expression did not show a significant relationship with disease-free survival in multivariate analysis (*P*=0.1589258). Age (*P*=0.0244879) and chemotherapy (*P*=0.0005936) were significantly related to disease-free survival.

ANCOVA analysis of overall survival according to BCL2 expression and clinicopathologic characteristics

Results of multivariate analysis (ANCOVA) between overall survival and BCL2 expression and clinicopathologic characteristics are shown in **Table 11**. BCL2 expression was not significantly related to overall survival in multivariate analysis (*P*=0.2337589) whereas chemotherapy (p=0.0003207) and age (p=0.0166602) were related to overall survival.

ANCOVA analysis of disease-free survival according to BCL2 expression and clinicopathologic characteristics

Results of multivariate analysis (ANCOVA) between disease-free survival and BCL2 expres-

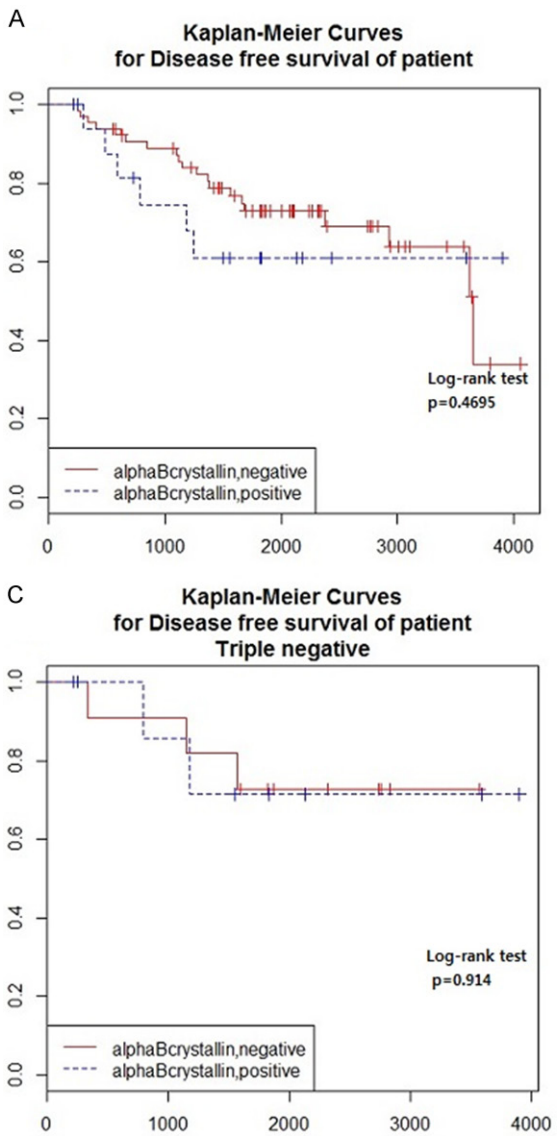

Figure 4. Kaplan-Meier curves and Log rank tests for disease-free survival according to alpha B crystallin expression were performed. A. Alpha B crystallin showed no relationship to disease-free survival in total infiltrating ductal carcinoma (P=0. 4695). B. Alpha B crystallin showed no relationship to disease-free survival in infiltrating ductal carcinoma, luminal type (P=0.3026). C. Alpha B crystallin showed no relationship to disease-free survival in triple negative carcinoma (P=0. 914).

sion and clinicopathologic characteristics are shown in **Table 12**. BCL2 expression did not show a significant relationship with disease-free survival in multivariate analysis (*P*=0.87-40983). However, age (*P*=0.0264587), chemotherapy (P=0.0006969), radiation therapy (*P*=0.0241012), and N stage (*P*=0.0228228) were significantly related to disease-free survival.

Alpha B crystallin expression and survival

Kaplan-Meier curves for survival according to alpha B crystallin expression are shown in **Figures 3** and **4**. Log rank tests were performed to evaluate the relationship between alpha B expression and survival. Alpha B crystallin showed no relationship to overall survival (total breast cancer group: *P*=0.3829; luminal cancer group: *P*=0.1729; triple negative group: *P*=0.804). In addition, alpha B crystallin also showed no relationship with disease-free survival (total breast cancer group: P=0.4695; luminal cancer group: *P*=0.3026; triple negative group: *P*=0.914).

BCL2 expression and survival

Kaplan-Meier curves for survival according to BCL2 expression are shown in **Figures 5** and **6**. Log rank tests were performed to evaluate the relationship between BCL2 and survival. BCL2 showed no relationship with overall survival in the total breast cancer group (*P*=0.0677) and triple negative group (*P*=0.2018). However, in the luminal cancer group BCL2 showed a statistically significant correlation with overall survival (*P*=0.01848). Similarly, BCL2 showed no relationship with disease-free survival in the

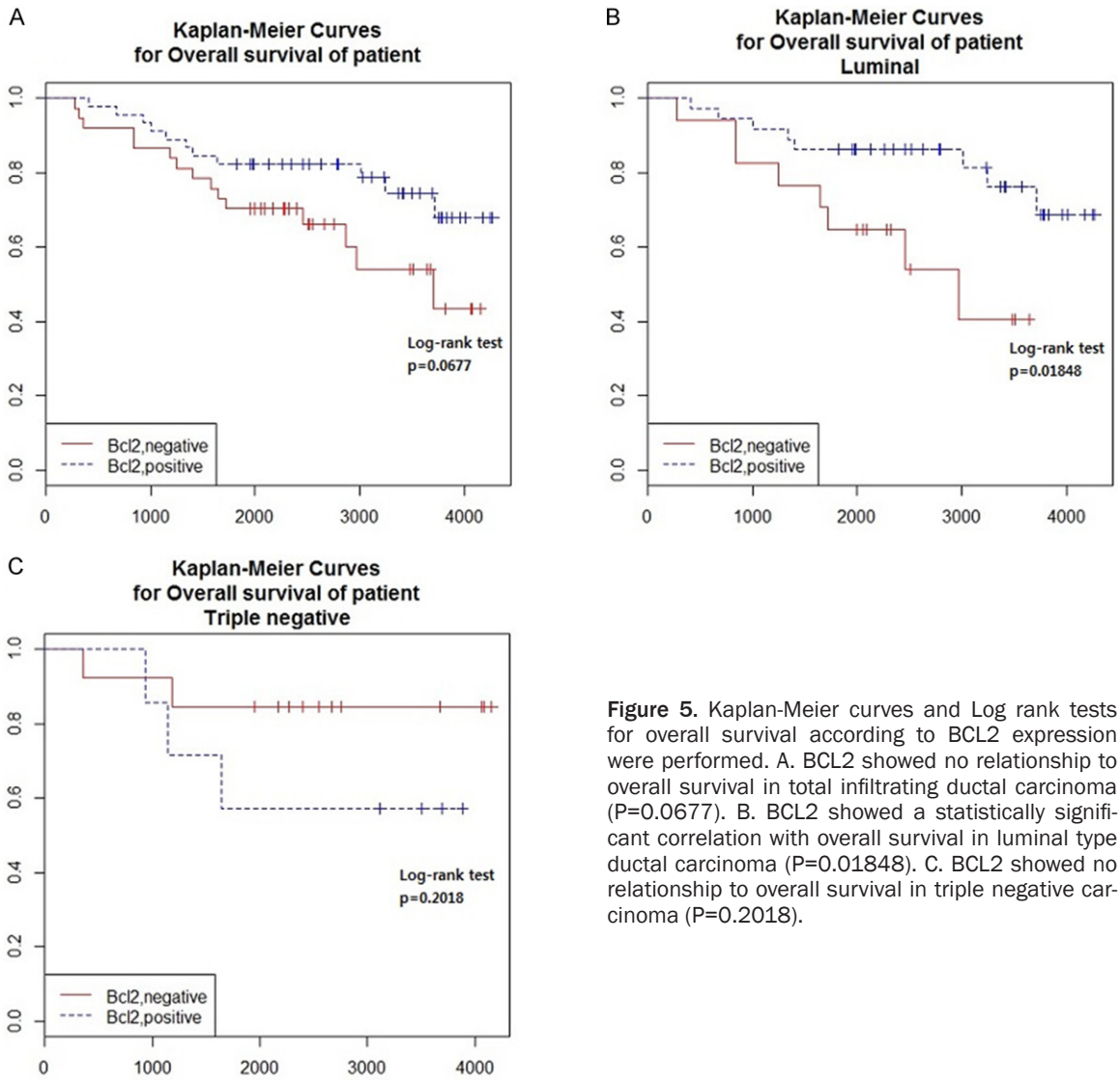

Figure 5. Kaplan-Meier curves and Log rank tests for overall survival according to BCL2 expression were performed. A. BCL2 showed no relationship to overall survival in total infiltrating ductal carcinoma (P=0.0677). B. BCL2 showed a statistically significant correlation with overall survival in luminal type ductal carcinoma (P=0.01848). C. BCL2 showed no relationship to overall survival in triple negative carcinoma (P=0.2018).

total breast cancer group (*P*=0.1203) and triple negative group (*P*=0.307), but was significantly correlated with disease-free survival in the luminal cancer group (*P*=0.03643).

Correlation analysis between alpha B crystallin protein and BCL2 protein

In correlation analyses, expression of the two proteins showed a weak negative correlation: Pearson's product-moment correlation: -0.2335857, Kendall's rank correlation tau: -0.1871347, Spearman's rank correlation rho: -0.2157422 (**Table 13**).

Discussion

Alpha-crystallin is a major protein of the vertebrate eye lens and is also found in several types of cancer, especially head and neck squamous cell carcinoma and breast carcinoma [1, 15, 16]. There are two alpha crystallin subtypes, alpha A (acidic) crystallin and alpha B (basic) crystallin. Alpha B crystallin is a 175-amino acid protein encoded by the *CRYAB* gene on chromosome 11. Alpha B crystallin is a small heat-shock protein and functions as a molecular chaperone. It selectively binds denatured or improperly folded proteins and prevents protein aggregation [17]. Apoptosis is modulated by various proteins. Members of the BCL2 family and heat-shock proteins have important functions in apoptosis. BCL2 proteins are present on mitochondrial, nuclear, and endoplasmic reticulum membranes, where they perform antiapoptotic functions. In contrast, Bax, which is localized in the cytoplasm and

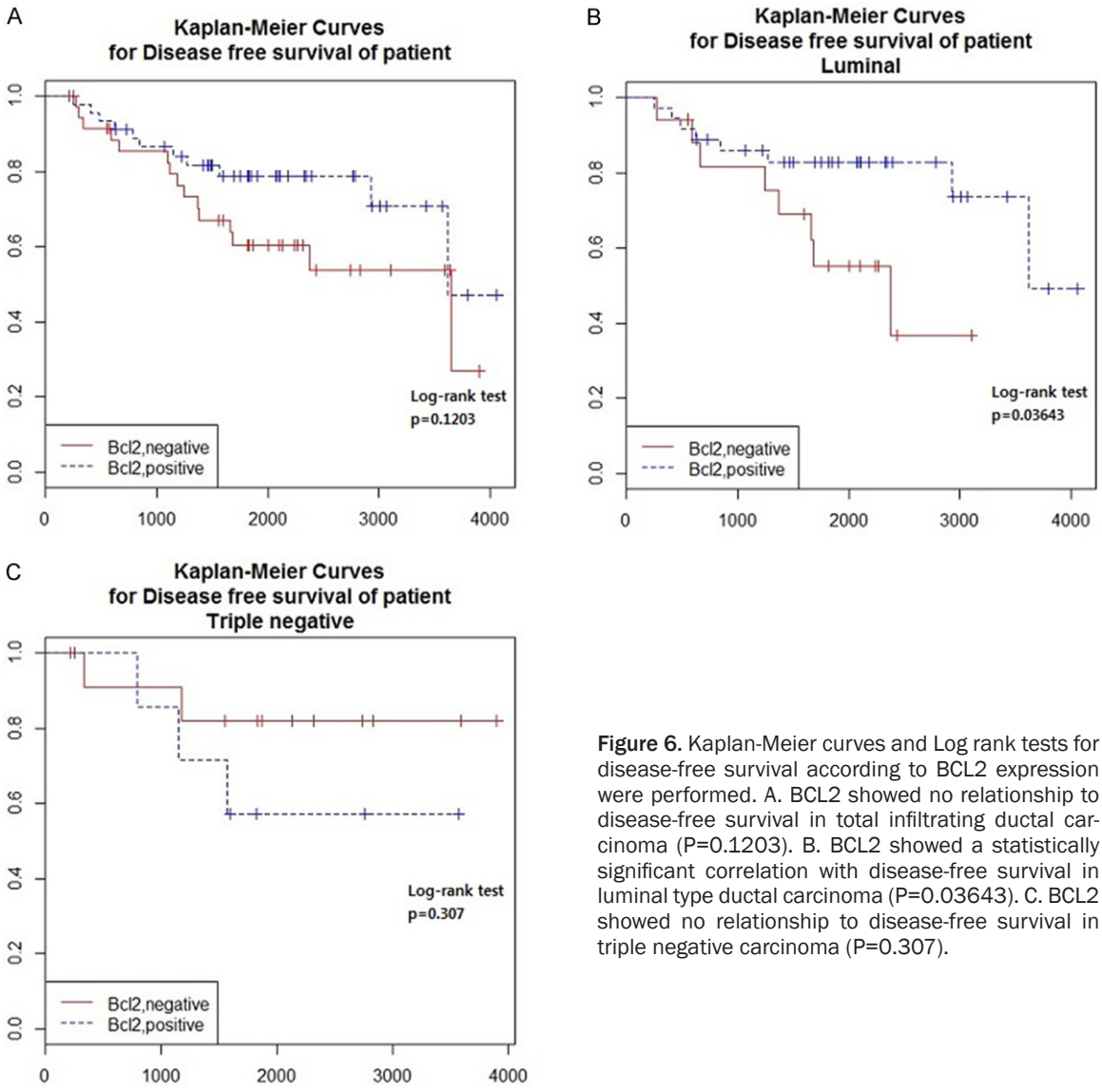

Figure 6. Kaplan-Meier curves and Log rank tests for disease-free survival according to BCL2 expression were performed. A. BCL2 showed no relationship to disease-free survival in total infiltrating ductal carcinoma (P=0.1203). B. BCL2 showed a statistically significant correlation with disease-free survival in luminal type ductal carcinoma (P=0.03643). C. BCL2 showed no relationship to disease-free survival in triple negative carcinoma (P=0.307).

is loosely attached to the mitochondrial membrane, induces apoptosis and Bcl-X_s stimulates apoptosis by inactivation of antiapoptotic BCL2 family members [6, 18]. BCL2 protein is a well-known antiapoptotic regulator that sustains cell survival rather than promoting cell proliferation. BCL2 protein expression in tumorigenesis was first described in non-Hodgkin's lymphoma, in which overexpression of BCL2 leads to lymphoma as a result of the chromosomal translocation [14, 18]. However, overexpression of BCL2 in breast cancer has been associated with a favorable prognosis. The mechanism underlying this paradoxical effect of BCL2 is still unknown [7-9]. Together with the BCL2 family, heat shock proteins also have an important role in apoptosis. Alpha B crystallin represses oxidative stress-induced apoptosis through interaction with procaspase-3 and partially processed procaspase-3 to prevent caspase-3 activation [10]. A previous study showed that expression of alpha B crystallin protein correlated with TRAIL (tumor necrosis factor-related apoptosis inducing ligand) expression in a panel of human cancer cell lines. Expression of alpha B crystallin protects cancer cells from TRAIL-induced caspase-3 activation and apoptosis *in vitro* [19]. Experimental results using cell cultures indicated that overexpression of alpha B crystallin provoked anchorage-independent growth, loss of polarity, decreased apoptosis, cell proliferation, broken structure, and increased migration and invasion in two mammary epithelial cell lines (MCF-10A and

MCF-12A). These findings suggest that alpha B crystallin functions as an oncoprotein [13].

In the present study, expression of alpha B crystallin was observed more frequently in triple negative cancer (9/20, 45%) than in luminal type cancer (8/53, 15.1%, P=0.02161). BCL2 tended to be more highly expressed in luminal type cancer than in HER2 and triple negative cancer types (luminal: 36/53, 68%, HER2: 2/9, 22%, triple negative: 7/20 35%, P=0.008652). In contrast to previous studies [7-9], BCL2 was not associated with survival in multivariate analysis using ANCOVA. Thus, in our study BCL2 was not an independent prognostic indicator. In univariate analysis of luminal type, BCL2 showed a relationship with overall survival (P=0.02819) but the reason for this is suspected to be the confounding effect of a lower T stage in the BCL2 positive group (T1: 44%, T2: 66%) than in the negative group (T1: 17.6%, T2: 64.7%, T3: 5.9%, T4: 11.8%). In univariate analysis of alpha B crystallin expression, the positive group showed a shorter overall survival in both the total breast cancer group (P=0.01646) and luminal type cancer group (P=0.01034). Furthermore, in multivariate analysis using ANCOVA, alpha B crystallin remained related to short overall survival (P=0.017173). These findings suggest that alpha B crystallin is an independent prognostic factor of infiltrating ductal carcinoma.

In rabbit lens epithelial cells, the human *BCL2* gene downregulated alpha B crystallin and induced apoptosis [10]. BCL2 protein is known to be a positive prognostic marker of breast cancer whereas alpha B crystallin is a negative prognostic marker. Given these findings, we studied the relationship between BCL2 protein expression and alpha B crystallin protein expression. Univariate analysis showed statistical significance in the total breast cancer group (P=0.01463). In correlation analysis, the two proteins showed a weak negative correlation (Pearson's product-moment correlation: -0.2335857, Kendall's rank correlation tau: -0.1871347, Spearman's rank correlation rho: -0.2157422). However, these findings were not sufficient to demonstrate a correlation between alpha B crystallin and BCL2.

Our study has the limitations of the small number of cases and the restriction of immunohistochemical staining as the only method of protein detection. Despite these limitations, our results will contribute to future research on alpha B crystallin and the prognosis of breast cancer.

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Disclosure of conflict of interest

None.

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