Next, LVTEs in the 3 tumor areas identified by HE staining were stained with D2-40 to determine whether they were true LVTEs to compare the positive predictive value of LVTEs identified by HE staining and by D2-40 staining (Fig. 2E, H, K, N, and P).

To determine the lymph vessel density, we searched for 4 to 5 D2-40–positive lymph vessel hot spots in each tumor area under low-power magnification (×40) and then counted the D2-40–positive lymph vessels in the highest D2-40–positive lymph vessel hot spot as the lymph vessel density, that is, as number per unit area of the tumor (Fig. 2C).

Consecutive histologic sections were cut from the HE- and D2-40–stained specimens to confirm that the LVTEs identified by HE staining in each tumor area were also D2-40 positive. Two investigators (CY and TH) used HE and D2-40 staining to identify the LVTEs and determine lymph vessel density in each tumor area of all IDCs, and whenever there was a discrepancy, they reexamined the slides together to reach a consensus.

2.4. Prognosis and statistical analysis

Survival was evaluated by follow-up for a median period of 101 months as of January 2005. The tumor recurred in 50 patients, and 39 patients had died of their disease.

We used D2-40 staining to confirm that the number of LVTEs identified by HE staining in each tumor area was the true number and analyzed the positive predictive value, sensitivity, and false-negative rates of the numbers of LVTEs based on the results of D2-40 staining. We also investigated the reason for failure to detect LVTEs by HE staining in each tumor area. We then analyzed the sensitivity, specificity, and positive and negative predictive value of the presence of LVTEs detected by HE staining in each tumor area of the IDCs in comparison with the LVTEs detected by D2-40 staining. In addition, correlations between the numbers of LVTEs identified by HE or D2-40 staining in the intratumor area and in the advance area or nontumor area were analyzed by calculating Pearson linear correlation coefficients, and the numbers of LVTEs identified by HE staining and D2-40 staining in corresponding tumor areas were also analyzed for calculations by Pearson linear correlation coefficient.

Univariate analyses for a significant association with nodal metastasis, tumor recurrence, or death were performed by using the following parameters: (1) presence of at least

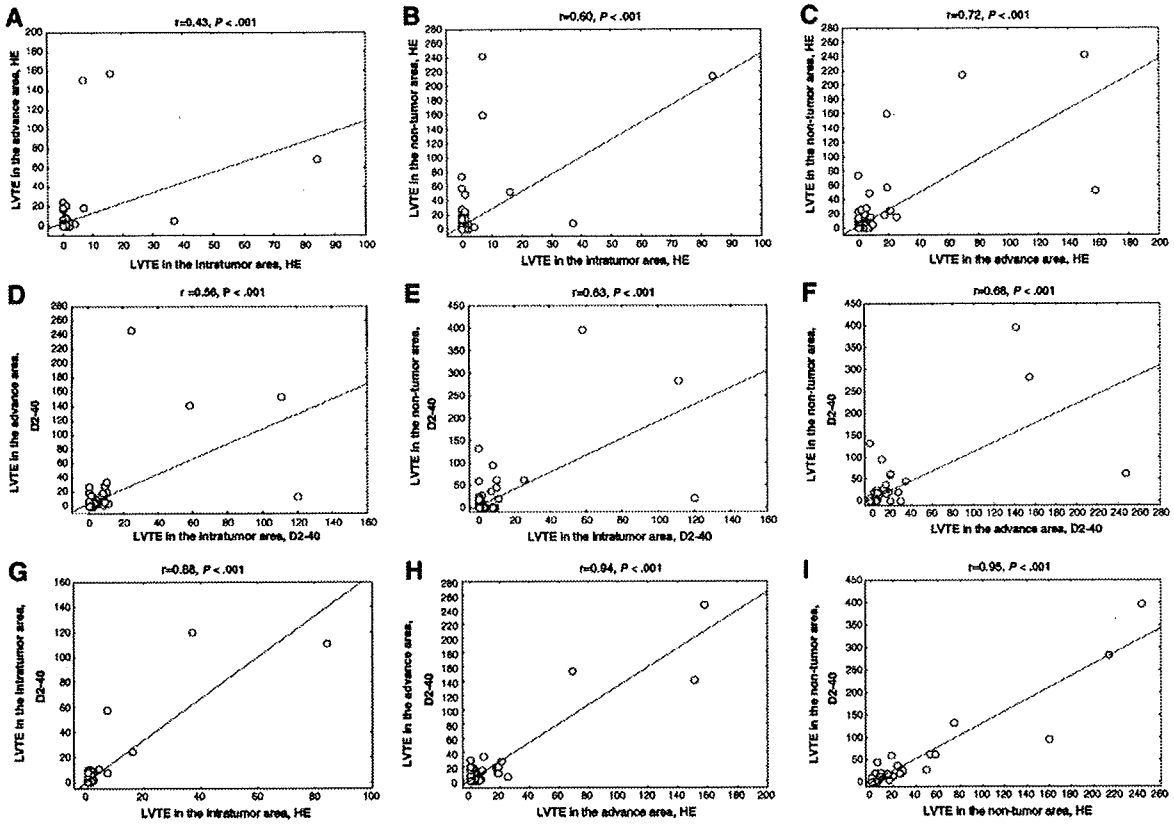


Fig. 3 Pearson coefficient for correlations between the numbers of LVTEs identified by HE staining and by D2-40 staining in the intratumor area, in the advance area, and in the nontumor stroma area of IDCs (A-I). The numbers of LVTEs in the 3 tumor areas were significantly correlated with each other, independent of the staining method used (A-F). The numbers of LVTEs in the corresponding tumor areas were also significantly correlated with each other (G-I).

Table 3 Number of cases with a true LVTE according to tumor area

	Total	No. of cases with a D-LVTE					
	151	Intratumor		Advance		Nontumor	
No. of cases with an H-LVTE		30	121				
Intratumor area		+ (%)	− (%)	+ (%)	− (%)	+ (%)	− (%)
+	19	16 (84)	3 (16)				
−	132	14 (11)	118 (89)				
Sensitivity, 54%; specificity, 98%; positive predictive value, 84%; negative predictive value, 89%							
Advance area				69	82		
+	52			44 (85)	8 (15)		
−	99			25 (25)	74 (75)		
Sensitivity, 64%; specificity, 90%; positive predictive value, 85%; negative predictive value, 75%							
Nontumor area						36	115
+	35					30 (86)	5 (14)
−	116					6 (5)	110 (95)
Sensitivity, 83%; specificity, 95%; positive predictive value, 86%; negative predictive value, 95%							

Mean no. of H- or D-LVTE in the advance area or in the nontumor area in cases with an LVTE identified by the intratumor area in HE staining or D2-40 staining

	Cases with an H-LVTE in the intratumor area			Cases with a D-LVTE in the intratumor area		
	Absent	Present	*P*	Absent	Present	*P*
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
No. of LVTE in the advance area	5.4 ± 3.3	23.9 ± 48.7	<.001	1.7 ± 4.2	36.6 ± 67.5	<.001
No. of LVTE in the nontumor area	5.9 ± 9.1	42.4 ± 75.6	<.001	3.0 ± 14.1	55.1 ± 105.0	<.001

Abbreviations: H-LVTE, an LVTE identified by HE staining; D-LVTE, an LVTE identified by D2-40 staining; +, number of cases with an LVTE identified by HE or D2-40 staining; −, number of cases in which no LVTEs were identified by HE or D2-40 staining.

1 LVTE detected by HE or D2-40 staining, (2) D2-40 lymph vessel density, (3) age (≤40 versus >40 years), (4) menopausal status (premenopause versus postmenopause), (5) invasive tumor size (≤20 versus >20 mm), (6) histologic grade (grade 1 or 2 versus grade 3) [13], (7) fibrotic focus (absent versus present) [14,15], (8) blood vessel invasion (absent versus present), (9) lymph node status (negative versus positive), (10) estrogen receptor (ER)/progesterone receptor (PR) status (either or both positive versus both negative), and (11) pTNM stages [16]. The median lymph vessel density value was used as the cutoff value in each tumor area, and because the median value in the intratumor area was "0," "1" was used as the cutoff value of the intratumor area. Clinicopathologic parameters, the presence or the absence of an LVTE identified by HE or D2-40, and D2-40 lymph vessel density, which were significantly associated with nodal metastasis or patient outcome in the univariate analyses, were then entered into the multivariate analyses using the logistic regression model [17] or the Cox proportional hazard regression model [18] by the step-down method until all remaining factors were significant at a *P* value of less than .05. Because only 6 patients with IDC without nodal metastasis died of their disease, we could not perform a multivariate analysis. In addition, because vessel

Table 4 Multivariate analyses for lymph node metastasis in all cases (151 cases)

Model 1: LVTEs identified by HE staining					*Model 2: LVTEs identified by D2-40 staining*				
Parameter	Cases	LNMR (%)	RR (95% CI)	*P*	Parameter	Cases	LNMR (%)	RR (95% CI)	*P*
LVTEs in the nontumor stroma area					LVTEs in the nontumor stroma area				
Absent	116	53 (46)	Referent		Absent	115	54 (47)	Referent	
Present	35	30 (86)	3.5 (3.0-24.4)	<.001	Present	36	29 (81)	6.5 (2.5-17.0)	.004
Fibrotic focus					Fibrotic focus				
Absent	65	27 (42)	Referent		Absent	65	27 (42)	Referent	
Present	86	56 (65)	2.6 (1.2-5.5)	.008	Present	86	56 (65)	3.0 (1.4-6.3)	.003
Blood vessel invasion					Blood vessel invasion				
Absent	127	64 (50)	Referent		Absent	127	64 (50)	Referent	
Present	24	19 (79)	4.7 (1.5-14.4)	.008	Present	24	19 (79)	4.6 (1.5-13.9)	.006

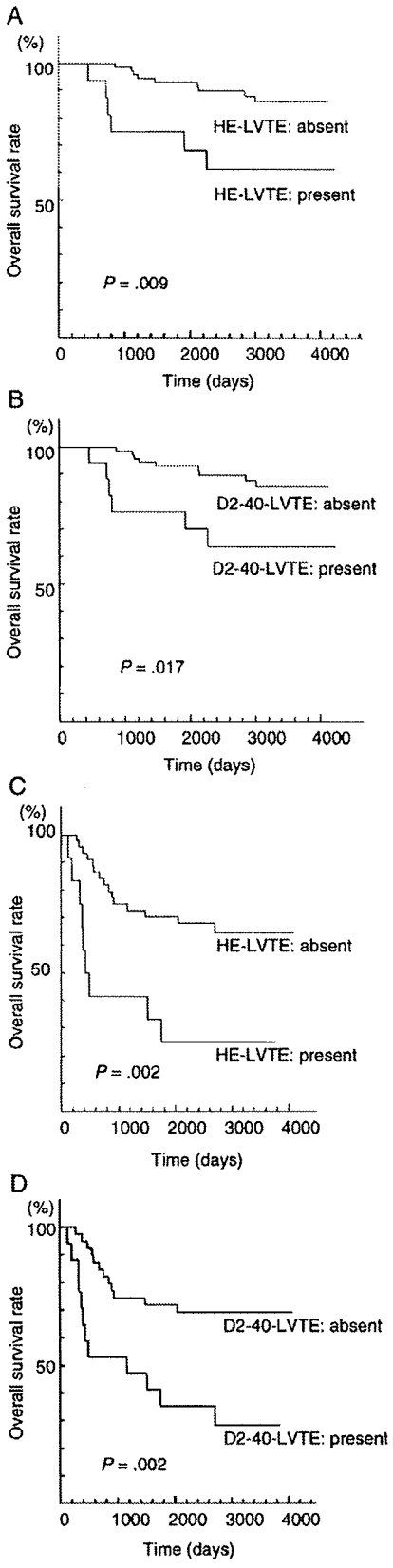
Abbreviations: LNMR, lymph node metastasis rate; RR, relative risk; CI, confidence interval.

Table 5 Multivariate analyses for tumor recurrence and death according to ER and PR status

Model 1: LVTEs identified by HE staining					Model 2: LVTEs identified by D2-40 staining				
ER or PR positive or both positive (90 cases)									
Parameter	Cases	TRR (%)	HR (95% CI)	*P*	Parameter	Cases	TRR (%)	HR (95% CI)	*P*
LVTEs in the nontumor stroma area					LVTEs in the nontumor stroma area				
Absent	73	15 (21)	Referent		Absent	72	34 (47)	Referent	
Present	17	11 (65)	3.9 (1.8-8.6)	<.001	Present	18	15 (83)	3.2 (1:4-7.1)	.004
Tumor size (mm)					Tumor size (mm)				
≤20	32	4 (13)	Referent		≤20	32	4 (13)	Referent	
>20	58	22 (38)	3.2 (1.1-9.5)	.032	>20	58	22 (38)	3.8 (1.3-11.0)	.015
Parameter	Cases	MR (%)	HR (95% CI)	*P*	Parameter	Cases	MR (%)	HR (95% CI)	*P*
Histologic grade					Histologic grade				
1 and 2	68	7 (10)	Referent		1 and 2	68	7 (10)	Referent	
3	22	8 (36)	3.0 (1.5-5.7)	.001	3	22	8 (36)	3.3 (1.7-6.4)	<.001
Lymph node metastasis					Lymph node metastasis				
Absent	41	1 (2)	Referent		Absent	41	1 (2)	Referent	
Present	49	14 (29)	3.6 (1.5-8.3)	.003	Present	49	14 (29)	2.7 (1.1-6.6)	.033
ER- and PR-negative cases (57 cases)									
Parameter	Cases	TRR (%)	HR (95% CI)	*P*	Parameter	Cases	TRR (%)	HR (95% CI)	*P*
LVTEs in the intratumor area					LVTEs in the advance area				
Absent	45	15 (33)	Referent		Absent	29	6 (21)	Referent	
Present	12	9 (75)	2.7 (1.1-6.6)	.031	Present	28	18 (64)	3.7 (1.4-9.7)	.009
Histologic grade					Histologic grade				
1 and 2	34	10 (29)	Referent		1 and 2	34	10 (29)	Referent	
3	23	14 (61)	5.4 (2.0-14.3)	.001	3	23	14 (61)	5.9 (2.2-16.3)	.001
Lymph node metastasis					Lymph node metastasis				
Absent	23	5 (22)	Referent		Absent	23	5 (22)	Referent	
Present	34	19 (56)	6.6 (2.0-21.2)	.002	Present	34	19 (56)	5.4 (1.8-16.4)	.003
Fibrotic focus					Fibrotic focus				
Absent	20	4 (20)	Referent		Absent	20	4 (20)	Referent	
Present	37	20 (54)	3.8 (1.2-11.9)	.021	Present	37	20 (54)	3.7 (1.1-12.1)	.029
Tumor size (mm)					Tumor size (mm)				
≤20	23	4 (17)	Referent		≤20	23	4 (17)	Referent	
>20	34	20 (59)	3.2 (1.0-10.1)	.043	>20	34	20 (59)	2.7 (1.1-6.6)	.031
Parameter	Cases	MR (%)	HR (95% CI)	*P*	Parameter	Cases	MR (%)	HR (95% CI)	*P*
LVTEs in the intratumor area					LVTEs in the intratumor area				
Absent	45	15 (33)	Referent		Absent	40	12 (30)	Referent	
Present	12	9 (75)	3.0 (1.2-7.4)	.015	Present	17	12 (71)	4.1 (1.7-9.9)	.002
Histologic grade					Histologic grade				
1 and 2	34	10 (29)	Referent		1 and 2	34	10 (29)	Referent	
3	23	14 (61)	3.8 (1.5-9.2)	.009	3	23	14 (61)	3.8 (1.5-9.2)	.004
					Tumor size (mm)				
					≤20	23	4 (17)	Referent	
					>20	34	20 (59)	4.6 (1.4-15.0)	.010

Abbreviations: TRR, tumor recurrence rate; MR, mortality rate; CI, confidence interval.

invasion (the combined assessment of lymph vessel invasion and blood vessel invasion) has recently been reported to be a very important prognostic factor for patients with IDC [19], we also analyzed the prognostic power of vessel invasion in the 3 tumor areas in the multivariate analyses. Because of the possibility of several IDCs with an LVTE identified by HE staining also containing LVTEs identified by D2-40 staining, the presence of at least 1 LVTE identified by HE and by D2-40 stain was analyzed separately in the multivariate analyses (model 1, HE staining; model 2, D2-40 staining). Crude disease-free survival curves and overall survival curves were drawn by



the Kaplan-Meier method [20]. All P values reported are 2 sided, and the significance level was set at $P < .05$. All analyses were performed with Statistica/Windows software (StatSoft, Tulsa, Okla).

3. Results

3.1. Maximum, minimum, median, and mean numbers of LVTEs identified by HE staining and D2-40 staining according to tumor area

The largest number of cases in which an LVTE was detected by HE or D2-40 staining was observed in the advance area, and the smallest number of cases that had an LVTE was observed in the intratumor area (Table 1). The highest maximum number of LVTEs identified by HE or D2-40 staining was in the nontumor area, and it was followed by the advance area and then the intratumor area, and the mean numbers of LVTEs showed a similar tendency.

The highest maximum D2-40 lymph vessel density was in the advance area, but the highest mean and median D2-40 lymph vessel density was in the nontumor area.

3.2. Cumulative actual numbers of LVTEs identified by HE and D2-40 staining according to tumor area

The cumulative actual numbers of LVTEs identified by HE and D2-40 increased from the intratumor area to the nontumor area, and D2-40 detected larger numbers of LVTEs than HE staining in every tumor area (Table 2). The sensitivity of HE staining for detection of LVTEs increased from the intratumor area to the nontumor area, and the results of this study clearly demonstrated LVTEs identified by HE staining in each tumor area had a very high positive predictive value, with the highest positive predictive value being in the intratumor area. The results of this study clearly demonstrated that almost all the true LVTEs in the intratumor area were detected as stroma-invasive tumor nests by HE staining, whereas the largest numbers of true LVTEs in the nontumor areas were detected in the form of angiovessel tumor emboli. In the HE-stained specimens true LVTEs in the advanced area were misdiagnosed as stroma-invasive tumor nests or angiovessel tumor emboli.

Fig. 4 A and B, Disease-free survival curves of patients with IDC with HE- and D2-40–stained LVTEs, respectively, that is, with ER or PR positive or positive for both. The IDC group with an LVTE in the nontumor area identified by HE or D2-40 staining had a shorter disease-free survival time than the IDC group without any LVTEs in the nontumor area ($P = .009$ and $P = .017$). C and D, Overall survival curves according to LVTE detection by HE staining and D2-40 staining in patients with IDC that is both ER and PR negative. The IDC group with an LVTE in the intratumor area identified by HE or D2-40 staining had a shorter overall survival time than the IDC group with no LVTEs in the intratumor area ($P = .002$ and $P = .002$).

Table 6 Multivariate analyses for tumor recurrence and death according to nodal status

Model 1: LVTEs identified by HE staining					Model 2: LVTEs identified by D2-40 staining				
Parameter	Cases	TRR (%)	HR (95% CI)	*P*	Parameter	Cases	TRR (%)	HR (95% CI)	*P*
Lymph node metastasis–negative cases (65 cases)									
LVTEs in the nontumor stroma area					LVTEs in the nontumor stroma area				
Absent	60	8 (13)	Referent		Absent	58	8 (14)	Referent	
Present	5	3 (60)	20.9 (3.2-137.2)	.002	Present	7	3 (43)	12.3 (1.7-87.4)	0.011
Fibrotic focus					Fibrotic focus				
Absent	37	4 (11)	Referent		Absent	37	4 (11)	Referent	
Present	28	7 (25)	6.9 (1.3-37.8)	.027	Present	28	7 (25)	7.0 (1.2-41.4)	0.033
Lymph node metastasis–positive cases (83 cases)									
Histologic grade					LVTEs in the advance area				
1 and 2	56	18 (32)	Referent		Absent	37	13 (35)	Referent	
3	27	20 (74)	3.1 (1.5-6.1)	.001	Present	46	25 (54)	2.8 (1.4-5.9)	.004
ER and PR					Histologic grade				
Negative	34	19 (56)	Referent		1 and 2	56	18 (32)	Referent	
Positive	49	19 (39)	0.3 (0.2-0.7)	.002	3	27	20 (74)	4.3 (2.1-9.0)	<.001
Tumor size (mm)					ER and PR				
≤20	23	4 (17)	Referent		Negative	34	19 (56)	Referent	
>20	60	34 (57)	3.7 (1.2-11.2)	.018	Positive	49	19 (39)	0.3 (0.2-0.7)	.002
Fibrotic focus					Tumor size (mm)				
Absent	27	8 (30)	Referent		≤20	23	4 (17)	Referent	
Present	56	30 (54)	2.4 (1.1-5.3)	.035	>20	60	34 (57)	3.9 (1.3-11.3)	.015
					Fibrotic focus				
					Absent	27	8 (30)	Referent	
					Present	56	30 (54)	2.5 (1.1-5.5)	.030
Parameter	Cases	MR (%)	HR (95% CI)	*P*	Parameter	Cases	MR (%)	HR (95% CI)	*P*
LVTEs in the intratumor area					LVTEs in the intratumor area				
Absent	67	22 (33)	Referent		Absent	60	17 (28)	Referent	
Present	16	10 (63)	2.3 (1.1-5.0)	.031	Present	23	15 (65)	3.3 (1.6-6.6)	.001
Histologic grade					Histologic grade				
1 and 2	56	14 (25)	Referent		1 and 2	56	14 (25)	Referent	
3	27	18 (67)	4.0 (1.9-8.2)	<.001	3	27	18 (67)	4.4 (2.1-9.0)	<.001
ER and PR					ER and PR				
Negative	34	18 (53)	Referent		Negative	34	18 (53)	Referent	
Positive	49	14 (29)	0.3 (0.2-0.7)	.003	Positive	49	14 (29)	0.4 (0.2-0.8)	.007

Abbreviations: TRR, tumor recurrence rate; MR, mortality rate; CI, confidence interval.

The numbers of LVTEs identified by HE and D2-40 staining in the intratumor area were significantly correlated with the number of LVTEs identified by HE and D2-40 staining in the advance and in the nontumor area (Fig. 3A-F). In addition, the numbers of LVTEs identified by HE staining in each area were significantly correlated with the numbers of LVTEs identified by D2-40 staining (Fig. 3G-I).

In terms of the concordance between the results of LVTE identified by HE and D2-40 staining, very high specificity, positive predictive values, and negative predictive values were obtained in cases with true LVTE in each tumor observed in this study (Table 3). In addition, cases in which an LVTE identified by HE or D2-40 staining in the intratumor area had significantly higher mean numbers of LVTEs identified by HE or D2-40 staining in the advance area and in the nontumor area than in which cases no LVTEs were identified by HE or D2-40 staining (Table 3).

3.3. Multivariate analyses for lymph node metastasis, tumor recurrence, and death

Multivariate analyses clearly demonstrated that identification of LVTEs by HE staining or D2-40 staining and the presence of a fibrotic focus or blood vessel invasion were significantly associated with the presence of lymph node metastasis (Table 4).

The multivariate analyses showed that in IDCs that were ER or PR positive or positive for both and in which HE or D2-40 staining detected an LVTE, the presence of an LVTE in the nontumor area and tumor size greater than 20 mm significantly increased the hazard rates (HRs) of tumor

Table 7 Multivariate analyses for tumor recurrence and death according to ER and PR status

Model 1: LVTEs identified by HE staining and blood vessel invasion					Model 2: LVTEs identified by D2-40 staining and blood vessel invasion				
Parameter	Cases	TRR (%)	HR (95% CI)	*P*	Parameter	Cases	TRR (%)	HR (95% CI)	*P*
ER or PR positive or both positive (90 cases)									
LVTEs in the nontumor stroma area and blood vessel invasion					Tumor size (mm)				
Absent	60	12 (20)	Referent		≤20	32	4 (13)	Referent	
Present	30	14 (47)	2.2 (1.1-4.2)	.022	>20	58	22 (38)	3.5 (1.2-10.2)	.023
Tumor size (mm)									
≤20	32	4 (13)	Referent						
>20	58	22 (38)	3.2 (1.1-9.4)	.036					
ER and PR negative (57 cases)									
LVTEs in the intratumor area and blood vessel invasion					LVTEs in the advance area and blood vessel invasion				
Absent	37	10 (27)	Referent		Absent	23	2 (9)	Referent	
Present	20	14 (70)	2.9 (1.3-6.2)	.006	Present	34	22 (65)	3.4 (1.6-7.1)	.001
Histologic grade					Histologic grade				
1 and 2	34	10 (29)	Referent		1 and 2	34	10 (29)	Referent	
3	23	14 (61)	6.2 (2.4-16.0)	<.001	3	23	14 (61)	7.6 (2.8-20.1)	<.001
Lymph node metastasis					Lymph node metastasis				
Absent	23	5 (22)	Referent		Absent	23	5 (22)	Referent	
Present	34	19 (56)	5.5 (1.7-17.6)	.004	Present	34	19 (56)	4.7 (1.4-15.1)	.010
Parameter	Cases	MR (%)	HR (95% CI)	*P*	Parameter	Cases	MR (%)	HR (95% CI)	*P*
LVTEs in the intratumor area and blood vessel invasion					LVTEs in the intratumor area and blood vessel invasion				
Absent	37	10 (27)	Referent		Absent	33	8 (24)	Referent	
Present	20	14 (70)	3.4 (1.6-7.4)	.002	Present	24	16 (67)	3.1 (1.7-5.9)	<.001
Histologic grade					Histologic grade				
1 and 2	34	10 (29)	Referent		3 and 2	34	10 (29)	Referent	
3	23	14 (61)	4.5 (1.8-11.2)	<.001	1	23	14 (61)	3.9 (1.6-9.5)	.003
Lymph node metastasis					Tumor size (mm)				
Absent	22	5 (23)	Referent		≤20	23	4 (17)	Referent	
Present	34	18 (53)	3.3 (1.2-9.4)	.024	>20	34	20 (59)	4.2 (1.4-12.5)	.011

Abbreviations: TRR, tumor recurrence rate; MR, mortality rate; CI, confidence interval.

recurrence (Table 5, Fig. 4A and B). The presence of an LVTE identified by HE or D2-40 staining failed to increase the HR for tumor death, but histologic grade 3 and the presence of nodal metastasis significantly increased the HRs for tumor death (Table 5).

The multivariate analyses showed that in IDCs that were both ER and PR negative and wherein HE-staining identified an LVTE, the presence of an LVTE in the intratumor area significantly increased the HRs of tumor recurrence (Table 5). On the other hand, in IDCs in which D2-40 staining detected an LVTE, a significant increase in the HR for tumor recurrence was observed when an LVTE was present in the advance area (Table 5). The presence of an LVTE identified by HE or D2-40 staining in the intratumor area significantly increased the HRs for tumor death in the multivariate analyses (Table 5, Fig. 4C and D).

In IDCs without nodal metastasis and in which HE or D2-40 staining detected an LVTE, the presence of an LVTE in the nontumor area and the presence of a fibrotic focus significantly increased the HRs of tumor recurrence (Table 6).

Although no significant association between the presence of an LVTE identified by HE staining and tumor recurrence was observed in IDCs with nodal metastasis, the presence of an LVTE, identified by D2-40 staining in the advance area as well as histologic grade 3, ER/PR-negative status, tumor size greater than 20 mm, and presence of a fibrotic focus, significantly increased the HRs of tumor recurrence in the multivariate analysis (Table 6). As for tumor death, the presence of an LVTE in the intratumor area, identified by HE or D2-40 staining, significantly increased the HRs of tumor death in the multivariate analysis (Table 6).

Combined assessment of the presence of an LVTE and the presence of blood vessel invasion revealed that the presence of an LVTE identified by HE staining in the nontumor stroma area in IDCs that were ER or PR positive or positive for both remained significantly associated with tumor recurrence in the multivariate analysis, but that the presence of a lymph vessel invasion identified by D2-40 staining in the nontumor stroma area lost its significant association with tumor recurrence (Table 7). Histologic grade 3 and the presence of lymph node metastasis

Table 8 Multivariate analyses for tumor recurrence and death according to nodal status

Model 1: LVTEs identified by HE staining and blood vessel invasion					Model 2: LVTEs identified by D2-40 staining and blood vessel invasion				
Parameter	Cases	TRR (%)	HR (95% CI)	*P*	Parameter	Cases	TRR (%)	HR (95% CI)	*P*
Lymph node metastasis–positive cases (83 cases)									
Histologic grade					LVTEs in the intratumor area and blood vessel invasion				
1 and 2	56	18 (32)	Referent		Absent	45	14 (31)	Referent	
3	27	20 (74)	3.1 (1.5-6.1)	.001	Present	38	24 (63)	2.2 (1.3-3.7)	.004
ER and PR					Histologic grade				
Negative	34	19 (56)	Referent		1 and 2	56	18 (32)	Referent	
Positive	49	19 (39)	0.3 (0.2-0.7)	.002	3	27	20 (74)	4.3 (2.2-8.4)	<.001
Tumor size (mm)					ER and PR				
≤20	23	4 (17)	Referent		Negative	34	19 (56)	Referent	
>20	60	34 (57)	3.7 (1.2-11.2)	.018	Positive	49	19 (39)	0.4 (0.2-0.8)	.011
Fibrotic focus									
Absent	27	8 (30)	Referent						
Present	56	30 (54)	2.4 (1.1-5.3)	.035					
Parameter	Cases	MR (%)	HR (95% CI)	*P*	Parameter	Cases	MR (%)	HR (95% CI)	*P*
LVTEs in the intratumor area and blood vessel invasion					LVTEs in the intratumor area and blood vessel invasion				
Absent	50	14 (28)	Referent		Absent	45	10 (22)	Referent	
Present	33	18 (55)	2.2 (1.2-4.2)	.015	Present	38	22 (58)	2.5 (1.4-4.3)	.001
Histologic grade					Histologic grade				
1 and 2	56	14 (25)	Referent		1 and 2	56	14 (25)	Referent	
3	27	18 (67)	4.0 (2.0-8.4)	<.001	3	27	18 (67)	4.6 (2.2-9.3)	<.001
ER and PR					ER and PR				
Negative	34	18 (53)	Referent		Negative	34	18 (53)	Referent	
Positive	49	14 (29)	0.3 (0.2-0.7)	.002	Positive	49	14 (29)	0.3 (0.2-0.7)	.004

Abbreviations: TRR, tumor recurrence rate; MR, mortality rate; CI, confidence interval.

significantly increased the HRs of tumor death (data not shown).

In IDCs that were both ER and PR negative, the combined presence of a lymph vessel invasion and blood vessel invasion showed that the same tumor area of the presence of an LVTE identified by HE or D2-40 staining significantly increased the HRs of tumor recurrence or death in the multivariate analyses (Table 7).

In IDCs without nodal metastasis, the combined presence of an LVTE identified by HE or D2-40 staining and blood vessel invasion failed to significantly increase the HRs of tumor recurrence in the multivariate analyses (data not shown). The combined assessment of the presence of an LVTE identified by HE or D2-40 staining and the presence of blood vessel invasion in IDCs with nodal metastasis showed that the same tumor area of the presence of an LVTE identified by HE or D2-40 also significantly increased the HRs of tumor recurrence or death in the multivariate analyses (Table 8).

4. Discussion

The results of this study clearly demonstrated that LVTEs were actually present in the intratumor area of IDCs and that the cumulative numbers of LVTEs identified by HE staining and D2-40 staining gradually increased from the intratumor area to the nontumor area. Indeed, LVTEs were detected by staining with D2-40 in each tumor area that were not detected by HE staining alone, for example, approximately 60% of IDC LVTEs in the intratumor area. However, the results of this study also clearly demonstrated a very high positive predictive value of LVTEs in each tumor area, especially in the intratumor area (95%), and significant correlations were observed between the numbers of LVTEs identified by HE staining and by D2-40 staining in each of the 3 areas. This strongly suggests that pathologists can fairly accurately determine whether LVTEs are present in each tumor area, if they are going to strictly determine whether any LVTEs are present in each tumor area. Furthermore, because the presence of a true LVTE by D2-40 staining can be confirmed, if pathologists find tumor nests that they suspect of being LVTEs in any of the tumor areas, especially in the intratumor area, they should perform D2-40 staining to accurately determine whether an LVTE is present.

From the standpoint of an individual case with the presence or the absence of an LVTE identified by HE staining in each tumor area, although the sensitivity values of each tumor area were not high, the positive or negative

predictive value of the presence or absence of an LVTE in each tumor area in all of the IDCs was very high. We can therefore conclude that pathologists can fairly accurately determine whether LVTEs are present in each tumor area in every patient with IDC by HE staining alone, and that was confirmed by the fact that in this study, the presence of an LVTE in any of the tumor areas had similar prognostic value, independent of the staining method used. These findings also strongly suggest that LVTEs identified by HE staining alone in each tumor area could very well be extremely important prognostic parameters for patients with IDC when all of the LVTEs cannot be identified. Because it would be difficult to routinely perform D2-40 staining of all IDCs to accurately count the LVTEs or identify an LVTE, it can be concluded that a routine method for determining whether an LVTE is present in each tumor area in each IDC would probably be adequate to accurately predict the outcome of patients with IDC, and detection of the presence of an LVTE in the intratumor area or nontumor area by HE staining is a very useful prognostic factor for patients with IDCs, independent of their ER/PR status or nodal status.

Although our approach to the assessment of LVTEs would probably enable pathologists to perform a high rate of agreement between the results of examination for the presence of LVTEs in each tumor area in HE- and D2-40–stained specimens, this study also showed very important evidence to judge whether there are LVTEs in the intratumor area in IDCs. In this study, cases with an LVTE in the intratumor area had significantly higher mean numbers of LVTEs in the advance area and in the nontumor stroma area, independent of the staining method. This strongly suggests a high probability that cases with many LVTEs in the advance area or in the nontumor stroma area have LVTEs in the intratumor area. Thus, when pathologists encounter IDCs, like an inflammatory breast cancer, with many LVTEs in the advance area or in the nontumor stroma area, they should carefully examine the intratumor area for the presence of an LVTE.

We studied the value of the combined examination of tumors for the presence of an LVTE and the presence of a blood vessel tumor embolus, because it has been reported that their presence is an important prognostic parameter for patients with IDCs [19]. However, the results of the combined examination were not better than the results of the examination for the presence of an LVTE alone in this study. Because the presence of blood vessel invasion had no predictive value for tumor recurrence or death in the multivariate analyses in this study, the results of the combined assessment of the presence of a lymph vessel invasion and the presence of a blood vessel invasion failed to add prognostic power to the results of the assessment of the presence of an LVTE alone. Thus, the results of this study clearly show that combined assessment of the presence of an LVTE and the presence of a blood vessel tumor embolus does not always provide greater prognostic power than assessment of the presence of an LVTE alone.

In contrast to the number of LVTEs, the mean D2-40 lymph vessel density values in the 3 tumor areas decreased from the nontumor area to the intratumor area, and lymph vessel density determined by D2-40 staining in the 3 tumor areas was of no significance for predicting the outcome of the patients with IDC in this study. Although it has been reported that lymph vessel density may or may not be a good prognostic parameter for patients with breast cancer [21-23] and that lymphangiogenesis may or may not occur in breast cancer tissues [24], our results clearly demonstrate no prognostic value of lymph vessel density for patients with IDC, and that lymphangiogenesis did not occur in our patients with IDC of the breast.

In conclusion, this study clearly demonstrated (1) that intratumoral LVTEs are present in IDCs, and (2) that the presence of LVTEs in the intratumor area and nontumor area has prognostic significance, independent of the stain used. Although it is somewhat difficult to determine whether intratumoral LVTEs are present by HE staining alone, to accurately assess the true malignant potential of IDCs of the breast, if pathologists find tumor nests that they suspect of being LVTEs in the intratumor area, they should examine the tumor for intratumoral LVTEs by using a combination of HE and D2-40 staining.

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Smaller Regional Volumes of Brain Gray and White Matter Demonstrated in Breast Cancer Survivors Exposed to Adjuvant Chemotherapy

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BACKGROUND. Previous studies have shown cognitive impairment in breast cancer survivors who were exposed to adjuvant chemotherapy. Neural damage by chemotherapy might have played some part in these findings. The current study explored the regional brain volume difference between breast cancer survivors exposed to adjuvant chemotherapy (C+) and those unexposed (C−).

METHODS. High-resolution 1.5-tesla brain magnetic resonance imaging (MRI) databases of breast cancer survivors and healthy controls were used. Brain images were preprocessed for optimal voxel-based morphometry. Comparisons of gray matter and white matter were performed between the C+ and the C− groups, by using MRI scans from within 1 year (the 1-year study, n = 51 and n = 55, respectively) or 3 years after their cancer surgery (the 3-year study, n = 73 and n = 59, respectively). As exploratory analyses, correlation analyses were performed between indices of the Wechsler Memory Scale-Revised and regional brain volume where the volumes were significantly smaller. As a reference, MRI scans of cancer survivors were compared with those of healthy controls (n = 55 for the 1-year study and n = 37 for the 3-year study).

RESULTS. The C+ patients had smaller gray matter and white matter including prefrontal, parahippocampal, and cingulate gyrus, and precuneus in the 1-year study. However, no difference was observed in the 3-year study. The volumes of the prefrontal, parahippocampal gyrus, and precuneus were significantly correlated with indices of attention/concentration and/or visual memory. Comparisons with healthy controls did not show any significant differences.

CONCLUSIONS. Adjuvant chemotherapy might have an influence on brain structure, which may account for previously observed cognitive impairments. ***Cancer* 2007;109:146–56.** © *2006 American Cancer Society.*

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The survival rate of breast cancer patients is increasing with the development of systemic chemotherapy. In this situation, management of long-term side effects of potentially curative breast cancer treatment is of substantial importance to optimal quality of life of breast cancer survivors. Recently, impairments of cognitive function, which is a prerequisite for functioning in daily life, have been recognized as a possible long-term adverse effect, which has been termed "chemobrain".[1,2] Previous reviews have shown that most of these studies have consistently indicated impairments of various cognitive domains in breast cancer survivors exposed to adjuvant chemotherapy.[3–6] However, the neural mechanisms have not been fully studied.

Neural impairments caused by chemotherapeutic agents as shown in animals may play a part in these mechanisms. Although chemotherapeutic agents were thought initially to have little ability to penetrate the blood-brain barrier, recent studies have indicated higher concentrations than were expected in cerebrospinal fluid and brain tissue.[7–9] Chemotherapeutic agents are hypothesized to have neurotoxic potential through their ability to interfere with DNA and RNA synthesis and function, inhibition of microtubule formation, and/or immunosuppressive properties.[10,11] In animals, intracerebroventricularly injected methotrexate was reported to cause learning and memory impairments and damage to the hippocampal CA4 region.[12] Injection of doxorubicin into the caudate-putamen indicated neuronal death in the ventral tegmentum and thalamus.[13] Intraperitoneal injection of cyclophosphamide produced lesions within the cortex, thalamus, hippocampal dentate gyrus, and caudate nucleus in a dose-dependent fashion.[14] In the same report, cyclophosphamide and methotrexate showed a concentration-dependent neurotoxic effect on neuronal cell cultures. Another study has demonstrated that free radicals are a possible mechanism for the toxic effect of chemotherapeutic agents.[15]

Recently, neuroimaging techniques have developed dramatically, thus enabling investigation of brain structure in humans. In a preliminary investigation that used structural magnetic resonance imaging (MRI), Saykin et al. reported regional brain volumes in breast and lymphoma cancer survivors who lived more than 5 years after their initial treatment.[10] Results suggest that chemotherapy may be associated with reductions in regional brain volume. However, a further study is needed with a comparison group of cancer patients unexposed to chemotherapy to control for the impact of cancer diagnosis. Contrary to results of the Saykin et al. investigation, our study showed no significant difference in regional and whole-brain volumes between breast cancer survivors exposed to adjuvant chemotherapy and those unexposed 3 years after their breast cancer surgery.[16] Although a previous study indicated long-term cognitive impairment,[17] by taking previous reports that show recovery of cognitive impairments over time into consideration,[4,18–20] adverse changes in the brain structure may recover.

In the current study, we explore the regional brain volume difference between cancer survivors exposed to adjuvant chemotherapy and those unexposed in a 2-study setting (the 1-year study of <1 year after surgery and the 3-year study of >3 years after surgery). Our hypothesis was that smaller regional brain volumes would be associated with adjuvant chemotherapy. For secondary analysis, associations were examined between memory functions (as 1 of the cognitive functions) and the regional brain volume, where the volumes are significantly smaller in cancer survivors exposed to adjuvant chemotherapy.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board and the Ethics Committee of the National Cancer Center of Japan and was performed after obtaining written informed consent from patients. This study was conducted by using 2 databases of brain MRI scans from breast cancer survivors. One database (Long-Term-Survivors Database) comprised brain MRI scans of patients 3 years after their breast cancer surgery.[16] The other database (Follow-up Database) comprised brain MRI scans from 3–15 months after patients' breast cancer surgery and additional scans from 2 years after their first scan.

Subjects and Procedures

The 1-year study used baseline data from the Follow-up Database (Fig. 1). Subjects were recruited during follow-up visits to the Division of Breast Surgery, National Cancer Center Hospital East. We selected all patients who underwent their breast cancer surgery and who survived >3–15 months. The inclusion criteria were 1) female sex to minimize sex-based brain differences and 2) ages between 18 and 55 years. Exclusion criteria were 1) a history of cancer other than breast cancer or double cancer, 2) bilateral breast cancer, 3) clear evidence of residual or recurrent cancer, 4) current chemotherapy or radiation therapy, 5) a history of any neurological disorders, traumatic brain injury, or psychiatric disorders other than affective and anxiety disorders, 6) psychotropic

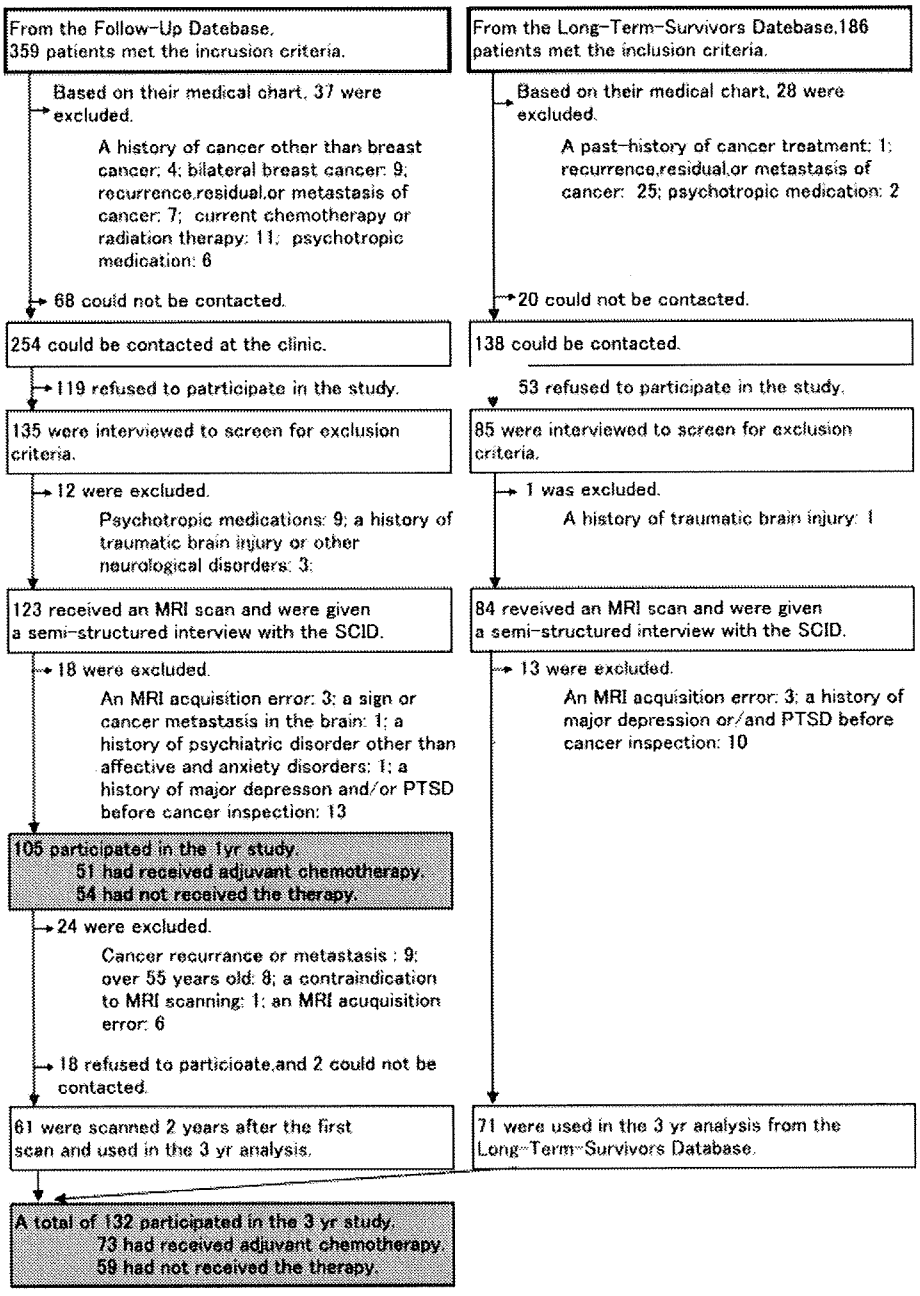

FIGURE 1. Subject sampling in the 1-year study and the 3-year study. SCID indicates the Structured Clinical Interview for DSM-IV Axis I Disorder, clinician version; MRI, magnetic resonance imaging; PTSD, post-traumatic stress disorder. In the 1-year study, the age and medical factors significantly differ between the 105 subjects and the 190 patients who did not participate because of our mistake to make contact with patients, patient refusal to participate, or MRI examination error were positive lymph node metastases found in their surgical tissue (31% versus 44%, $\chi^2 = 4.4$, $P = .04$), poor histological grade (27% versus 56%, $\chi^2 = 21.1$, $P < .01$), and receiving adjuvant chemotherapy (49% versus 62%, $\chi^2 = 5.1$, $P = .02$), respectively. In the 3-year study, age and medical factors that differed significantly between 132 subjects who participated in the study and 300 cancer survivors who did not participate were age (mean 44 years versus 46 years, $t = 3.7$, $P < .001$), presence of positive lymph node metastases (32% versus 43%, $\chi^2 = 4.9$, $P = .03$), poor histological grade (37% versus 49%, $\chi^2 = 4.9$, $P = .03$), and receiving hormonal therapy (35% versus 23%, $\chi^2 = 7.0$, $P = .01$), respectively.

TABLE 1
Demographic Information of Cancer Patients With and Without Adjuvant Chemotherapy, and Healthy Control Samples in the 1-Year and 3-Year Studies

	Sample 1 (1-Year study)			Sample 2 (3-Year study)		
	Patients		Healthy controls	Patients		Healthy controls
Characteristics	Adjuvant chemotherapy + (n = 51)	Adjuvant chemotherapy − (n = 54)	(n = 55)	Adjuvant chemotherapy + (n = 73)	Adjuvant chemotherapy − (n = 59)	(n = 37)
Age, mean ± SD, y	47.3 ± 5.2	46.3 ± 6.1	46.2 ± 6.7	48.2 ± 5.6	48.4 ± 4.8	48.0 ± 6.4
Handedness: right-handedness, no. (%)	51 (100)	51 (94.4)	51 (92.7)	73 (100)	55 (93.2)*	33 (89.2)
Height, mean ± SD, cm	155.0 ± 5.6	157.9 ± 5.8*	157.2 ± 5.0	156.4 ± 5.6	157.5 ± 6.0	156.6 ± 5.2
Weight, mean ± SD, kg	54.8 ± 6.6	56.9 ± 8.6	54.1 ± 7.9	55.1 ± 6.5	58.2 ± 8.7*	53.9 ± 8.0§
Education, mean ± SD, y	13.2 ± 1.7	13.2 ± 2.0	14.1 ± 1.9§	12.8 ± 1.7	13.2 ± 2.0	14.1 ± 1.7
Smoking, no. (%)	5 (9.8)	6 (11.1)	2 (3.6)	12 (16.4)	7 (11.9)	2 (5.4)
Accumulated alcohol consumption, mean ± SD, g × 10^3	27 ± 87	39 ± 59	29 ± 59	16 ± 42	47 ± 75†	38 ± 66
Postmenopausal, no. (%)	40 (78.4)	20 (37.0)†	16 (29.1)§	47 (64.4)	19 (32.2)†	15 (40.5)
Performance status: 0, no. (%)	30 (60.0)	43 (81.1)*	36 (97.3)§	67 (94.4)	57 (98.3)†	35 (97.2)
Clinical stage: 0–I, no. (%)	14 (27.5)	25 (46.3)*	NA	14 (19.2)	30 (50.8)†	NA
Lymphnode metastasis, positive, no. (%)	29 (56.9)	4 (7.4)†	NA	41 (56.2)	1 (1.7)†	NA
Histological type, no. (%)						
Carcinoma in situ	2 (3.9)	4 (7.4)	NA	1 (1.4)	4 (6.8)	NA
Invasive carcinoma	42 (82.4)	41 (75.9)	NA	61 (83.6)	49 (83.1)	NA
Special type	7 (13.7)	9 (16.7)	NA	11 (15.1)	6 (10.2)	NA
Histological grade: poor, no. (%)	21 (41.2)	7 (13.0)†	NA	36 (49.3)	13 (22.0)†	NA
Surgical type: partial mastectomy, no. (%)	25 (49.0)	32 (59.3)	NA	27 (37.0)	30 (50.8)	NA
Axillary lymphadectomy, no. (%)	42 (82.4)	28 (51.9)†	NA	68 (93.2)	43 (72.9)†	NA
Days after surgery, mean ± SD, d	345 ± 71	234 ± 103†	NA	1641 ± 360	1416 ± 316	NA
Protocol of adjuvant chemotherapy, no. (%)						
AC	3 (5.9)	NA	NA	15 (20.5)	NA	NA
CMF	40 (78.4)	NA	NA	37 (50.7)	NA	NA
EC	2 (3.9)	NA	NA	1 (1.4)	NA	NA
PTX	2 (3.9)	NA	NA	1 (1.4)	NA	NA
5-FU	0 (0)	NA	NA	9 (12.3)	NA	NA
5′-DFUR	0 (0)	NA	NA	1 (1.4)	NA	NA
HCFU	0 (0)	NA	NA	2 (2.7)	NA	NA
UFT	5 (9.8)	NA	NA	7 (9.6)	NA	NA
Days after adjuvant chemotherapy, mean ± SD, d	119 ± 47	NA	NA	1189 ± 359	NA	NA
Hormonal therapy	20 (39.2)	11 (20.4)*	NA	21 (28.8)	5 (8.5)†	NA
Radiation therapy, no. (%)	25 (49.0)	26 (48.1)	NA	23 (31.5)	19 (32.2)	NA
WMS-R index, mean ± SD						
Attention	99.4 ± 12.5	99.5 ± 11.5	99.6 ± 13.0	98.6 ± 10.4	103.0 ± 11.1*	NA
Verbal memory	96.9 ± 13.0	101.7 ± 14.5	99.2 ± 14.4	100.4 ± 15.6	103.3 ± 14.7	NA
Visual memory	101.9 ± 12.1	102.7 ± 11.4	101.4 ± 10.3	103.7 ± 10.4	104.1 ± 12.7	NA
Delayed recall	100.3 ± 10.4	102.5 ± 12.2	100.7 ± 12.6	103.9 ± 12.6	105.5 ± 11.5	NA
History of major depression, No. (%)	6 (11.8)	2 (3.7)	NA	20 (27.4)	8 (13.6)	0 (0)
History of PTSD, No. (%)	5 (9.8)	4 (7.4)	NA	5 (6.8)	2 (3.4)	0 (0)

NA indicates not applicable; PTSD, post-traumatic stress disorder; AC, regimen with doxorubicin and cyclophosphamide; CMF, regimen with cyclophosphamide, methotrexate and fluorouracil; EC, regimen with epirubicin and cyclophosphamide; PTX, paclitaxel; 5-FU, fluorouracil; 5′-DFUR, doxifluridine; HCFU, carmofur; UFT, tegafur/uracil; WMS-R, the Wechsler Memory Scale-Revised.

* Indicates significant difference ($P < .05$) between adjuvant chemotherapy group and no-adjuvant chemotherapy group.

† Indicates significant difference ($P < .01$) between adjuvant chemotherapy group and no-adjuvant chemotherapy group.

§ Indicates significant difference ($P < .01$) between cancer patients group and healthy control group.

medication taken within 1 month before participation in the study, 7) a history of substance abuse or dependence, 8) a family history of early dementia, 9) any physical symptoms that interfered with daily life, 10) possible dementia defined as a score of <24 on the Mini-Mental State Examination,[21,22] 11) a history of major depression and/or post-traumatic stress disorder (PTSD) before inspection for cancer diagnosis to exclude regional brain volume changes brought about by these disorders,[23] and 12) any contraindication to undergoing an MRI scan.

For the 3-year study, subjects were collected from the Follow-Up Database and the Long-Term-Survivor Database. From the Follow-Up Database, 105 subjects who participated in the 1-year study were asked to participate in the follow-up more than 2 years after their 1-year study.[16] Figure 1 indicates a summary of the recruitment of participants to the 1-year study and to the 3-year study.

We recruited healthy subjects, who lived in the same geographic areas as the patients, by using advertisements in the local newspaper. The inclusion and exclusion criteria were the same as those for cancer patients except for the requirement of a history of breast cancer surgery. Fifty-five healthy controls participated in the 1-year study. After 2 years, 37 of 55 healthy controls participated again in the 3-year study.

Neuropsychological Measurements

The Wechsler Memory Scale-Revised Japanese version was performed. The Wechsler Memory Scale-Revised (WMS-R),[24] a memory function scale validated in Japanese,[25,26] consists of indices of Attention/Concentration, Immediate Visual Memory, Immediate Verbal Memory, and Delayed Recall to estimate memory function. This scale is among the most generalized and widely used in the world.

Image Data Processing for Optimized Voxel-based Morphometry

MRI scans were conducted on a 1.5-tesla MRI unit (Signa Scanner, GE Medical Systems, Milwaukee, Wis), with 3-dimensional, spoiled gradient-recalled acquisition of 1.5-mm contiguous sections under the following conditions: field of view = 230 mm, matrix = 256 × 256 pixels, repetition time = 25 milliseconds, echo time = 5 milliseconds, and flip angle = 45°.[16]

The theory and algorithm of voxel-based morphometry (VBM) for Statistical Parametric Mapping 2 (SPM2) software (Wellcome Department of Cognitive Neurology, London, UK) have been well documented.[27] VBM was carried out by using an optimized method.[28] First, optimized study-specific template sets for the 1-year study and for the 3-year study comprising a T1 image and a priori gray matter, white matter, and cerebrospinal fluid images were created on the basis of brain images of participants in the 1-year study and the 3-year study, respectively. All scans were spatially normalized to customized templates, and then they were smoothed with an 8-mm, full-width half-maximum (FWHM) smoothing kernel, followed by averaging to create customized templates. Next, for the study group MRI scans, a brain extraction procedure that incorporated a segmentation step was used to remove nonbrain tissue from the MRI images.[28,29] Extracted gray matter and white matter images were normalized to the gray matter and white matter templates.[27,30] The normalization parameters were then reapplied to the original structural images to maximize optimal segmentation of fully normalized images, and these normalized images were segmented into gray matter/white matter and cerebrospinal fluid/noncerebrospinal fluid partitions.[31] Segmented images were modulated by the Jacobian determinants from spatial normalization to correct for volume changes that were introduced during nonlinear spatial transformations.[28] Finally, images were smoothed with a 12-mm FWHM kernel.[27,32]

Statistical Analysis

Student t test, Mann-Whitney U test, or χ^2 tests were used for comparison of background and medical factors. α levels were set at $P < .05$ (2-tailed).

By using SPM2, group differences in each of the gray matter and white matter scans were compared between the cancer patients exposed to their adjuvant chemotherapy and those unexposed, by using ANCOVA models, respectively, with age, alcohol consumption, intracranial volume, and background characteristics significantly different between these 2 groups (in the 1-year study, number of days after surgery and current hormonal therapy; in the 3-year study, handedness and current hormonal therapy) as nuisance variables. The intracranial volumes (sum of the gray matter, white matter, and cerebrospinal fluid volumes) were calculated from non-normalized segmented images during optimized-VBM preprocessing. Height and weight were not included as nuisance variables because intracranial volumes were modeled. Medical factors that seemed to be causes or results of adjuvant chemotherapy were not included as nuisance variables to avoid overmatching between the 2 groups. The groups were compared

using statistical t-test contrasts within SPM2. The distribution of morphological differences across each of the total gray matter or white matter was assessed initially on a voxel-by-voxel basis; clusters of over 400 voxels were used to suppress small clusters possibly arising by chance, and a threshold of $P < .001$ was used, uncorrected for multiple comparisons. Inference was centered on differences that achieved a significance of $P < .05$, after family-wise error correction for multiple comparisons.[33] In all analyses, we reported the Montreal Neurological Institute (MNI) coordinates of voxels of statistical significance.[34]

To see the effect of cancer on the brain structure as a reference, MRI scans of cancer survivors were compared with those of healthy controls, by using ANCOVA models with age, alcohol consumption, intracranial volume, and background characteristics significantly different between these 2 groups (in the 1-year study, year of education and menopausal state; in the 3-year study, no additional covariate) as nuisance variables.

For subanalyses, we examined the correlations between indices of the WMS-R and regional brain volume of the voxel where cancer survivors exposed to adjuvant chemotherapy had a significantly smaller brain region. Regional brain volumes of the voxels were calculated by using the region of interest (ROI) function in the SPM2 software as a substitution for the regional brain volume index.

RESULTS

Table 1 shows the background and medical factors of each group in both the 1-year study and the 3-year study. Eight percent of the survivors in the 1-year study and 8% of those in the 3-year study received tegafur and uracil (UFT) for <80 days. Ten percent of survivors in the 1-year study and 7% in the 3-year study received 5 of 6 cycles of their cyclophosphamide, methotrexate, and 5-fluorouracil regimen, and others completed their regimen in the 1-year study and in the 3-year study, respectively. In other cases, quantities of each of the administered chemotherapeutic agents complied with the protocols of each regimen.

The peak voxel coordinates of the smaller regions in cancer survivors exposed to adjuvant chemotherapy compared with those unexposed using corrected $P < .05$ are presented in Table 2. Figures 2 and 3 indicate superimposed images of the statistical t map (regional brain volume in cancer survivors exposed to adjuvant chemotherapy less than regional brain volume in those unexposed) on the template T1 image in the 1-year study. There was no significantly bigger region in cancer survivors exposed to adjuvant chemotherapy in the 1-year study. As an ad hoc analysis, we performed comparisons of gray matter and white matter between cancer survivors exposed to a cyclophosphamide, methotrexate, and 5-fluorouracil regimen (n = 40) and those unexposed to any adjuvant chemotherapy (n = 54). The distributions of regional brain volume difference were similar to those observed in the primary comparisons in the 1-year study (data not shown). There were no significantly smaller regions in gray matter and white matter when we used a corrected $P < .05$ in cancer survivors exposed to adjuvant chemotherapy in the 3-year study, as shown in Table 2.

In referential analyses, there were no significantly smaller or bigger regions in gray matter and white matter between cancer survivors and healthy controls in the 1-year study and in the 3-year study.

Table 3 indicates that significant correlations between memory functions and regional brain volumes

TABLE 2
Regions of Smaller Gray Matter and Nearest Gray Matter to Smaller White Matter in Breast Cancer Survivors With Adjuvant Chemotherapy Compared With Those Without Adjuvant Chemotherapy

	Coordinates of peak difference						
	x	y	z	Side	t score*	Corrected P	Region†
1-year study (3 to 15 months after breast cancer surgery)							
Gray matter	30	66	8	Right	4.77	.031	Middle frontal gyrus
	10	71	4	Right	4.73	.035	Superior frontal gyrus
	13	65	−12	Right	4.66	.045	Superior frontal gyrus
	21	−40	−11	Right	4.63	.048	Parahippocampal gyrus
White matter	−11	−60	64	Left	5.38	.005	Precuneus
	35	43	30	Right	5.12	.013	Middle frontal gyrus
	−13	−33	−5	Left	4.97	.023	Parahippocampal gyrus
	10	49	−1	Right	4.95	.025	Cingulate gyrus
	−10	49	44	Left	4.93	.026	Middle frontal gyrus
3-year study (27 to 39 months after breast cancer surgery)							
Gray matter							None
White matter							None

* All scores significant ($P < .05$) after family-wise error correction for multiple comparisons over each area of gray or white matter.
† Gray matter or nearest gray matter regions were indicated.

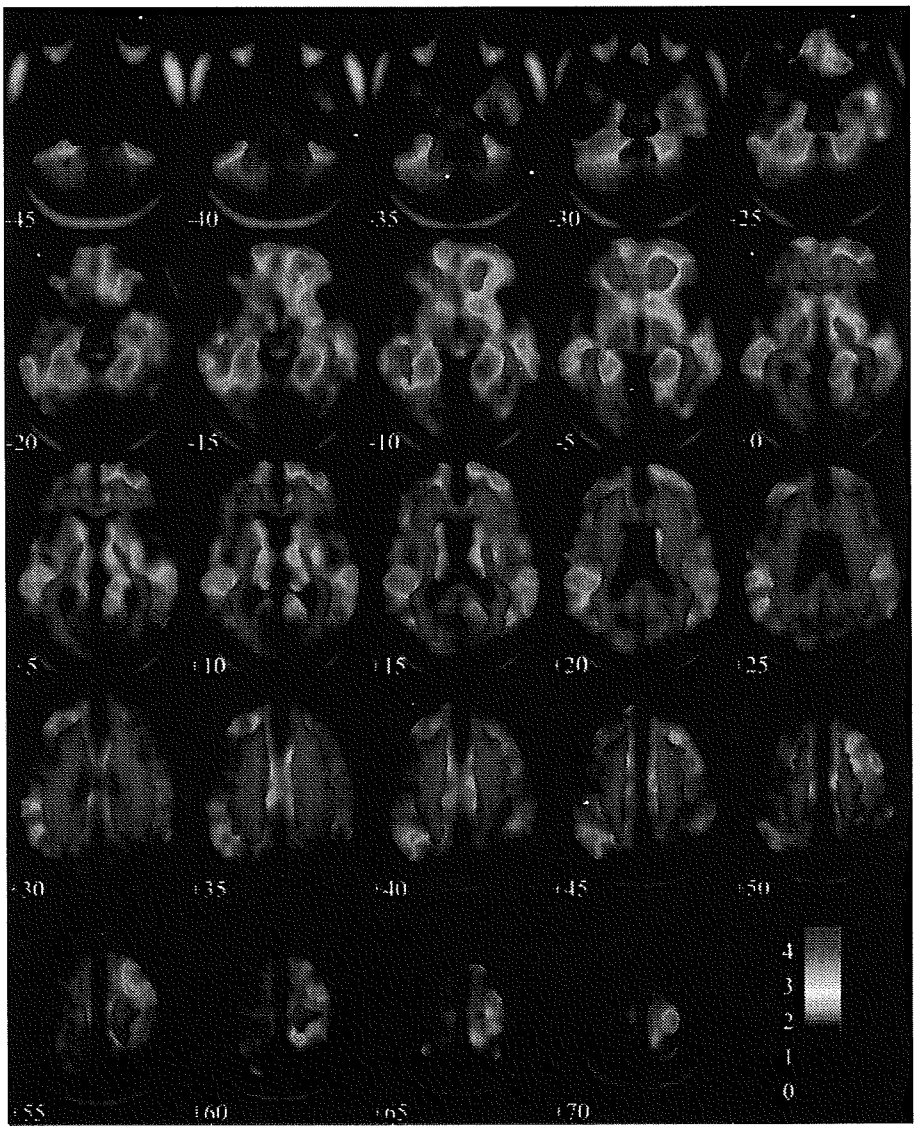

FIGURE 2. The superimposition of the *t*-value map of smaller gray matter in cancer survivors exposed to their adjuvant chemotherapy compared with those unexposed onto coronal slices of the customized T1 template image in the 1-year study. The color bar indicates the *t* value.

at the coordinates are significantly smaller in cancer survivors exposed to adjuvant chemotherapy in the 1-year study.

DISCUSSION

The current study showed smaller right prefrontal and parahippocampal gyrus in cancer survivors who were exposed to adjuvant chemotherapy before the mean of 4 months, compared with those unexposed. These volume differences were not found in cancer survivors at a mean of 4.2 years after completion of their adjuvant chemotherapy. In subanalyses, the volumes of the right superior frontal gyearus, 1 of the smaller regions in cancer survivors exposed to adjuvant chemotherapy, were associated with memory functions. These results indicate a potential effect of adjuvant chemotherapy on brain structure, and the change of the brain structure may be associated with memory function.

A previous report using VBM in 10 breast cancer and 2 lymphoma survivors (>5 years) exposed to chemotherapy showed smaller regional gray matter and cortical and subcortical white matter brain

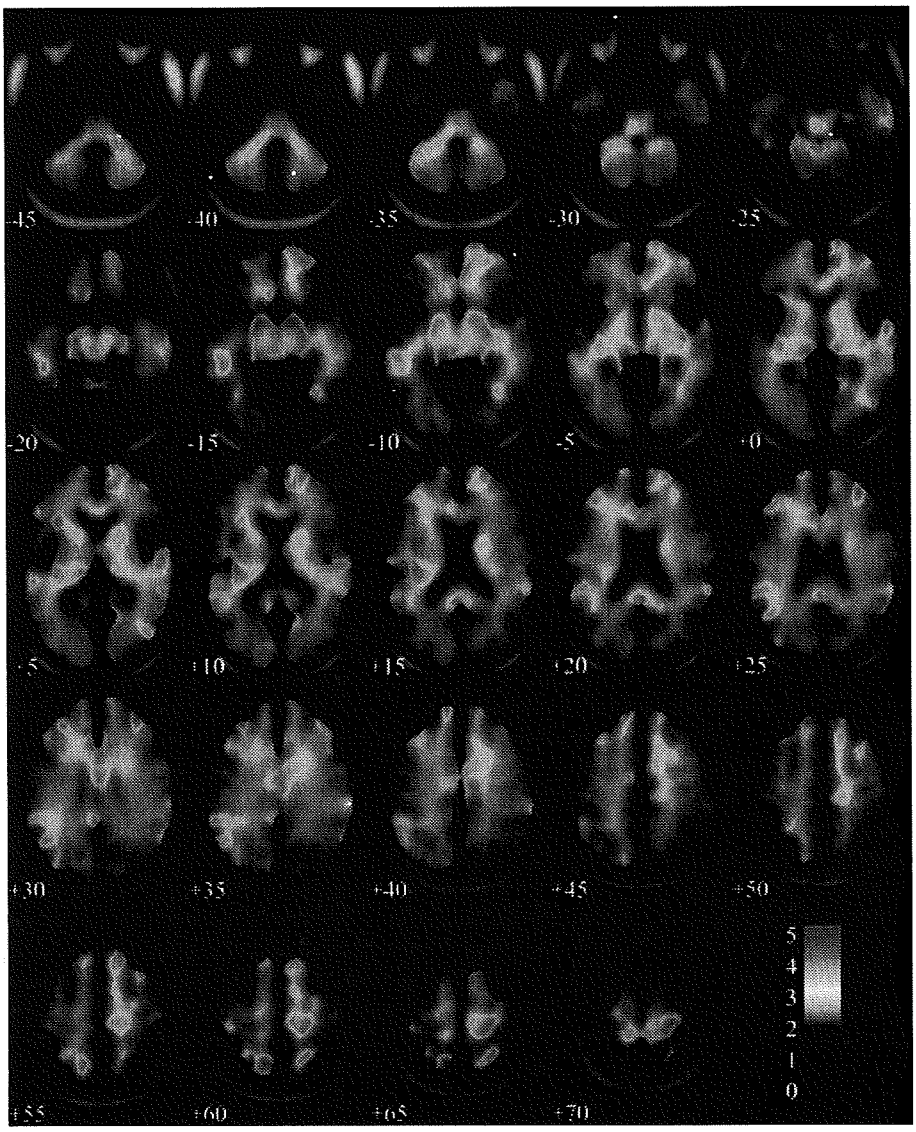

FIGURE 3. The superimposition of the *t*-value map of smaller white matter in cancer survivors exposed to their adjuvant chemotherapy compared with those unexposed onto coronal slices of the customized T1 template image in the 1-year study. The color bar indicates the *t* value.

regions compared with healthy controls.[10] Chemotherapeutic agents included in the previous report were similar to those in the current study. Contrary to results of the previous report, the results of the current study did not show any significant difference in regional brain volume as shown in the 3-year study. These findings were consistent with our previous study where we used a manual tracing method, which is a different method from the VBM, to measure regional brain volume.[16] That study indicated that there were no significantly smaller hippocampal and amygdalar volumes among breast cancer survivors who had survived >3 years since their surgery. This difference in results may be caused by differences in the methods, such as number and characteristics of subjects, comparisons with cancer survivors unexposed to chemotherapy, and/or use of corrections for multiple comparisons, as in the current study.

The current study indicated regional brain volume differences in the superior and middle frontal gyri, parahippocampal gyrus, cingulate gyrus, and

TABLE 3
Correlations Between Memory Functions and the Regional Brain Volume in the 1-Year Study

	x-y-z coordinate (Region)								
	Gray matter regions				White matter regions				
	30 66 8 (mFG)	10 71 4 (sFG)	13 65 −12 (sFG)	21 −40 11 (pHG)	11 60 64 (Pc)	35 43 30 (mFG)	13 33 −5 (pHG)	10 49−1 (CG)	−10 49 44 (mFG)
WMS-R index									
Attention/concentration	0.15	0.25*	0.08	0.12	0.21*	−0.03	0.06	0.09	0.12
Visual memory	0.10	0.24*	0.12	0.20*	0.13	−0.02	0.11	0.04	0.08
Verbal memory	−0.01	0.11	0.09	0.06	0.16	0.05	0.03	0.02	0.01
Delayed recall	−0.03	0.09	−0.01	−0.01	0.13	−0.02	−0.02	−0.08	−0.08

WMS-R indicates the Wechsler Memory Scale-Revised; mFG, middle frontal gyrus; sFG, superior frontal gyrus; pHG, parahippocampal gyrus; Pc, precuneus; CG, cingulate gyrus. Data indicate *r* value of the Pearson correlation test.
* Indicates significant association ($P < .05$).

precuneus. The significantly smaller volume of the superior frontal gyrus in the current study was correlated with the attention/concentration and visual memory indices of the WMS-R. The prefrontal cortex, including superior and middle frontal gyri, has been reported to have roles in various functions including memory, planning, execution, monitoring of cognitive processing and behavior, and inhibition and change in circumstantial behavior.[35] Not all, but many, of the studies in cancer survivors exposed to adjuvant chemotherapy have reported impairments in various cognitive domains including attention/concentration and visual memory functions.[3–6] The structural differences of the superior and middle prefrontal gyrus may partly account for some of the previously reported cognitive impairments and complaints referred to as "chemobrain". A previous positron emission tomography study of breast cancer survivors in whom the researchers had found significant neurocognitive changes associated with adjuvant chemotherapy, including impairment of verbal learning, demonstrated hypometabolism in the superior frontal gyearus. In addition to the prefrontal cortex, the parahippocampal gyrus is associated with cognitive functions, such as memory function.[36] Recently, the precuneus was also thought to have important roles in self-centered mental imagery strategies and episodic memory retrieval,[37] and these concepts lead us to suppose the potential engagement of structural changes in these brain regions in cognitive impairments caused by adjuvant chemotherapy.

The distribution of the regional brain volume differences observed in the 1-year study did not reappear in the 3-year study. Results from the 1-year and 3-year studies can lead us to speculate that the brain volume change related to adjuvant chemotherapy may well recover over the course of time. Although a previous report showed cognitive impairments in cancer survivors even after a long period following completion of adjuvant chemotherapy,[17] several longitudinal studies[18–20] and a review article[4] have demonstrated recovery from cognitive impairment in breast cancer survivors exposed to adjuvant chemotherapy. Regional brain structural changes and cognitive impairments observed in cancer survivors exposed to adjuvant chemotherapy may recover in time.

In reference comparisons between cancer survivors and healthy controls both in the 1-year study and in the 3-year study, there were no significant differences in regional brain volume. These results support the idea that cancer had little influence on the main analyses of the current study. We did not include healthy controls in the primary comparisons. We did not make a model, such as a 2-factorial ANCOVA in which 1 factor is cancer survivors versus healthy controls and the other factor is whether chemotherapy was received or not, because of the lack of any healthy controls exposed to adjuvant chemotherapy.

The current study has several limitations. 1) Background and medical factors were entered into statistical models as nuisance variables, and medical factors usually used to judge the application of adjuvant chemotherapy and factors reported as a result of adjuvant chemotherapy were not entered to avoid overadjustment. Given potential biases, results need to be interpreted with caution. 2) Effects of each regimen or each chemotherapeutic agent on regional brain volumes were unclear. 3) Pathophysiological mechanisms of volume differences were unclear. The

other reason we did not explore effects of each chemotherapeutic agent in the study setting was that interactions between each chemotherapeutic agent may exist and may make our inference difficult. 4) The VBM has several limitations. A method with higher sensitivity, such as a manual tracing method like those reported previously,[16] should be used. 5) The current study did not have any specific functional targets related to each of the detected regions. Functions related to brain regions significantly different in volume from those in the current study need to be examined by using specific neuropsychological tasks and neuroimaging of brain function.

In conclusion, the current study showed significantly smaller regional brain volumes in areas related to cognitive functions in cancer survivors who received adjuvant chemotherapy. The smaller regional brain volumes were not observed at more than 3 years after completion of adjuvant chemotherapy. Results lead to the idea that adjuvant chemotherapy could have a temporary effect on brain structure. These findings can provide new insights for future research to improve the quality of life of cancer patients who receive adjuvant chemotherapy.

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