practice and no additional burdens were imposed on patients. Hence, ethical approval was not required.

Statistical Methods

The χ^2 test (Pearson statistic) was used to determine the differences in clinical and pathological factors between two groups of patients. A P value of <0.01 was considered statistically significant.

The follow-up duration was calculated as the length of time between the date of diagnosis and the date of death or last contact. Disease-free survival (DFS) was defined as the time from surgical resection to the first of any of the following events: locoregional relapse, distant relapse, second primary breast cancer, any second (non-breast) malignancy or death from any cause. Locoregional relapse was defined as the reappearance of cancer in the ipsilateral breast, chest wall or regional lymph nodes. We classified distant relapse into two categories depending on metastatic sites: non-visceral (soft-tissue and/or bone) or visceral (including lung, liver, brain and other organs). Overall survival (OS) was defined as the time from surgical resection to death due to any cause, regardless of recurrence. DFS and OS curves were drawn by the Kaplan–Meier method and were compared among patient subsets using the log-rank test.

In univariate analyses, the following prognostic factors were evaluated for their potential associations with DFS and OS: age at the time of diagnosis, familial breast cancer, pT, pN, histological type, histological grade, LVI, BVI, tumor subtype stratified by HR and HER2 status, operative procedure, administration of radiation therapy and adjuvant systemic therapy. ER, PgR and HER2 were excluded from the prognostic analyses for DFS and OS because these factors are closely related to tumor subtype. Multivariate analysis of potential prognostic factors was performed to generate a Cox proportional hazards model. Multivariate models were created using age at diagnosis and other variables that showed significant association ($P < 0.01$) with DFS or OS on univariate analysis. All tests were two-tailed, with $P < 0.01$ being taken as an indicator of statistical significance. The statistical software SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Patient Characteristics

Out of a total of 3944 patients who underwent surgery at the National Cancer Center Hospital, Tokyo, Japan, between January 1990 and December 2004, 242 patients were eligible for this study. Of which 99 (41.0%) were aged <35 years at diagnosis, and 143 (59.0%) were aged between 35 and 39 years (Table 1). The median age at diagnosis was 36 years (range 22–39 years). The distribution of various clinicopathological factors did not differ significantly between the two age groups. PgR positivity was observed in a higher percentage of patients aged 35–39 years than in those aged <35 years, but the proportion of patients falling into each of these four tumor subtypes did not differ significantly between the two groups. Sixty-nine percent of the 242 patients were classified as HR+HER2−, 10.3% were HR+HER2+, 5.8% were HR−HER2+ and 14.9% were HR−HER2− (triple-negative).

During a median follow-up of 80 months (range 5–186 months), 86 patients (35.5%) experienced DFS events [second primary breast cancer 3.7%; locoregional relapse 7.4%; distant relapse 24.4% (non-visceral 8.7%; visceral 15.7%)] and 51 patients (21.1%) died. No significant difference was found in DFS and OS between patients aged <35 years and those aged 35–39 years (Fig. 1). We did not also find a significant difference in frequency of occurrence of various DFS events between the two age groups (Table 1).

Univariate Analyses

For breast cancer patients under 40 years old, univariate analyses showed that significant adverse factors associated with both DFS and OS included higher T stage (pT3–4), positive lymph nodes (pN1–3), grade 3, extensive LVI, BVI, triple-negative status and adjuvant chemotherapy (Tables 2 and 3). With regard to adjuvant chemotherapy, patients who were treated with chemotherapy had significantly worse DFS and OS. No significant difference in survival was observed between the familial breast cancer group and the non-familial group.

Multivariate Analyses

For all patients under the age of 40, multivariate analyses identified positive axillary lymph nodes (pN1–pN3) and triple-negative status as independent factors associated with poor DFS and OS (Tables 2 and 3, and Fig. 2). Age, represented as either a categorical or a continuous variable, was not an independent prognostic factor in multivariate analyses. The independent factors negatively influencing DFS included pN1 (hazard ratio 3.69, 95% CI 1.61–8.47), pN2–pN3 (hazard ratio 6.55, 95% CI 2.72–15.75) and triple-negative status (hazard ratio 2.45, 95% CI 1.37–4.36). The independent adverse factors affecting OS included pN1 (hazard ratio 6.00, 95% CI 1.77–20.35), pN2–pN3 (hazard ratio 7.95, 95% CI 2.31–27.37), the presence of BVI (hazard ratio 2.88, 95% CI 1.35–6.13) and triple-negative status (hazard ratio 4.25, 95% CI 2.08–8.72).

For patients aged <35, multivariate analyses indicated that positive axillary lymph nodes (pN1–pN3) and triple-negative status were the independent factors associated with poor DFS and OS (Table 4). For those aged 35–39, triple-negative status was the only independent adverse prognostic factor identified. Axillary lymph node status was not found to be an independent factor, probably due to the

Table 1. Clinicopathological characteristics of breast cancer patients under 40 years old ($n = 242$)

Variable	All patients		Aged <35		Aged 35–39		P^a
	($n = 242$)	(%)	($n = 99$)	(%)	($n = 143$)	(%)	
Familial breast cancer							
No	192	79.3	78	78.8	114	79.7	
Yes	50	20.7	21	21.2	29	20.3	0.492
Primary tumor							
pT1	25	10.3	11	11.1	14	9.8	
pT2	78	32.2	37	37.4	41	28.7	
pT3	112	46.3	40	40.4	72	50.3	
pT4	27	11.1	11	11.1	16	11.2	0.436
Regional lymph node							
pN0	127	52.5	55	55.5	72	50.3	
pN1	65	26.9	21	21.2	44	30.8	
pN2	34	14.0	16	16.2	18	12.6	
pN3	16	6.6	7	7.1	9	6.3	0.411
Histological type							
Invasive ductal carcinoma	221	91.3	95	96.0	126	88.1	
Invasive lobular carcinoma	5	2.1	1	1.0	4	2.8	
Others	16	6.6	3	3.0	13	9.0	0.103
Histological grade							
Grade 1	14	5.8	3	3.0	11	7.7	
Grade 2	84	34.8	31	31.3	53	37.1	
Grade 3	144	59.5	65	65.7	79	55.2	0.148
Lymph vessel invasion							
Absent	98	40.5	46	46.5	52	36.4	
Focal–moderate	135	55.8	50	50.5	85	59.4	
Extensive	9	3.7	3	3.0	6	4.2	0.283
Blood vessel invasion							
Absent	222	91.7	89	89.9	133	93.0	
Present	20	8.3	10	10.1	10	7.0	0.264
Estrogen receptor							
Negative	78	32.2	35	35.4	43	30.1	
Positive	164	67.8	64	64.6	100	69.9	0.234
Progesterone receptor							
Negative	63	26.0	34	34.3	29	20.3	
Positive	179	74.0	65	65.7	114	79.7	0.014
HER2 receptor							
Negative	203	83.9	81	81.8	122	85.3	
Positive	39	16.1	18	18.2	21	14.7	0.290
Subtype							
HR+HER2−	167	69.0	61	61.6	106	74.1	
HR+HER2+	25	10.3	11	11.1	14	9.8	
HR−HER2+	14	5.8	7	7.0	7	4.9	

Continued

Table 1. *Continued*

Variable	All patients		Aged <35		Aged 35–39		P^a
	($n = 242$)	(%)	($n = 99$)	(%)	($n = 143$)	(%)	
HR−HER2− (triple-negative)	36	14.9	20	20.3	16	11.2	0.165
Operative procedure							
Breast-conserving surgery	87	36.0	40	40.4	47	32.9	
Mastectomy	155	64.0	59	59.6	96	67.1	0.230
Radiation							
No	163	67.4	66	66.6	97	67.8	
Yes	79	32.6	33	33.3	46	32.2	0.479
Adjuvant endocrine therapy							
No	84	34.7	38	38.4	46	32.2	
Yes	158	65.3	61	61.6	97	67.8	0.318
Adjuvant chemotherapy							
No	89	36.8	35	35.4	54	37.8	
Yes	153	63.2	64	64.6	89	62.2	0.702
DFS event							
None	156	64.5	68	68.7	88	61.5	
Second primary breast cancer	9	3.7	4	4.0	5	3.5	
Locoregional relapse	18	7.4	8	8.1	10	7.0	
Distant relapse—non-visceral	21	8.7	5	5.1	16	11.2	
Distant relapse—visceral	38	15.7	14	14.1	24	16.8	0.493

[a]χ^2 test.
HR, hormone receptor; DFS, disease-free survival.

subtraction of LVI and BVI, which significantly correlate with positive axillary lymph nodes (Table 4).

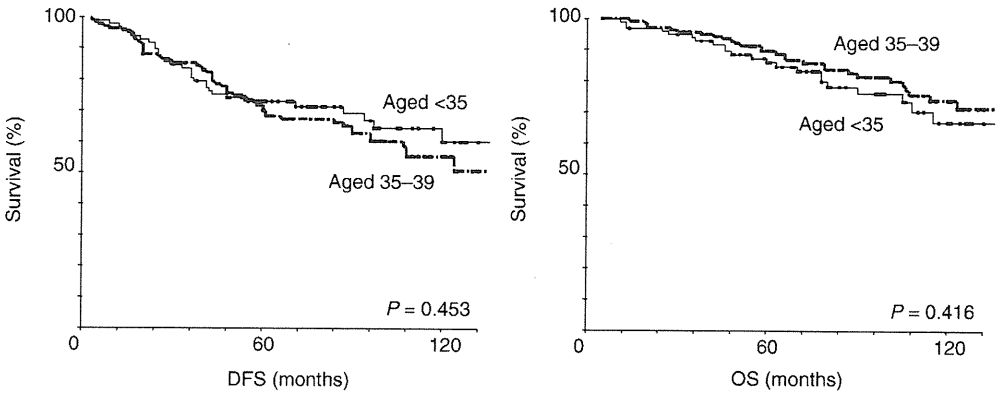
DISCUSSION

Although being 'young' has been reported to be a predictor of poor prognosis independent of other known factors (17–21), the definition of 'young' has varied across studies. The age of 35 years has been used as a cutoff age based on consensus in the international guidelines for treatment of primary breast cancer (1–5). However, the St Gallen international expert consensus panel discontinued the use of the threshold of 35 years of age as a risk category in 2009 (22).

The primary objective of this study was to verify whether breast cancer patients aged <35 at diagnosis have poorer prognoses than those aged 35–39 or to identify the prognostic value of age in younger premenopausal patients under 40 years old. Our results did not indicate any significant differences between patients aged <35 years and those aged 35–39 years in either DFS or OS, and age at diagnosis was not an independent factor associated with DFS or OS in our cohort of breast cancer patients younger than 40 years. We believe that these observations are reliable because the distribution of various clinical and pathological factors did not differ significantly between the two age groups.

A population-based study in Switzerland found no effect of young age on survival when accounting for breast tumor characteristics and treatment (23). A study by van de Vijver et al. (6) also demonstrated that, whereas gene-expression profile was a powerful predictor of disease outcome in younger women with breast cancer, age was not an independent prognostic factor. Younger premenopausal women have been reported to more frequently present with breast cancer marked by poor prognostic features such as higher T stage, positive lymph nodes, endocrine non-responsiveness, high grade, extensive PVI and high proliferating fraction than older premenopausal women (24–29). Kollias et al. (25) concluded that age itself had no influence on the prognosis of individuals because the association of poor prognosis with young age at diagnosis could be explained by a higher proportion of aggressive tumors.

Our present study of breast cancer patients under the age of 40 supports these observations and we consider that the age of <35 years at diagnosis is an unreasonable threshold to identify patients with primary breast cancer at high risk of relapse.



	5-year survival	10-year survival
Aged <35	72.9%	60.2%
Aged 35–39	70.1%	55.3%

	5-year survival	10-year survival
Aged <35	86.1%	66.9%
Aged 35–39	89.7%	73.9%

Figure 1. Kaplan–Meier curves of disease-free survival (DFS) and overall survival (OS) compared between breast cancer patients aged <35 years ($n = 99$) and aged 35–39 years ($n = 143$).

Table 2. Univariate and multivariate analyses of clinicopathological factors associated with disease-free survival in breast cancer patients under 40 years old ($n = 242$)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
Age						
<35	1	–	–	1	–	–
35–39	1.18	0.76–1.84	0.455	1.27	0.80–2.02	0.320
Regional lymph node						
pN0	1	–	–	1	–	–
pN1	2.93	1.69–5.10	<0.001	3.69	1.61–8.47	0.002
pN2–3	6.23	3.67–10.57	<0.001	6.55	2.72–15.75	<0.001
Lymph vessel invasion						
Absent	1	–	–	1	–	–
Focal–moderate	3.32	1.86–5.90	<0.001	2.29	1.19–4.38	0.013
Extensive	4.90	2.64–9.11	<0.001	2.10	0.95–4.65	0.066
Blood vessel invasion						
Absent	1	–	–	1	–	–
Present	3.90	2.23–6.84	<0.001	1.99	1.05–3.78	0.034
Subtype						
HR+HER2–	1	–	–	1	–	–
HR+HER2+	1.22	0.62–2.40	0.559	1.12	0.53–2.36	0.768
HR–HER2+	0.89	0.32–2.46	0.822	1.11	0.39–3.15	0.847
HR–HER2– (triple-negative)	2.16	1.25–3.73	0.006	2.45	1.37–4.36	0.002

95% CI, 95% confidence interval; HR, hormone receptor.
[a]Cox proportional hazards model.

Table 3. Univariate and multivariate analyses of clinicopathological factors associated with overall survival in breast cancer patients under 40 years old ($n = 242$)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
Age						
<35	1	–	–	1	–	–
35–39	0.80	0.46–1.34	0.418	0.86	0.47–1.57	0.617
Regional lymph node						
pN0	1	–	–	1	–	–
pN1	4.90	2.13–11.28	<0.001	6.00	1.77–20.35	0.004
pN2–3	10.47	4.72–23.24	<0.001	7.95	2.31–27.37	0.001
Lymph vessel invasion						
Absent	1	–	–	1	–	–
Focal–moderate	4.22	1.82–9.77	0.001	2.41	0.98–5.98	0.057
Extensive	7.71	3.23–18.41	<0.001	2.80	0.95– 8.26	0.063
Blood vessel invasion						
Absent	1	–	–	1	–	–
Present	5.69	3.02–10.73	0.077	2.88	1.35–6.13	0.006
Subtype						
HR+HER2–	1	–	–	1	–	–
HR+HER2+	0.92	0.32–2.62	0.876	0.73	0.24–2.22	0.584
HR–HER2+	1.33	0.41–4.38	0.636	1.64	0.46–5.85	0.445
HR–HER2– (triple-negative)	3.65	1.92–6.95	<0.001	4.25	2.08–8.72	<0.001

95% CI, 95% confidence interval; HR, hormone receptor.
[a]Cox proportional hazards model.

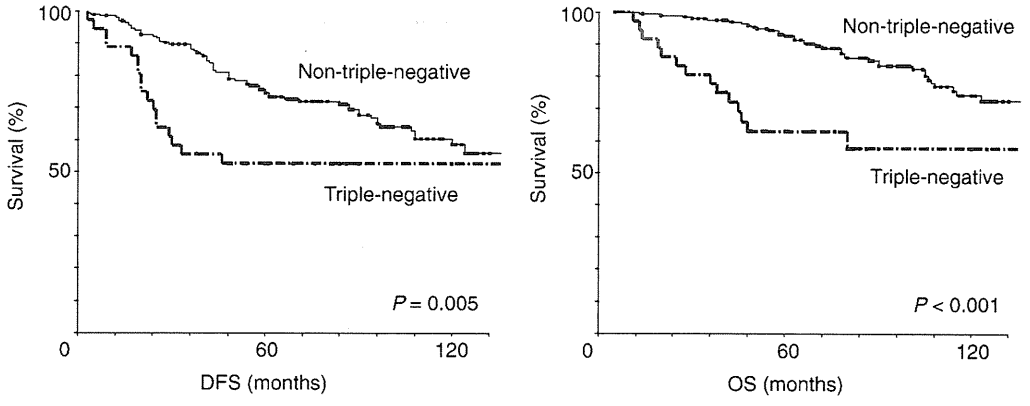
In contrast to our findings, de la Rochefordiere et al. (19) reported that, in a series of 1703 patients from a single institution, the relationship between recurrence hazard and age was best fitted by a log-linear function that indicated a 4% decrease in recurrence and a 2% decrease in death for every year of age in premenopausal women. Han and Kang also recently reported that in patients younger than 35 years, the risk of death rose by 5% for every year of decrease in age, whereas death risk did not vary significantly with age in patients aged 35 years or older (30).

What is more, our unpublished data confirms that breast cancer patients aged <40 years have poorer DFS than those aged 41–49 years (5-year DFS: 79 vs. 86%, $P = 0.04$), while no significant difference was found in OS (5-year OS: 86 vs. 90%, $P = 0.2$). However, there were a much greater number of patients aged 41–49 years compared with those aged <40 years, and the difference in sample number between the two groups was beyond the allowed limit. Therefore, we limited ourselves only to calculating DFS and OS for patients between 40 and 49 years of age. Anders et al. (31) documented similar findings that survival rate in patients who were diagnosed before the age of 40 years was worse when compared with that in older women.

These results indicate that age does have some impact on long-term outcome of patients. Our report and unpublished data suggest that other clinicopathological features rather than age at diagnosis should be used to determine individualized treatment courses for breast cancer patients under 40 years old, but not across all age groups. Further analyses are needed in order to assess the prognostic value of age at diagnosis in women with primary breast cancer across all age groups. However, this can still be a significant finding given that women are now commonly bearing children at older ages in Japan.

Our secondary objective in this study was to assess prognostic factors specific for younger premenopausal women with primary breast cancer. We found that the most important factors associated with poor DFS and OS in patients under the age of 40 were positive axillary lymph nodes (pN1–pN3) and triple-negative status. Triple-negative status was also an independent factor associated with worse DFS and OS in both age groups.

Previous studies have identified axillary lymph node status, HR and HER2 status, tumor size, histological grade, operative procedure, radiation therapy, adjuvant systemic therapy, family history of ovarian cancer and age <35 or 40



	5-year survival	10-year survival
Non-triple-negative	74.5%	58.8%
Triple-negative	52.6%	52.6%

	5-year survival	10-year survival
Non-triple-negative	92.7%	74.3%
Triple-negative	63.0%	57.8%

Figure 2. Kaplan–Meier curves of DFS and OS compared between triple-negative breast cancer patients ($n = 36$) and breast cancer patients whose tumors fall into one of the other three subtypes (non-triple-negative; $n = 206$).

Table 4. Multivariate analyses of clinicopathological factors associated with disease-free survival and overall survival for the two age groups; aged <35 vs. aged 35–39

Variable	Disease-free survival						Overall survival					
	Aged <35 ($n = 99$)			Aged 35–39 ($n = 143$)			Aged <35 ($n = 99$)			Aged 35–39 ($n = 143$)		
	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
Regional lymph node												
pN0	1	–	–	1	–	–	1	–	–	1	–	–
pN1	18.64	3.36–103.30	0.001	1.43	0.52–3.94	0.489	56.57	7.74–413.30	<0.001	1.30	0.32–5.37	0.715
pN2–3	11.86	1.95–72.00	0.007	3.72	1.28–10.83	0.016	52.95	5.55–505.71	0.001	1.94	0.46–8.25	0.368
Lymph vessel invasion												
Absent	1	–	–	1	–	–	1	–	–	1	–	–
Focal–moderate	1.82	0.62–5.32	0.277	2.32	1.00–5.40	0.051	0.86	0.24–3.12	0.816	6.06	1.22–30.10	0.028
Extensive	3.36	0.76–14.83	0.110	2.04	0.75–5.51	0.162	2.69	0.50–14.55	0.250	4.49	0.83–24.42	0.082
Blood vessel invasion												
Absent	1	–	–	1	–	–	1	–	–	1	–	–
Present	2.57	0.90–7.29	0.077	1.32	0.53–3.32	0.555	2.75	0.81–9.32	0.104	3.28	1.07–10.06	0.037
Subtype												
HR+HER2–	1	–	–	1	–	–	1	–	–	1	–	–
HR+HER2+	1.04	0.31–3.43	0.951	1.00	0.33–3.02	0.996	0.49	0.09–2.67	0.407	0.66	0.12–3.69	0.640
HR−HER2+	1.86	0.38–9.17	0.447	0.74	0.16–3.42	0.703	1.17	0.11–12.66	0.899	1.35	0.22–8.25	0.745
HR−HER2−(triple-negative)	3.80	1.39–10.42	0.009	3.16	1.42–7.01	0.005	7.58	2.18–26.37	0.001	7.64	2.66–21.94	<0.001

95% CI, 95% confidence interval; HR, hormone receptor.
[a]Cox proportional hazards model.

years as independent prognostic factors in younger premenopausal patients (17,19–21,23,24,27,28).

Axillary lymph node status in particular has been highlighted as a powerful independent prognostic parameter in women with primary breast cancer across all age groups. However, in the present study, axillary lymph node status was not an independent prognostic factor in patients aged 35–39 years. This discrepancy with previous studies is likely the result of the subtraction effects of LVI and BVI, which significantly correlate with positive axillary lymph nodes. We also observed that, in univariate analyses, patients who were treated with chemotherapy had significantly worse DFS and OS. This finding reflects the significantly higher proportion of positive axillary lymph nodes in those patients. Taken together, these results support axillary lymph node status as an important prognostic factor.

The triple-negative subtype or the basal-like subtype (defined immunohistochemically as ER negative, HER2 negative and cytokeratin 5/6 and/or HER1 positive) (32) is associated with aggressive histology and poor clinical outcome. In our study, triple-negative status was confirmed as a prognostic factor for poorer long-term outcome. The triple-negative subtype accounts for ~15% of the four tumor subtypes in the general population and for a higher percentage of breast cancer arising in African-American women (33,34) which is a contributing factor to their poorer prognosis (9). According to surveillance data from the Registration Committee of the Japanese Breast Cancer Society, the triple negative subtype accounts for 15.5% of breast cancers, with no difference in mean age at diagnosis among the four tumor subtypes (35). In our study of breast cancer patients under age 40, the proportion of patients falling into each of these four tumor subtypes was approximately the same as that in a representative population of Japanese women with breast cancer, and did not differ significantly between patients aged <35 and those aged 35–39 years. Further studies are needed to clarify the associations between the factors involved in triple-negative status, younger onset and poorer prognosis in patients with breast cancer.

In conclusion, our results did not indicate any significant differences between patients aged <35 years and those aged 35–39 years in either DFS or OS. In our cohort of breast cancer patients under the age of 40, the independent factors associated with poor DFS and OS included positive axillary lymph nodes (pN1–pN3) and triple-negative status, but not age at diagnosis. Adverse prognostic factors also did not differ considerably between the two age groups. Our findings suggest that other clinicopathological features rather than age should be used to determine individualized treatment courses for breast cancer patients younger than 40 years.

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Conflict of interest statement

None declared.

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A histopathological study for evaluation of therapeutic effects of radiofrequency ablation in patients with breast cancer

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Abstract
Purpose To reveal the rate of complete therapeutic effect of radiofrequency ablation (RFA) and its correlation with tumor size by the histopathological examination of surgically resected early breast cancers.
Methods For 28 patients who received RFA and subsequent surgical therapies for early breast cancer treatment, the effect of RFA was evaluated by both histopathological examination and nicotinamide adenine dinucleotide (NADH)-diaphorase staining of resected tumor specimens according to the criteria described by Seki et al. (this issue). The correlation of 100% RFA effect with tumor parameters including tumor size and the presence of extensive intraductal component (EIC) was examined.
Results The mean size and invasive size of the primary tumors were 2.21 cm (ranging from 0.6 to 5.0 cm) and 1.44 cm (ranging from 0 to 5.0 cm), respectively. By examining hematoxylin-eosin (HE) sections, the effectiveness of RFA was found to be 100% in 16 tumors (57%). However, the effectiveness of RFA was found to be 100% in 22 cases (79%) examined by NADH-diaphorase staining of frozen sections containing part of tumorous and nontumorous tissues. The accuracy of diagnosis of complete RFA effect using NADH-diaphorase staining with reference to HE was 79% (22 of 28) with 100% (16 of 16) sensitivity and 50% (6 of 12) specificity. The rate of 100% RFA effect by HE examination was higher in EIC(−) tumors (13 of 17, 76%) than in EIC(+) tumors (1 of 9, 11%) ($P = 0.0022$), and was higher in tumors of $\leq$1.5 cm (10 of 11, 91%) than in tumors of >1.5 cm (6 of 17, 35%; $P = 0.0034$). All five tumors of $\leq$1.0 cm showed 100% RFA effect, but 3 (27%) of 11 tumors of >1.0 and $\leq$2.0 cm and 9 (75%) of 12 tumors of >2.0 cm showed suboptimal RFA effect by HE.
Conclusions Tumor size of $\leq$1.5 cm, strictly $\leq$1.0 cm, could be an indication for RFA if a complete histological therapeutic effect is mandatory.

Keywords Radiofrequency ablation · Breast cancer · Therapeutic effect · NADH diaphorase

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Introduction

Histopathological evaluation of radiotherapeutic effects in patients' cancerous tissues, including esophageal or cervical cancers, was established in Japan in the 1960s [1]. This system evaluates the percentage of area with markedly altered, presumably nonviable cancer cells, and the area

Table 1 Pathological findings and therapeutic effects in 28 tumors subjected to radiofrequency ablation (RFA)

No./age	Permanent section												Frozen section	
	Histology	Grade	pN	ER	PR	HER2	Tumor size (cm)	Invasive size (cm)	Daughter nodule	EIC	Area with RFA effect (cm)	RFA effect (HE) (%)	Tumor size in sample	NADH effect (%)
1/52/L	muc	1	0	2	0	1	2.4	2.4	–	–	6.6	100	1.6	100
2/69/R	Pred DCIS	1	0	3	1	1	2.2	0.08	–	NA	3.2	100	0.8	100
3/79/R	IDC(pap)	2	0	3	3	0	2.1	2.1	–	–	6	100	1.2	100
4/67/L	IDC(sol)	3	0	0	0	0	2	1.7	–	–	4.2	100	1.7	100
5/64/R	IDC(sol)	3	0	0	0	1	1.7	1	–	–	5.5	100	1.3	100
6/68/L	IDC(sci)	3	0	0	0	0	1.6	1.6	–	–	3.5	100	1.3	100
7/54/L	IDC(pap)	1	0	3	1	1	1.5	0.8	–	+	5.8	100	0.3	100
8/62/L	IDC(sol)	2	0	3	3	0	1.5	1.5	+	–	2.7	100	1.3	100
9/53/R	IDC(sci)	2	0	3	3	0	1.3	1.3	–	–	2.8	100	1.1	100
10/36/R	IDC(pap)	1	0	2	0	0	1.2	1.2	–	–	3.4	100	0.9	100
11/82/R	IDC(sci)	1	0	2	3	1	1.1	1.6	–	–	4	100	1	100
12/47/L	IDC(pap)	1	0	3	3	1	1	0.7	–	–	1.9	100	1	100
13/66/R	DCIS	NA	0	0	0	1	0.9	0	–	NA	4	100	0.8	100
14/67/L	IDC(sci)	2	1	3	2	1	0.8	0.8	–	–	3.7	100	0.9	100
15/42/R	IDC(pap)	1	1	2	2	0	0.6	0.5	–	–	3	100	0.7	100
16/38/L	IDC(pap)	1	0	1	0	1	0.5	0.2	–	–	2.4	100	0.5	100
17/52/R	IDC(sci)	1	3	3	3	0	5	5	–	–	5	90–95	2	90–95
18/45/L	IDC(pap)	1	3	1	3	1	4.7	1.1	+	+	3.9	30	0.5	100
19/57/L	IDC(sol)	1	0	3	2	0	4.7	2.1	–	+	1.7	40	0.7	90
20/78/R	IDC(pap)	1	1	3	0	0	4.2	1.2	–	+	4.7	95	1.2	100
21/48/R	IDC(sci)	2	0	1	3	0	4	2.4	–	+	4	60	1.3	90
22/59/L	IDC(sol)	3	0	0	0	3	3.5	2.6	–	+	3.9	40–50	1.5	40–50
23/62/R	IDC(pap)	1	0	3	3	1	3.2	1.1	–	+	2.6	60–70	1.6	80
24/73/L	IDC(sci)	1	0	3	1	1	2.5	2.5	–	–	3.3	90	1.4	100
25/60/L	IDC(sol)	1	0	3	3	0	2.5	1	–	+	2.5	95	1	100
26/43/L	IDC(sci)	1	0	3	3	1	2	0.8	–	–	2.5	95	0.4	100
27/63/L	IDC(pap)	1	0	3	3	1	1.8	1.5	–	+	1.7	80	1.5	0
28/69/L	muc	1	0	3	0	0	1.5	1.5	–	–	5.5	95	1.3	100

Tumor size includes both invasive and intraductal components

DCIS ductal carcinoma in situ, *EIC* extensive intraductal component, *ER* estrogen receptor, *HE* hematoxylin-eosin, *HER2* human epidermal growth factor receptor 2, *IDC* invasive ductal carcinoma, *L* left, *muc* mucinous carcinoma, *NA* not applicable, *NADH* nicotinamide adenine dinucleotide-diaphorase, *pap* papillotubular carcinoma, *PR* progesterone receptor, *Pred DCIS* predominantly DCIS, *R* right, *sci* scirrhous carcinoma, *sol* solid-tubular carcinoma

from where cancer cells had disappeared [1]. The application of this classification was extended to evaluation of chemotherapeutic effects in 1985 and adopted in the 11th edition of the General Rules for the Gastric Cancer Study [2]. However, it was very difficult to determine correctly whether the altered cancer cells were viable.

In the NSABP-B-18 trial of neoadjuvant chemotherapy for patients with breast cancer, a histopathological evaluation system for chemotherapeutic effects was adopted solely on the basis of the presence or absence of invasive cancer cells, regardless of the degree of cellular degeneration or viability [3]. Thereafter, in different histopathological criteria used for evaluating therapeutic effects in breast cancers by using neoadjuvant chemotherapy, the evaluation was done usually on the basis of the presence of residual tumor cells, regardless of the extent of alteration of residual cancer cells [4, 5]. Because the sensitivity of cancer cells against chemo- or radiotherapy differs among cases, and the extent of therapeutic effects was shown to be correlated with patients' prognosis, accurate histopathological assessment of therapeutic effects has come to be required by clinical oncologists to pathologists as a routine activity. Because sometimes the pathological complete response (pCR) is set as the primary end point of clinical studies of neoadjuvant therapies to breast cancers [6–8], evaluation is frequently conducted in the form of a central pathological review [7, 8].

Recently, radiofrequency ablation (RFA) has been introduced in the field of breast cancer therapy [9, 10]. Because the effect of RFA is evaluated by cautery effect, which is almost common among tumors if the conditions of operation were uniform, establishment of common criteria for assessing therapeutic effects would be very useful to pathologists. In general, evaluation is performed by a combination of hematoxylin and eosin (HE) staining and nicotinamide adenine dinucleotide (NADH)-diaphorase

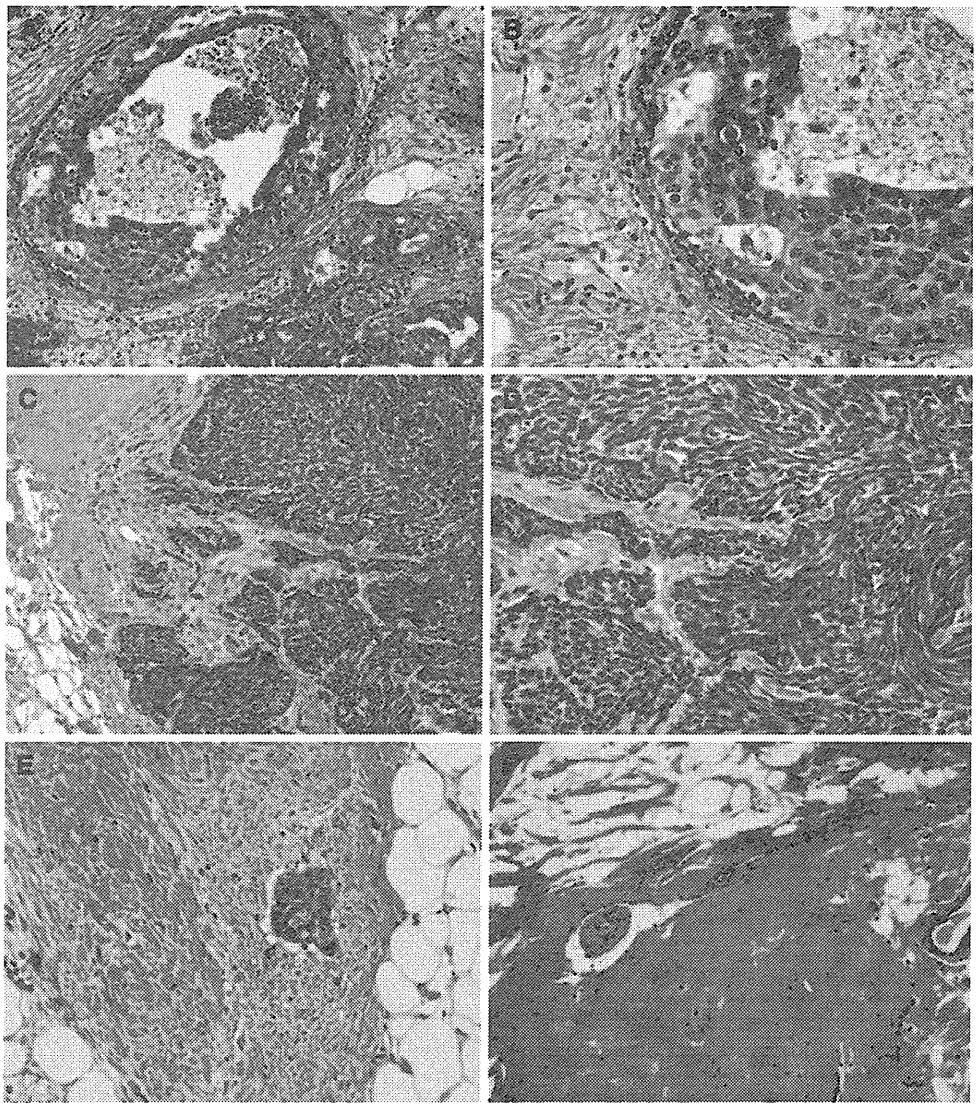

Fig. 1 Alterations in breast cancer and noncancerous tissues by radiofrequency ablation (RFA). **a**, **b** In situ component of a ductal carcinoma without RFA effect. Cancer cells and stromal lymphocytes are viable without degenerative changes, and the morphology of stromal collagen fiber is well preserved. **c**, **d** Invasive carcinoma component with RFA effect. Cancer cells and stromal cells show pyknotic "streaming" nuclei, unclear intercellular boundaries, and unclear nuclear and cytoplasmic morphological details. In stroma, collagen fibers show degenerative changes. **e** Breast stromal tissue without RFA effect. The morphology of stromal collagen fibers is well preserved. A cancer cell nest is also seen. **f** Noncancerous stromal tissue with RFA effect. In stroma, collagen fibers show degenerative changes resulting in dense homogeneous and highly eosinophilic features. ×100 in **a**, **c**, **e**, and **f**, and ×200 in **b** and **d**. ×100 in **a** and **c**, and ×200 in **b** and **d**

staining (reviewed in ref. [11]). Although several studies have shown that evaluation of the therapeutic effects by using NADH-diaphorase staining is useful, evaluation of therapeutic effects by RFA would become more reproducible and accurate if evaluations are effectively done using HE-stained sections of formalin-fixed, paraffin-embedded tissues. Seki et al. [11] have described criteria for histopathological evaluation of RFA effects in breast cancers.

In the present study, RFA effects assessed by HE staining were compared with those assessed by NADH-diaphorase staining of surgically resected breast-cancer tissues, which were obtained immediately after RFA application in a pilot study to examine the safety and efficacy of RFA [9, 11]. In addition, we examined the parameters that caused suboptimal histopathological therapeutic effects in the tumor treated by RFA.

Patients and methods

RFA study protocol

This study was approved by the Institutional Review Board for ethical issues in the National Cancer Center, Japan. All the patients provided written informed consent. The criteria for patient selection and the RFA protocol have been previously described [9]. Under ultrasound guidance, RFA was performed using the 17-gauge Valleylab RF Ablation System with Cool-tip Technology (Covidien, Energy-Based Devices, Interventional Oncology, Boulder, CO) [9]. Histochemical and histopathological examinations of specimens from 28 patients who underwent RFA for primary breast cancer and subsequent partial breast resection or mastectomy between June 2008 and May 2009 were performed. For RFA, radiofrequency energy was sufficient in 19, but the increase of the energy during the procedure was suboptimal in 2 cases (cases 23 and 27 in Table 1).

After ablation, the surgically resected specimens were subjected to sampling of tumor and control tissues for NADH-diaphorase staining. From the representative cut surface of the ablated tumor, at least two thinly sliced sections 2–3 cm in size were removed and prepared as frozen sections: one section contained an apparently ablated area and a representative cut surface of the main tumor, and the other contained non-tumor areas without RFA effect. These tissues were mounted in Cryo Mount I (Muto Pure Chemicals, Tokyo, Japan), immediately frozen on dry ice, and cut into 8- to 10-μm-thick sections. One of these sections was stained with HE, and the others were stored at −80°C until NADH-diaphorase staining.

Enzyme histopathological analysis of the ablated breast tumors was performed according to a method described by Seki et al. [11] and Imoto et al. [12]. The ablated areas were confirmed to contain dead cells, which were negative for the oxidation-reduction reaction mediated by NADH-diaphorase, whereas residual live cells stained blue. We compared the histopathological features of the stained with the unstained adjacent areas by using serial sections stained with both the NADH-diaphorase reaction and HE. The histopathological features attributable to the thermal effects of RFA were investigated by two pathologists.

The remaining surgically resected specimens were fixed in 10% formalin and processed for routine histopathological examination. For partially resected breast specimens, a total of ~20–30 tissue blocks were all sectioned. For total mastectomy specimens, more than 15 tissue blocks were made, all including entire tumor areas. If necessary, additional cutting was performed after initial histological examinations. The ablated areas and ablated tumorous areas were mapped on cut sections, and on the basis of this map, the percentage areas containing tumorous tissue was estimated by at least 2 pathologists.

For both frozen sections and formalin-fixed, paraffin-embedded sections stained with HE, RFA damage to the epithelial cells and fibrous stroma was histologically evaluated according to the criteria described by Seki et al. [11]. In brief, the area with RFA effect in HE-stained sections was histologically visualized as follows (Fig. 1).

Table 2 Summary of the profiles of 28 primary breast carcinomas subjected to radiofrequency ablation (RFA) and subsequent partial resection of the breast or mastectomy

Mean tumor size (total)	2.21 cm (0.6–5.0) (1.31 SD)
Mean tumor size (invasion)	1.44 cm (0–5.0) (1.00 SD)
Mean size of degenerated area by RFA	3.71 cm (1.7–6.0) (1.33 SD)
Incidence of 100% RFA effect by HE staining in primary tumor	16/28 (57%)
Incidence of 100% RFA effect by NADH-diaphorase staining in primary tumors	22/28 (79%)
Concordance between RFA effect by HE and RFA effect NADH-diaphorase staining	22/28 (79%)

Tumor size (total) includes both invasive and intraductal components

HE hematoxylin and eosin, *NADH* nicotinamide adenine dinucleotide, *SD* standard deviation

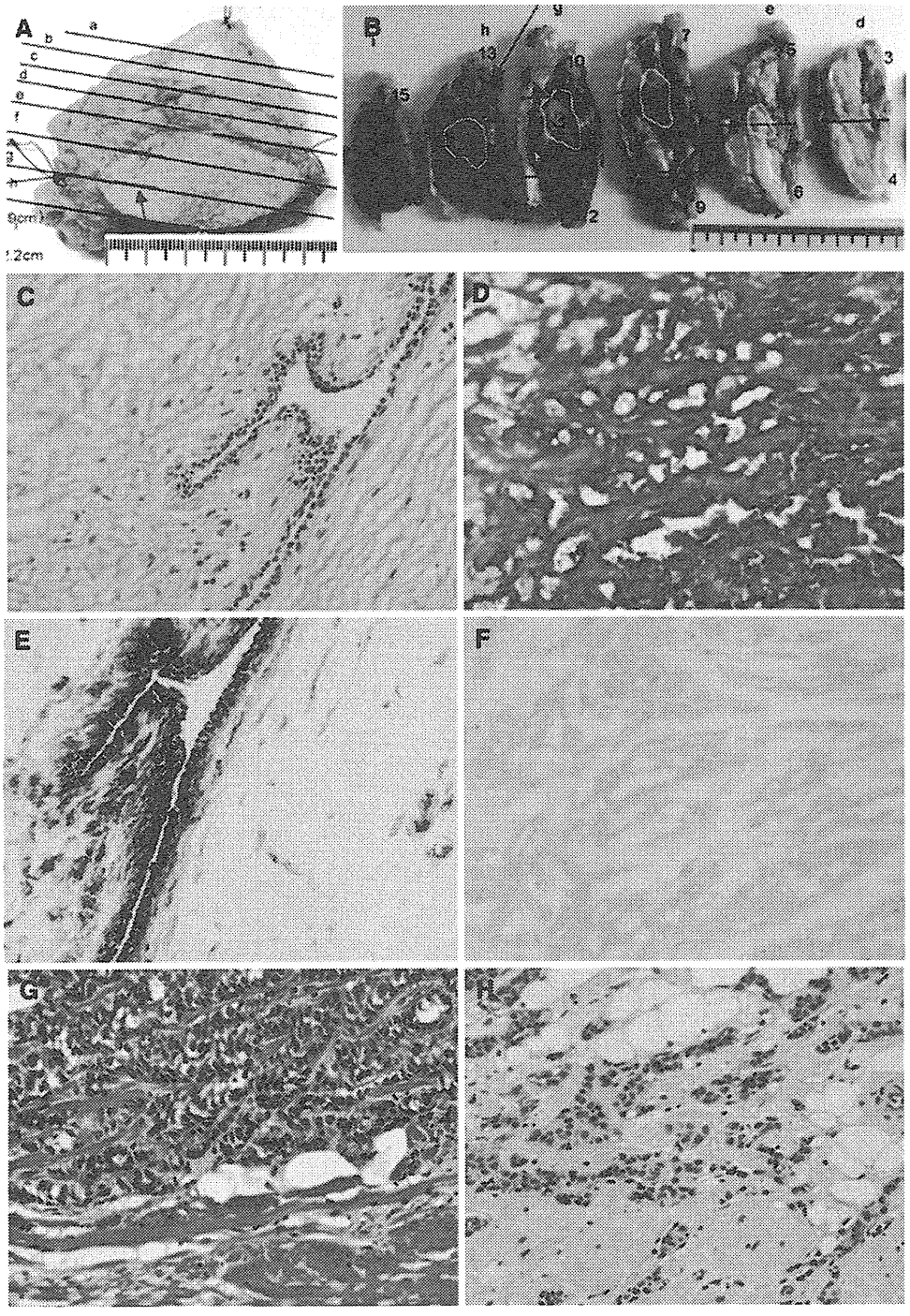

Fig. 2 A case with 100% effective RFA in the main tumor but no effect in the daughter lesion assessed by hematoxylin and eosin (HE) staining. **a**, **b** Surgically resected specimens. *Areas* in *red* represent invasive carcinoma components. Ablated areas are circumscribed in *green*. An *arrow* indicates the daughter lesion. **c**, **d** Frozen sections of the resected tissue specimens stained with HE. **c** Viable non-tumor area with no histopathological RFA effect. No degradation changes are seen in epithelial cells and stromal structures. **d** Tumor area with a strong histopathological RFA effect. Tumor tissues had lost intercellular boundaries and nuclear or cytoplasmic morphological details. Fibrous connective tissue is also highly degenerated into a densely homogeneous and highly eosinophilic structure. **e**, **f** Nicotinamide adenine dinucleotide (NADH)-diaphorase reaction of serial sections of **c** and **d**. **e** NADH diaphorase in a histopathologically viable area. **f** NADH diaphorase in an area with highly degenerated histopathological features. **g**, **h** Permanent sections stained with HE. **g** Histopathologically highly degenerated tumor area (*upper*) and stromal area (*lower*). **h** Histopathological features of the daughter lesions in which no RFA effect is seen. ×100

Epithelial cells, both cancerous and noncancerous, were characterized by elongated eosinophilic cytoplasm with pyknotic "streaming" nuclei. The intercellular boundaries and details of the nuclear and cytoplasmic morphology were unclear (Fig. 1a–d). Fibrous connective tissue also showed degenerative changes with dense homogeneous and highly eosinophilic features. The original delicate, wavy appearance entirely disappeared. Fibroblasts in the area also showed thermal degenerative changes identical to those seen in epithelial cells (Fig. 1e, f).

Statistical analysis

Statistical difference was analyzed by Fisher's exact test.

Results

The findings of pathological examination of the 28 tumors subjected to RFA are presented in Tables 1 and 2. In the Tables, tumor size or tumor size (total) means to the largest

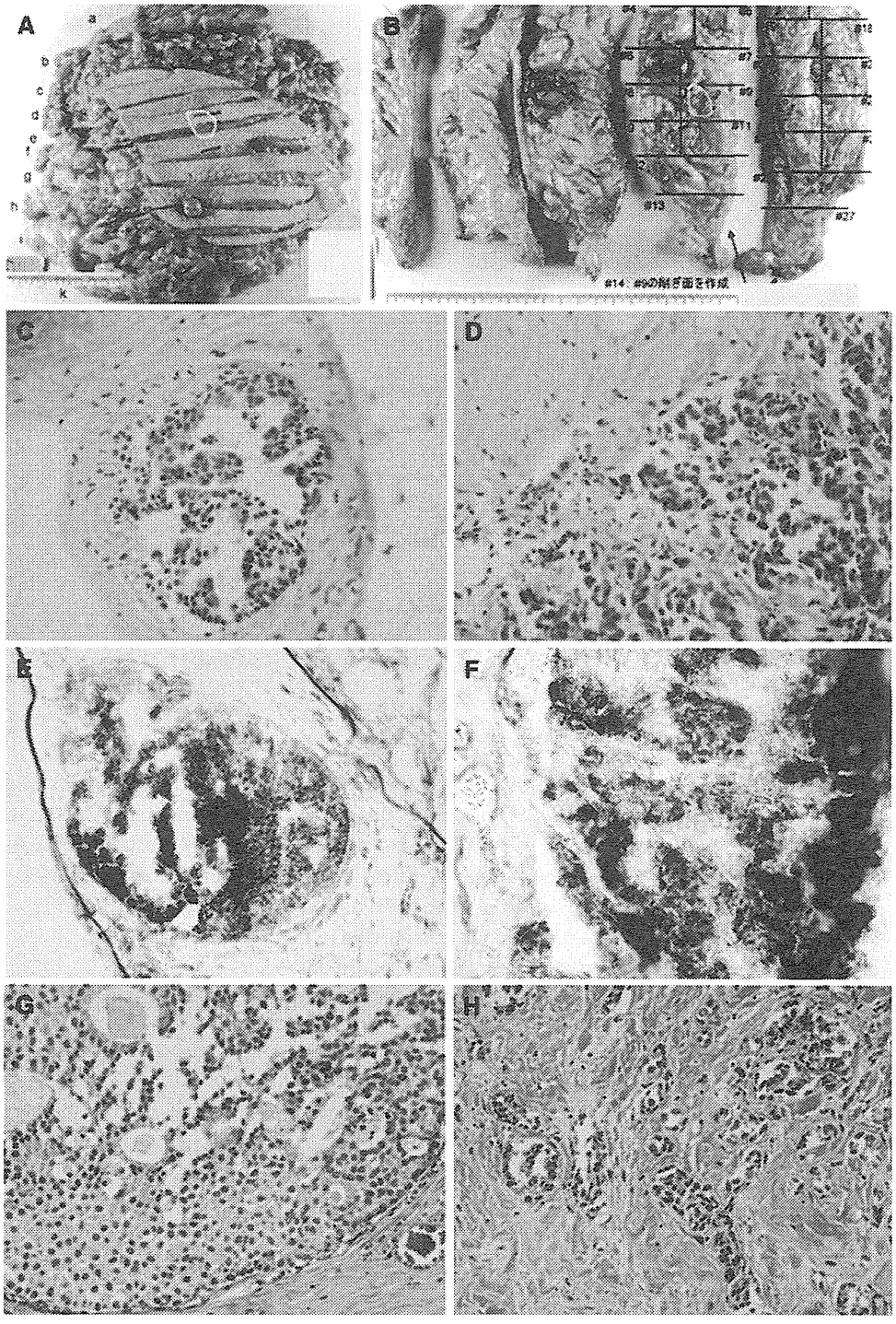

Fig. 3 A case in which histological evaluation of RFA effect was 80% by HE, but the effect was absent by NADH-diaphorase staining. **a**, **b** Surgically resected specimens. *Areas* in *red* and *blue* represent invasive carcinoma and ductal carcinoma in situ (DCIS), respectively. Ablated areas are represented in *yellow*. **c**, **d** Frozen sections of the tumor tissue stained with HE. **c** A viable area without histopathological RFA effect. No degradation changes are seen in either tumor or stromal tissues. **d** An area with histopathological RFA effect. The cells of the tumor tissue show elongated eosinophilic cytoplasm with pyknotic nuclei. **e**, **f** NADH-diaphorase reaction. The results of NADH-diaphorase staining were positive in both histologically viable and nonviable tissue areas in series with **c** and **d**. **g**, **h** Formalin-fixed, paraffin-embedded sections stained with HE. **g** Histopathologically viable DCIS area. **h** Histopathologically highly degenerated invasive carcinoma area. Findings are the same as those in **d**; ×100 in **c** and **e**; ×200 in **d**, **f**, **g** and **h**

diameter of the tumor, including invasive carcinoma and intraductal carcinoma components and including both ablated and non-ablated tumor area. Histological grades of the tumors were 1, 2, and 3 in 18, 5, and 4, respectively. Grading of one tumor could not be determined because of marked degenerative changes. The mean tumor size, including both invasive and noninvasive components, was 2.21 cm, ranging from 0.6 to 5.0 cm. The mean size of invasive components was 1.44 cm, ranging from 0 [ductal carcinoma in situ (DCIS)] to 5.0 cm. An extensive intraductal component (EIC) was positive in 9 tumors, but negative in 17 tumors. One case of DCIS and one case of invasive ductal carcinoma with a predominantly DCIS component were included in the study.

The mean size of the degenerated areas by RFA, including both tumorous and the surrounding nontumorous

Table 3 Factors correlated with 100% radiofrequency ablation (RFA) effect by hematoxylin eosin (HE) findings

Factor	Number of tumors (%) RFA effect by HE			P
	Total	100% Effect	<100% Effect	
Extensive intraductal component (EIC)				
EIC(+)	9	1 (11)	8 (89)	0.0022
EIC(−)	17	13 (76)	4 (24)	
DCIS	2	2 (100)	0 (0)	
Tumor size (total, cutoff 1.5 cm)[a]				
≤1.5 cm	11	10 (91)	1 (9)	0.0034
>1.5 cm	17	6 (35)	11 (65)	
Tumor size (total, cutoff 1.0 cm and 2.0 cm)				
≤1.0 cm	5	5 (100)	0 (0)	0.0037
>1.0 cm, ≤2.0 cm	11	8 (73)	3 (27)	
>2.0 cm	12	3 (25)	9 (75)	

Tumor size (total) includes both invasive and intraductal components

DCIS ductal carcinoma in situ, *EIC* extensive intraductal component

[a] One tumor of ≤1.5 cm but <100% RFA effect was 1.5 cm in diameter, and the effect was 95% by HE and 100% by NADH-diaphorase staining

tissues, was 3.71 cm, ranging from 1.7 to 6.0 cm. Incidence of a 100% effective RFA by HE was 57% (16 of 28) in primary tumors. Of these 16 cases, 1 had a daughter nodule, and because RFA was performed only for the main tumor, the therapeutic effect was 100% for the main tumor, but the effect was absent for the daughter nodule (Fig. 2). By HE, in tumors with incomplete RFA effect, the area of RFA effect was evaluated to be ≥90% in 6, whereas the area was evaluated to be <90% in 6, including 2 tumors (cases 23 and 27 in Table 1), which were potentially subjected to a suboptimal increase in energy during the RFA procedure.

Nicotinamide adenine dinucleotide-diaphorase staining was positive for all sections containing nonablated areas. In the ablated tissues, tumorous tissue, and surrounding non-tumorous tissue, the size of tumor detected by the NADH-diaphorase staining varied from 0.3 to 2.0 cm (mean 1.10 cm). The incidence of 100% effective RFA detected by NADH-diaphorase staining was 79% (22 of 28) in the primary tumors. The therapeutic effect evaluated by NADH-diaphorase staining was 100% in 22 tumors, <100% but ≥90% in 3 tumors. For the other 3 tumors, the RFA effect evaluated by NADH-diaphorase staining was ≤80%: 2 of these were patients 23 and 27, in whom the radiofrequency energy did not increase optimally. In case 27, histological evaluation of the RFA effect was 80% by HE, but the effect was absent by NADH-diaphorase staining because the results of the latter staining were positive in the entire specimen examined (Fig. 3). Concordance between HE findings and NADH findings of the RFA effect was 79% (22 of 28). The specificity and sensitivity of NADH-diaphorase results (complete or incomplete RFA effect) to HE results (complete or incomplete RFA effect) were 100% (16 of 16) and 50% (6 of 12), respectively.

A 100% effective RFA evaluated by HE staining was correlated with EIC(−) and tumor size (Table 3). A 100% effective RFA was observed in 13 (76%) of the 17 EIC(−) tumors, whereas a 100% effective RFA was observed in only 1 (11%) of the 9 EIC(+) tumors ($P = 0.0022$; Fig. 4). Two cases of DCIS showed 100% RFA effect.

Likewise, a 100% effective RFA evaluated by HE staining was correlated with tumor size, including intraductal component (Table 3): a 100% effective RFA was observed in 10 (91%) of 11 tumors of ≤1.5 cm in diameter, whereas a 100% effective RFA was observed in only 6 (35%) of 17 tumors of >1.5 cm in diameter ($P = 0.0034$). One case of ≤1.5 cm, but <100% effective RFA was 1.5 cm in diameter, and the effect was observed in 95% of the area by HE and 100% of the area by NADH-diaphorase staining.

When tumor size, including intraductal component, was stratified into three categories, 100% effects of RFA were detected in all 5 (100%), 3 (27%) of 11, and 9 (75%) of 12 in tumor groups of tumor size ≤1.0 cm, >1.0 cm but ≤2.0 cm, and >2.0 cm, respectively (Table 3). The rate of a 100% effect of RFA was significantly higher in the patients with a tumor of ≤1.0 cm in size than in those with a tumor of >1.0 cm ($P = 0.0037$).

Discussion

In this study, we evaluated the RFA effect by both HE and NADH-diaphorase staining in 28 primary breast cancers resected from patients immediately after RFA procedures. As studied by Seki et al. [11], therapeutic effects of RFA evaluated by HE were mostly concordant with the loss of cellular viability evaluated by NADH-diaphorase staining. Because the area examined in HE-stained sections was wider (2.21 cm on an average) than the area examined in NADH-diaphorase-stained sections (1.10 cm on an average), the percentage of 100% effective RFA became lower in the former (57%) than in the latter (79%). In 1 case (case 27), there was a discrepancy in the RFA effect between the HE and NADH staining in frozen sections, but examinations of the tumor tissue in formalin-fixed sections stained with HE revealed that HE findings were concordant between frozen sections and the formalin-fixed ones. From these results, only frozen-sectioning examination did not completely and accurately clarify the status of the RFA effect in the entire tumor tissue.

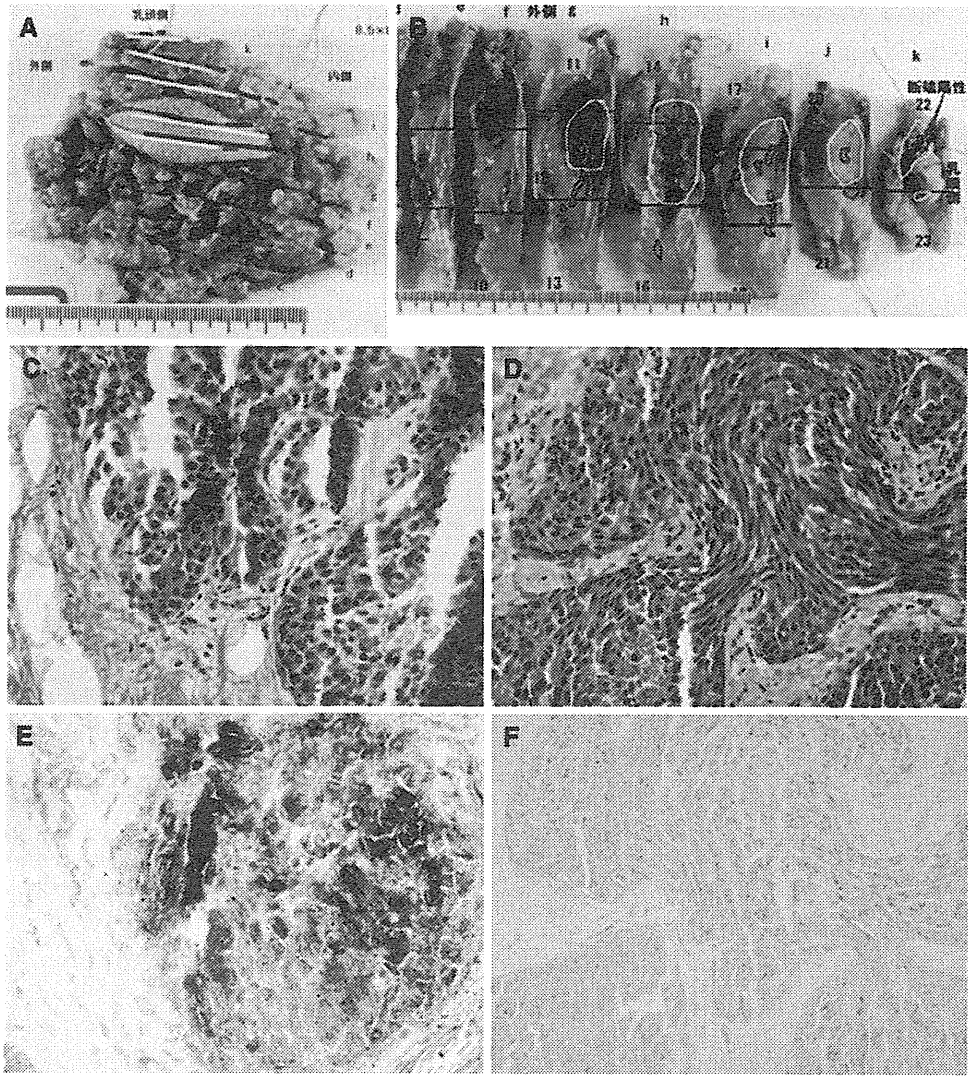

Fig. 4 A case of breast carcinoma, extensive intraductal component (EIC)(+), with 60% effective RFA detected by HE staining of the sections. **a**, **b** Surgically resected specimens. *Areas* in *red* and *blue* represent invasive carcinoma and DCIS, respectively. Ablated areas are represented in *yellow*. **c**, **d** Frozen sections of the tumor tissue stained with HE. **c** Viable area without histopathological RFA effect. No degradative changes are seen in both tumor and stromal tissues. **d** An area with histopathological RFA effect. The cells of the tumor tissue show elongated eosinophilic cytoplasms with pyknotic "streaming" nuclei, unclear intercellular boundaries, and unclear morphological details in the nuclear or cytoplasmic features. Fibrous connective tissue also shows degenerative changes, resulting in dense homogeneous and highly eosinophilic features. **e**, **f** NADH-diaphorase reaction. **e** The results of NADH-diaphorase staining in histopathologically viable areas are positive. **f** NADH diaphorase in the area with histopathological RFA effect shows no reaction. ×200

Nonetheless, if the tumor size was small (≤1.5 cm) and the tumor lacked the EIC component, the proportion of cases with complete RFA effect became very high. In particular, complete RFA effects were observed in all tumors with a diameter of ≤1.0 cm. In these cases, if RFA therapy was conducted with subsequent follow-ups, examination of the therapeutic effect by means of core-needle/mammotome biopsy would be potentially sufficient. In contrast, the ratio of suboptimal RFA effect was high in the tumors sized 2.0 cm or larger. Even in the tumors of >1.0 cm but ≤2.0 cm in size microscopically, 27% of cases did not show a 100% of RFA effect. From the present data, tumor size of ≤1.5 cm, strictly ≤1.0 cm, could be an indication for RFA if a complete histological therapeutic effect is mandatory.

There are still challenges in determining the therapeutic effects of RFA. Judgments of the RFA therapeutic effects between HE and NADH-diaphorase staining, even by examining serial tissue sections, do not always agree. In the Chiba Cancer Center, RFA therapy and subsequent follow-up revealed cases in which HE findings showed effectiveness, but the results of NADH-diaphorase staining were positive, or cases in which HE showed no changes but the results of NADH-diaphorase staining were negative (Yamamoto N., personal communication). Data acquisition from a larger number of cases and establishment of uniform criteria for evaluation of histopathology determining therapeutic RFA effects, including researching on how the NADH-diaphorase findings should be incorporated into such criteria, are important next stages of research.

From Table 1, the diameter of the area with a RFA effect usually exceeds the tumor size by several times, including the intraductal component. The effect of RFA appeared to extend in a radial direction. We need to be concerned about the effects of this technique on the superficial and deep sides of the mammary gland. Histologically, 1 of 28 patients suffered ulceration by the heat injury on the overlying skin (no. 3 in Table 1). Pectoral

muscle was not resected in any of the patients, but in 11 of the 28 patients, the deepest area of the resected specimen widely showed a RFA effect (e.g., Fig. 4). In these patients, it is unclear if thr pectoral muscle suffered significant injury from RFA, and close follow-up is necessary.

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Conflict of interest The authors and their immediate family members have no conflicts of interest.

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Histopathological effect of radiofrequency ablation therapy for primary breast cancer, with special reference to changes in cancer cells and stromal structure and a comparison with enzyme histochemistry

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Abstract Radiofrequency ablation (RFA) therapy is expected to be applicable to small breast cancers, but no criteria for its histopathological effect have yet been established. Using samples obtained from 15 patients who had undergone RFA and subsequent mastectomy, we compared the histopathological changes in the ablated area with the results of histochemical staining based on the reduction of nitroblue tetrazolium chloride (NBT) by nicotinamide adenine dinucleotide (NADH) diaphorase in frozen tissue sections, and looked for histological changes indicative of the effect of RFA on breast cancer. Grossly, the ablated area in most of the tumors was rough, gritty, less moist, and surrounded by a red congestive limbic zone. The ablated area showed no staining by the NADH diaphorase reaction, and cancer cells in the area showed marked destruction characterized by an unclear intercellular boundary, elongated eosinophilic cytoplasm, pyknotic "streaming" nuclei, and a poorly defined nuclear and cytoplasmic texture. At the same time, fibrous connective tissue also showed degenerative changes, becoming densely homogeneous with loss of its delicate wavy structure. The area in which RFA appeared to have been histopathologically effective was mostly concordant with the area in which the NADH diaphorase reaction was negative. In the periphery of the ablated area, however, cellular changes caused by RFA were less marked, although the NADH diaphorase reaction was visualized with NBT. A larger number of cases should be examined in order to establish criteria for the histopathological effect of RFA on breast cancer.

Keywords Breast cancer · Radiofrequency ablation therapy · NADH diaphorase reaction · Histopathological criteria for therapeutic effect

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Introduction

Radiofrequency ablation (RFA) therapy is expected to be applicable to small breast cancers as an effective and safe curative treatment of choice. However, no criteria for defining its therapeutic effect have yet been established. The majority of previous studies have employed histopathological examination of hematoxylin–eosin (HE)-stained sections and the histochemical technique for visualizing the reduction of nitroblue tetrazolium chloride (NBT) by nicotinamide adenine dinucleotide (NADH) diaphorase in frozen sections.

The NAD+/NADH redox reaction is one of the most important in living biologic systems. NADH diaphorase activity judged from the reduction of NBT to formazan via oxidation of NADH is a reliable marker of cell viability. Assay of NADH diaphorase is performed histochemically using fresh frozen tissue sections. When reduced NADH is

oxidized by NADH diaphorase, free electrons are transferred to NBT, which becomes reduced and converted to the blue, water-insoluble dye formazan (Fig. 1). NADH diaphorase becomes bound to the structural components of the cell, thereby permitting histochemical visualization of its intracellular location by the use of NBT. Only viable cells have active diaphorase, whereas this activity seems to subside immediately after cell death.

On the other hand, several previous reports have described criteria for evaluation of the RFA effect. Jeffrey et al. [1] considered the presence of pyknotic nuclei and increased intensity of eosinophilic staining to be characteristics of tissue cautery due to heating. Earashi et al. [2] applied histopathological criteria for assessment of therapeutic response described in the "General Rules for Clinical and Pathological Recording of Breast Cancer". However, no histopathological criteria for the therapeutic effect of RFA have yet been established.

In the present study, on the basis of a comparison of histopathological changes in the ablated area with the results of histochemical assay with NADH diaphorase, we attempted to characterize the histological changes in breast cancer induced by RFA.

Patients and methods

RFA study protocol

Patient selection and the RFA protocol have been described previously [3]. Histochemical and histopathological examinations were performed on specimens from 15 patients who had undergone RFA for primary breast cancer and subsequent mastectomy between June 2006 and May 2007.

Pathological analysis

After ablation, the surgically resected specimen was cut at the maximum diameter of the ablated breast tumor (Fig. 2). Both the ablated and non-ablated areas of each tumor and adjacent tissue were grossly evaluated, focusing particularly on the features of coagulation, congestion, and elasticity. Slices of tissue, each including apparently ablated and non-ablated areas, were obtained and mounted in optimal cutting temperature (OCT) compound. The tissue was then immediately frozen in liquid nitrogen, and cut into sections 8- to 10-μm thick. One of these sections was immediately stained with HE, and the others were stored at −80°C until NADH diaphorase-NBT studies. The remaining surgically resected specimens were fixed in 10% formalin and processed for routine histopathological examination.

Enzyme histochemical analysis

For enzyme histopathological analysis of ablated breast tumors, frozen tissue sections were incubated for 1 h at 37°C in a solution consisting of 0.8 mg/mL reduced β-NADH (Sigma), 0.5 mg/mL nitroblue tetrazolium (Sigma), and 0.05 M Tris-buffered saline (pH 7.4) (Fig. 3). Each slide was fixed in 10% formalin for 30 min and washed in distilled water for 2 min, then glass coverslips were applied with an aqueous medium.

Mapping and evaluation

Ablated cells were confirmed to be non-viable by their negativity for the oxidation–reduction reaction mediated by NADH diaphorase, whereas residual viable cells were stained blue. By referring to serial sections stained with

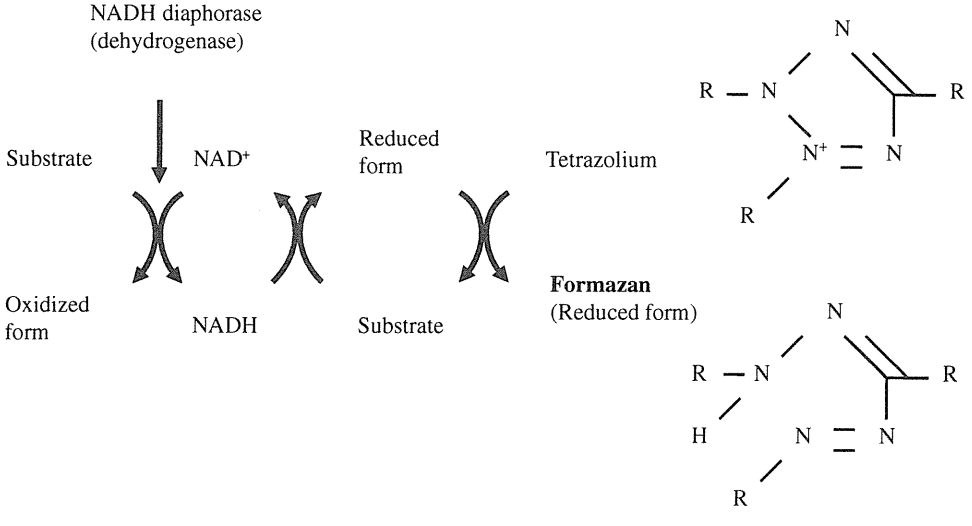

Fig. 1 Nicotinamide adenine dinucleotide (NADH) redox circuit

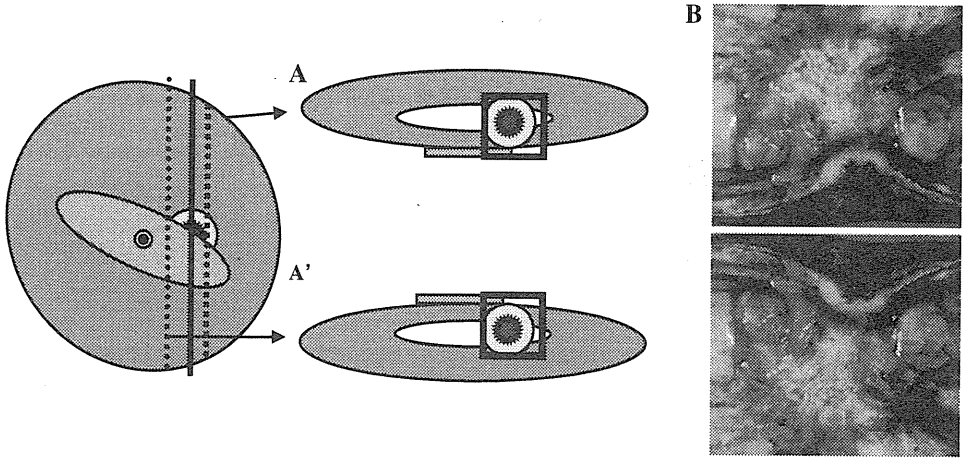

Fig. 2 Schematic representation of tissue specimens used for evaluation of the histopathological effect of radiofrequency ablation (RFA) to primary breast cancer. **a** An ablated tumor cut at the maximum diameter. The tumor in cut section **a** is taken for NADH diaphorase staining. The tumor in cut section **a′**, the mirror image of section **a**, is taken for routine formalin-fixed and paraffin-embedded blocks. **b** Gross features of the ablated tumor. A congestive limbic zone encircles the ablated area containing the tumor

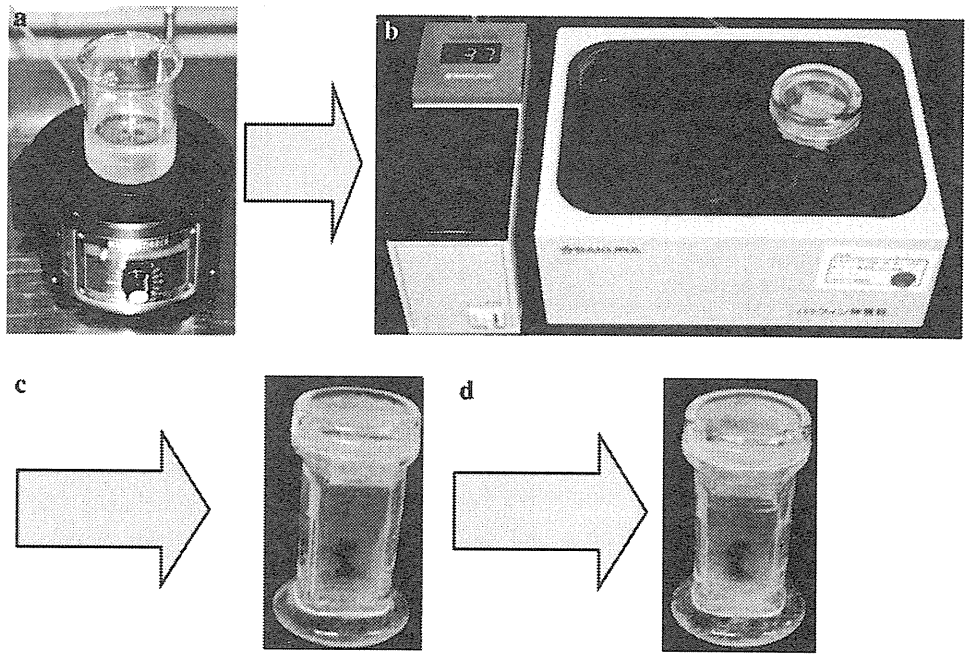

Fig. 3 Preparation of reagents for histochemical assay of nicotinamide adenine dinucleotide (NADH) diaphorase activity. **a** Adjustment of NADH medium. The incubation medium consists of 0.8 mg/mL reduced β-NADH (Sigma), 0.5 mg/mL nitroblue tetrazolium, and 0.05 M Tris-buffered saline (pH 7.4), mixed at 37°C. **b** Fresh frozen tissue sections are incubated in the NADH medium in a water bath at 37°C for 1 h. **c** The tissue sections are washed in distilled water for 2 min. **d** These sections are subsequently fixed in 10% formalin for 30 min

both the NADH diaphorase reaction and HE, we compared the histopathological features of stained and adjacent non-stained areas. Gross and histological features attributable to the thermal effects of RFA were investigated by two pathologists (K.S. and H.T.).

Results

Gross examination

At the cut surface including the tumor, the ablated area felt firmer and more fragile than the surrounding non-ablated area. The cut surface of the ablated area composed of tumor and fibrous stroma was rough, gritty, and less moist, forming a round, flat surface surrounded by swollen fresh, unablated mammary, and fibroadipose tissue (Fig. 4a). In the central zone of the ablated area, the tumor and fibrous connective tissue were grayish-white to tan in color, forming a fissure or small cavity around the needle track (Fig. 4a). Coagulated non-tumor fibroadipose tissue was also firm and had changed to a tan-yellowish color. A red congestive limbic zone surrounded the ablated area (indicated with dots in Fig. 4a). These congestive rings were observed in 14 of 15 cases.