Table 2 Association between nuclear *BUB1* immunohistochemical status and clinicopathological parameters in 104 breast carcinomas

	Nuclear *BUB1* status		*P* value
	+ (n=42)	− (n=62)	
Patient age[a] (years)	54.2±1.6	56.0±1.5	0.44
Menopausal status			
Premenopausal	17 (16%)	21 (20%)	0.49
Postmenopausal	25 (24%)	41 (39%)	
Stage			
I	6 (6%)	23 (22%)	**0.0070**
II	19 (18%)	28 (27%)	
III	7 (7%)	8 (7%)	
IV	10 (10%)	3 (3%)	
Pathological tumor factor (pT)			
pT1	11(11%)	30 (29%)	**0.023**
pT2-4	31 (30%)	32 (31%)	
Lymph node metastasis			
Positive	25 (24%)	22 (21%)	**0.016**
Negative	17 (16%)	40 (38%)	
Distant metastasis			
Positive	10 (10%)	3 (3%)	**0.041**
Negative	32 (31%)	59 (57%)	
Histological grade			
1 (well)	1 (1%)	19 (18%)	**0.009**
2 (moderate)	21 (20%)	27 (26%)	
3 (poor)	20 (19%)	16 (15%)	
ER status			
Positive	32 (31%)	50 (48%)	0.58
Negative	10 (10%)	12 (12%)	
PR LI[a] (%)	28.0±3.7	21.5±4.6	0.27
HER2 status			
Positive	14 (14%)	12 (12%)	0.13
Negative	28 (27%)	50 (48%)	
Ki-67 LI[a] (%)	26.8±2.7	14.6±1.9	**0.0003**
Cytoplasmic *BUB1* status			
Positive	32 (31%)	28 (27%)	**0.0017**
Negative	10 (10%)	34 (33%)	
γ-Tubulin immunoreactivity			
Low	16 (15%)	18 (17%)	0.46
Moderate	15 (14%)	21 (20%)	
High	11 (11%)	23 (22%)	

P values less than 0.05 were considered significant and described as boldface

[a] Data are presented as mean±SEM. All other values represent the number of cases and percentage

Breast cancer-specific survival curves of *BUB1* status were summarized in Fig. 3c and d. A significantly positive correlation (P=0.0007) was detected between nuclear *BUB1* status and adverse clinical outcome of the patients examined, but cytoplasmic *BUB1* status was not associated (P=0.72). In the univariate analysis (Table 6), nuclear *BUB1* status (P=0.011), histological grade (P=0.018), Ki-67 LI (P=0.026), and lymph node metastasis (P=0.043) were all detected as significant prognostic variables for breast cancer-specific survival in this study. However, a following multivariate analysis revealed that only nuclear *BUB1* status was independent prognostic factor with a relative risk over 1.0 (P=0.043), whereas histological grade (P=0.21), Ki-67 LI (P=0.75), and lymph node metastasis (P=0.087) were all not significant.

In our present study, 51 patients received tamoxifen therapy following surgery as an adjuvant treatment in ER-positive stages I-III breast carcinoma cases, and nuclear *BUB1* status was significantly associated with an increased risk of recurrence in these patients (P=0.0079) (Fig. 4a). Similar tendency was detected between nuclear *BUB1* status and breast cancer-specific survival of the patients, although *P* value did not reach statistical significance (P=0.14). Significant association between nuclear *BUB1* status and clinical outcome of the patients was also detected in 67 patients who received adjuvant chemotherapy (P=0.0001 for disease-free survival (Fig. 4b) and P=0.0028 for breast cancer-specific survival). Nuclear *BUB1* status was significantly associated with an increased risk of recurrence (Fig. 4c) and worse prognosis in the ER-negative stages I-III cases (n=19), although *P* values were not available because no patient had recurrent disease or died in the group of these nuclear *BUB1*-negative cases.

Discussion

Results of our present study demonstrated that *BUB1* expression level was significantly associated with Ki-67 LI in the breast carcinoma cells, and similar tendency was also detected in *BUB1B*, *MAD2*, *CDC20*, and *TTK*. Yuan et al. [14] previously reported that mRNA levels of mitotic checkpoint genes, such as *BUB1*, *BUB1B*, *BUB3*, *MAD1*, *MAD2*, *CDC20*, and *TTK*, were almost uniformly increased in breast carcinoma cell lines compared with MCF10A and mammary epithelial cells. Overexpression of *BUB1*, *BUB1B*, *BUB3* [23, 24], and *MAD2* [25] was also reported in the gastric carcinoma cells. In particular, Grabsch et al. [24] did report a positive association between *BUB1*, *BUB1B*, or *BUB3* and Ki-67 mRNA levels in the gastric carcinoma. Association between *BUB1* mRNA level and Ki-67 LI was also reported in the salivary gland tumors [15]. Results of these studies above are all consistent with those of our present study. However, *MAD1* expression tended to be inversely associated with Ki-67 LI in our present study. Han et al. [26] reported that MAD1 expression was significantly reduced in poorly differentiated breast carcinomas, which may partly explain our present finding. These results

Table 3 Association between nuclear *BUB1* status and clinicopathological parameters according to ER status in 104 breast carcinomas

Variable	Nuclear *BUB1* status (positive/negative)	
	ER-positive group (n=82)	ER-negative group (n=22)
Patient age	0.49	0.84
Menopausal status	0.75	0.43
Stage	**0.0081**	**0.025**
pT	**0.034**	0.19
Lymph node metastasis	**0.018**	0.57
Distant metastasis	**0.032**	**0.041**
Histological grade	**0.0006**	0.53
HER2 status	0.083	0.94
Ki-67 LI	**0.0005**	0.28
Cytoplasmic *BUB1* status	**0.0015**	0.39
γ-Tubulin immunoreactivity	0.55	0.42

Data are presented as P values. P values less than 0.05 were considered significant, and described as boldface

Table 4 Association between cytoplasmic *BUB1* immunohistochemical status and clinicopathological parameters in 104 breast carcinomas

	Cytoplasmic *BUB1status*		P value
	+ (n=60)	− (n=44)	
Patient age[a] (years)	55.9±1.4	54.5±1.8	0.53
Menopausal status			
Premenopausal	18 (17%)	20 (19%)	0.11
Postmenopausal	42 (40%)	24 (23%)	
Stage			
I	12 (12%)	17 (16%)	0.11
II	30 (29%)	17 (16%)	
III	8 (8%)	7 (7%)	
IV	10 (10%)	3 (3%)	
Pathological tumor factor (pT)			
pT1	39 (38%)	24 (23%)	0.28
pT2-4	21 (20%)	20 (19%)	
Lymph node metastasis			
Positive	28 (27%)	19 (18%)	0.72
Negative	32 (16%)	25 (24%)	
Distant metastasis			
Positive	10 (10%)	3 (3%)	0.13
Negative	50 (48%)	41 (39%)	
Histological grade			
1 (well)	8 (8%)	12 (12%)	0.19
2 (moderate)	29 (28%)	19 (18%)	
3 (poor)	23 (22%)	13 (13%)	
ER status			
Positive	49 (47%)	33 (32%)	0.41
Negative	11 (11%)	11 (12%)	
PR LI[a] (%)	26.8±4.1	23.1±3.8	0.53
HER2 status			
Positive	16 (15%)	10 (10%)	0.74
Negative	44 (42%)	34 (33%)	
Ki-67 LI[a] (%)	22.4±2.2	15.8±2.5	0.052
γ-Tubulin immunoreactivity			
Low	16 (15%)	18 (17%)	0.14
Moderate	20 (19%)	16 (15%)	
High	24 (23%)	10 (10%)	

P values less than 0.05 were considered significant

[a] Data are presented as mean ± SEM. All other values represent the number of cases and percentage

also indicated that amounts of mitotic checkpoint proteins were increased in their expression in breast carcinoma cells according to their proliferative activity, and in particular, BUB1 was most pronouncedly increased among these proteins.

This is a first study to demonstrate immunolocalization of *BUB1* in human breast cancer patients. *BUB1* immunoreactivity was detected in both the nuclei and/or cytoplasm of the carcinoma cells. *BUB1* protein is involved in the spindle assembly checkpoints, and therefore, its intracellular localization is postulated to be the nucleus. Grabsch et al. [16] demonstrated nuclear *BUB1* immunolocalization in the gastric carcinoma cells, which is consistent with our present findings. However, cytoplasmic immunolocalization was also reported in some mitotic checkpoint proteins in carcinoma cells. For instances, cytoplasmic *BUB1B* immunoreactivity was detected in the breast [14] and colon [27] carcinomas, and cytoplasmic MAD2 immunolocalization was shown in the colon [28] and gastric [29] carcinomas. In addition, Burum-Auensen et al. [30] reported that subcellular localization of *BUB1B* shifted from the cytoplasm to nucleus during the malignant transformation. Results of our present study did demonstrate that *BUB1* expression was correlated with Ki-67 LI in the microarray analysis, and nuclear *BUB1* immunoreactivity was also associated with Ki-67 LI and cytoplasmic *BUB1* status. Therefore, *BUB1* immunoreactivity is required to be evaluated in the nucleus in the breast carcinoma tissues.

In our present study, nuclear *BUB1* immunoreactivity was positively associated with stage, pT, lymph node metastasis, distant metastasis, histological grade, and Ki-67 LI in the 104 breast cancer patients. Shigeishi et al. [15] reported that *BUB1* protein level evaluated by immunoblot analysis was significantly associated with stage (P=0.02) and marginally associated with pT (P=0.11) or lymph node metastasis (P=0.14) in ten salivary gland carcinomas, which is consistent with results of our present study. Results of our present study also revealed that nuclear *BUB1* status was not significantly associated with γ-tubulin immunoreactivity,

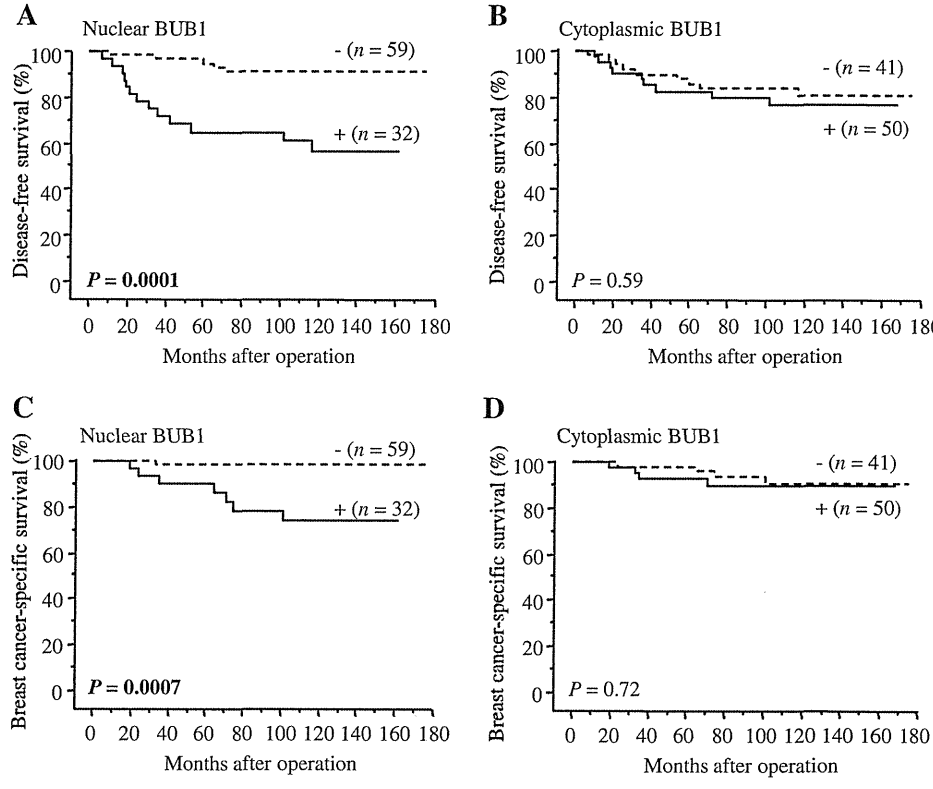

Fig. 3 Disease-free (**a**, **b**) and breast cancer-specific survival (**c**, **d**) of stages I-III breast carcinoma patients according to *BUB1* status studied by Kaplan–Meier method (n=91). Statistical analysis was evaluated by the log-rank test. P values less than 0.05 were considered significant and described as *boldface*

which is reported to reflect centrosome aberrations [22] or chromosomal changes [20] in the breast cancer. Grabsch et al. [16] previously reported that *BUB1* immunoreactivity was not associated with DNA ploidy or microsatellite instability in the gastric carcinoma, which is consistent with the findings in our present study. Decreased expression level of mitotic checkpoint proteins may result in defective spindle checkpoint controls, but further investigations are required to determine whether *BUB1* expression level reflects spindle checkpoint function or not in human malignancies. Overexpression of *BUB1* lead to chromosome instability of the cells [31], and BUB1 was also reported to negatively regulate p53-mediated early cell death [8, 32]. Therefore, *BUB1* may have various biological functions in addition to mitotic checkpoint and play important roles in the cell proliferation and/or progression of the breast carcinoma.

Results of our present study also indicated that an association between nuclear *BUB1* status and aggressive phenotype of breast carcinoma was more pronounced in ER-positive cases (Table 3). *BUB1* gene has a functional estrogen-responsive element at 4,500 bp from the most upstream mRNA 5'-end of the gene [33], and *BUB1* mRNA expression was upregulated by estradiol in MCF-7 breast carcinoma cells [34]. Ebata et al. [35] recently reported that expression profiles of estrogen-induced genes in ER-positive breast carcinomas were different between noninvasive and invasive cases, and

Table 5 Uni- and multivariate analyses of disease-free survival in stages I-III breast cancer patients examined

Variable	Univariate	Multivariate	
	P value	*P* value	Relative risk (95% CI)
Lymph node metastasis (positive/negative)	**0.0005**	**0.0022**	7.1 (2.0–25.1)
Nuclear *BUB1* status (positive/negative)	**0.0007**	**0.0056**	4.5 (1.6–13.0)
Pathological tumor factor (pT) (pT2-4/pT1)	**0.045**	0.39	
Adjuvant chemotherapy (yes/no)	0.15		
Ki-67 LI[a] (78%–0%)	0.23		
HER2 status (positive/negative)	0.29		
Cytoplasmic BUB1 status (positive/negative)	0.59		
Histological grade (3/1,2)	0.74		

Data considered significant (P<0.05) in the univariate analyses were described as boldface and were examined in the multivariate analyses

[a]Data were evaluated as continuous variables. All other data were evaluated as dichotomized variables

Table 6 Uni- and multivariate analyses of breast cancer-specific survival in stages I-III breast cancer patients examined

Variable	Univariate	Multivariate	
	P value	*P* value	Relative risk (95% CI)
Nuclear *BUB1* status (positive/negative)	**0.011**	**0.043**	9.4 (1.1–83.2)
Histological grade (3/1,2)	**0.018**	0.21	
Ki-67 LI[a] (78%–0%)	**0.026**	0.75	
Lymph node metastasis (positive/negative)	**0.043**	0.087	
Pathological tumor factor (pT) (pT2-4/pT1)	0.091		
HER2 status (positive/negative)	0.23		
Cytoplasmic BUB1 status (positive/negative)	0.72		

[a]Data were evaluated as continuous variables. All other data were evaluated as dichotomized variables

Data considered significant ($P < 0.05$) in the univariate analyses were described as boldface, and were examined in the multivariate analyses

BUB1 mRNA level was much higher in invasive carcinoma. Therefore, BUB1 may also play important roles especially in the estrogen-mediated progression of the breast carcinoma.

In our study, nuclear *BUB1* immunoreactivity was significantly associated with recurrence and aggressive clinical course in the breast cancer patients, and similar tendency was also detected in ER-positive cases that received tamoxifen therapy or chemotherapy. In addition, results of multivariate analyses clearly demonstrated that nuclear *BUB1* immunoreactivity was an independent prognostic factor for both recurrence and breast cancer-specific survival. Dai et al. [36] reported the occurrence of metastasis is strongly predicted by a homogeneous gene expression pattern almost entirely consisting of cell cycle genes within a subset of breast carcinoma patients characterized by relatively abundant ER expression for their age, and *BUB1* was included in these genes. In addition, Suzuki et al. [37] very recently identified *BUB1* as a gene associated with recurrence of ER-positive breast carcinomas patients who received tamoxifen as a result of microarray analysis. The nuclear *BUB1* status was not necessarily associated with ER status in the breast carcinoma in our study, which also indicated that nuclear *BUB1* immunoreactivity at the time of surgery may reflect the increased basal level of *BUB1* rather than the level induced by estrogens in the breast carcinoma, and residual carcinoma cells following surgical treatment in *BUB1*-positive breast carcinomas could still have the potential to rapidly grow and/or metastasize, despite of the tamoxifen

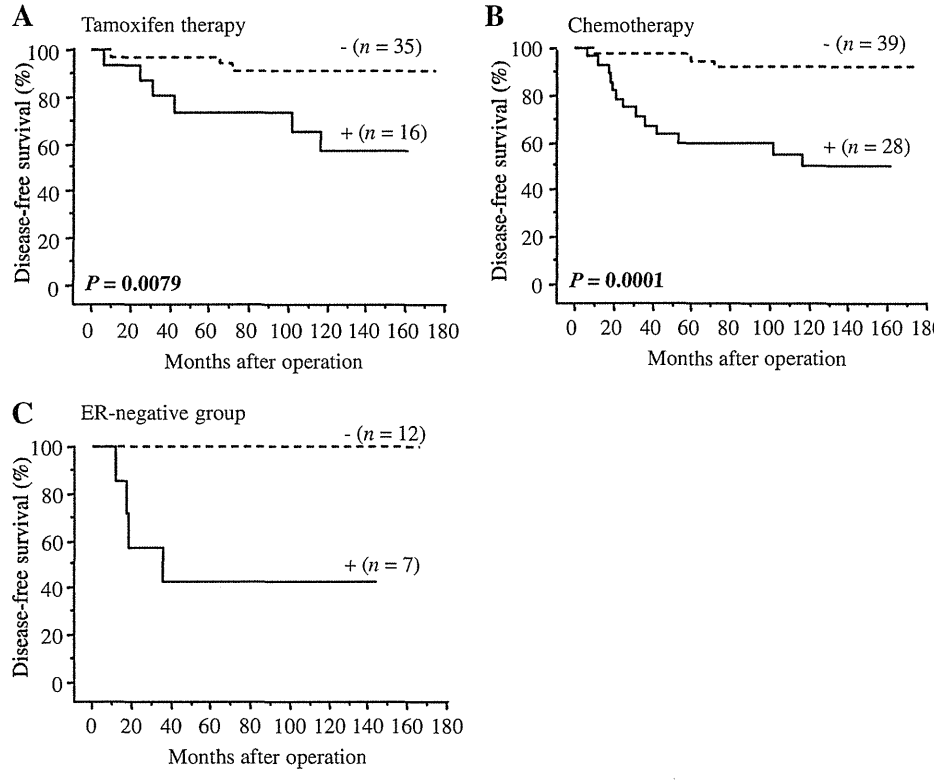

Fig. 4 Association between nuclear *BUB1* status and disease-free survival in a subset of stages I-III breast carcinoma cases (Kaplan–Meier method). **a** ER-positive breast carcinoma cases received tamoxifen therapy (n=51), **b** patients who received adjuvant chemotherapy (n=67), and **c** ER-negative breast carcinoma cases (n=19). Statistical analysis was evaluated by the log-rank test. *P* values less than 0.05 were considered significant and described as *boldface*. **c** *P* values were not available because no patient had recurrent disease in the group of nuclear *BUB1*-negative cases

or chemotherapy. The expression of other mitotic checkpoint protein *MAD2* was reported to be associated with resistance to neoadjuvant chemotherapy in the uterine cervical cancer [38], and an orally bioavailable *TTK* inhibitor (NMS-P715) selectively reduced carcinoma cell proliferation [39]. Results of our present study may serve as a starting point for clarification of biological functions and possible therapeutic potential of *BUB1* in breast carcinoma, but it awaits further investigations for clarification.

In summary, we examined expression profiles of mitotic checkpoint genes using microarray analysis. Results demonstrated that *BUB1* expression was closely associated with Ki-67 LI in the breast carcinoma cells. A subsequent immunohistochemical analysis did demonstrate that nuclear *BUB1* immunoreactivity was detected in 40% of breast carcinoma cases and was significantly associated with stage, pT, lymph node metastasis, distant metastasis, histological grade, Ki-67 LI, and cytoplasmic *BUB1* status of breast cancer cases. In addition, multivariate analysis further revealed that the nuclear BUB status was an independent prognostic factor of the patients. These findings all suggest that *BUB1* plays important roles in the proliferation and/or progression of breast carcinoma, and nuclear BUB1 immunoreactivity is a potent prognostic factor in the breast cancer patients regardless of ER status.

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Declaration of Interest We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Study of the effects of ocular hypotensive drugs on number of neurons in the retinal ganglion layer in a rat experimental glaucoma

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PURPOSE. *We investigated the effects of antiglaucomatous drugs on neurons in the retinal ganglion layer (RGL) in an experimental model of elevated intraocular pressure (IOP).*
METHODS. *Three episcleral veins of rats with normal IOP were cauterized. Three months later, we examined the effects on the number of neurons in the RGL as well as in rats submitted to treatment with timolol, latanoprost, or brimonidine. The IOP was measured using a calibrated Tono-Pen XL tonometer before and immediately after cauterization and every 2 weeks for the following 3 months as well as immediately before perfusion.*
RESULTS. *The IOP was 14.85±0.65 mmHg in the control group, whereas it was 1.25-fold higher (33.5±1.06 mmHg) in the experimental group. After treatment, the IOP returned to baseline levels. The mean number of neurons per mm² in the RGL was 33% lower in the experimental group (283±10 cells/mm²) compared with the control group (423±11 cells/mm²). In the groups treated with timolol, latanoprost, or brimonidine, the neuronal loss was less (331±10, 360±15, and 333±3 cells/mm², respectively), although values did not return to baseline levels.*
CONCLUSIONS. *This experimental model provokes an immediate, constant, and prolonged increase in IOP and the application of hypotensive agents affords a certain degree of protection to neurons in the RGL. (Eur J Ophthalmol 2009; 19: 963-70)*

KEY WORDS. *Brimonidine, Experimental glaucoma, Latanoprost, Number of neurons, Retinal ganglion layer, Timolol*

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INTRODUCTION

Glaucoma is an optic neuropathy associated with elevated intraocular pressure (IOP) and irreversible loss of retinal ganglion cells (RGC). The current pharmacologic therapy for open-angle glaucoma relies almost exclusively on drugs that lower IOP. The β-adrenoreceptor antagonists are one of the most important classes of drugs used clinically to lower elevated IOP in patients with glaucoma (1). In clinical studies, timolol has been shown to have preventative action against visual field loss progression, optic disc cupping, and decreased retinal thickness (1, 2). Timolol, a nonselective β-adrenoreceptor antagonist, lowers IOP by decreasing the formation of aqueous humor in the ciliary epithelium (3). Other drugs of choice include latanoprost and brimonidine. Latanoprost is a prostaglandin $F_{2\alpha}$ analogue that produces an ocular hypotensive effect (4-6). Its major mechanism of action is an increased uveoscleral outflow (7, 8). Another compound currently used in the control of pressure is brimonidine, a potent α-adrenergic agonist with high selectivity for the α_2 adrenergic receptor (9). Brimonidine has been shown to lower IOP safely and

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effectively in ocular hypertensive, glaucomatous, and normotensive eyes (10, 11). Brimonidine lowers IOP through a dual mechanism of action; it decreases aqueous production and increases uveoscleral outflow (12).

The purpose of this study was to compare the effects of 3 clinical drugs used in the treatment of glaucoma—timolol, latanoprost, and brimonidine—on survival of neurons in the retinal ganglion layer (RGL) after increased IOP, in order to compare our results with those reported by others using different glaucoma models (13-20).

These drugs were applied topically to rats submitted to chronic, elevated IOP and the effects evaluated after 3 months of treatment. The resulting data complete those from other researchers interested not only in stabilizing IOP at a lower level, but also in preventing the death of neurons.

MATERIALS AND METHODS

Subjects and measurement of IOP

Thirty-three adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 250–300 g at the beginning of the experiment were used. The animals were divided into 5 groups: control (n=6), experimental (n=8), and treated with timolol (n=8), latanoprost (n=6), or brimonidine (n=5). All animals were maintained and handled in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The IOP of both eyes was measured using a calibrated Tono-Pen XL tonometer (Mentor Ophthalmics, Inc., Norwell, MA) before and immediately after cauterization, and every 2 weeks for the following 3 months as well as immediately before perfusion. Rats were sedated by an intraperitoneal injection of 8% chloral hydrate (0.1 mL per 30 g body weight). A drop of topical anesthetic (proparacaine hydrochloride; Alcon Inc., Mississauga, Canada) was instilled and, with the eye under good illumination, the Tono-Pen was oriented perpendicular to the cornea and, using a swift and steady stroke, the tip was brought into contact with the cornea. Each IOP registered was an average of 3 consecutive measurements made at the same time of day (10 AM–12 PM), as well as immediately just before killing by perfusion.

Surgical procedure

One group of rats was not submitted to the surgical procedure and was used as a control group. The remainder of the animals were deeply anesthetized by intraperitoneal injection of 8% chloral hydrate (0.1 mL per 30 g body weight). Right eye limbus-draining veins were exposed by incising the conjunctiva and 3 of the 4 veins were cauterized, using a small vessel cauterizer (Ophthalmic Cautery-Cautere, Moria, Antony, France) (13, 14, 19, 21, 22). Special care was taken during surgery not to injure the limbal venous plexus as well as to minimize the amount of blood loss and damage to the conjunctiva and the underlying sclera. The blood supply to the retina remains unaffected in this surgery. After surgery, the eyes were treated during recovery with a topical antibiotic (Tobrex®, Alcon Cusí S.A., Barcelona, Spain).

Drug treatment

After the surgical procedure, the rats were then divided into 4 groups: an untreated group and 3 groups treated with timolol (Timoftol®, MSD de España S.A., Madrid, Spain), latanoprost (Xalatan®, Pharmacia España, S.A., Barcelona, Spain), or brimonidine (Alphagan®, Allergan S.A., Madrid, Spain), respectively. Treatment was started 2 weeks after inducing elevated IOP. Prior to starting the treatment, the IOP was measured in each group. We considered this period sufficient to simulate in our experimental animals the conditions usually found in the glaucomatous human eye, because an elevated IOP is not usually diagnosed until it has been present for some time. After determining that the IOP remained high during this period, we then instilled in the operated eye of each animal 2 drops per day of timolol or brimonidine and 1 drop per day of latanoprost for 3 months. All ocular tissues, including the cornea, lens, and sclera, appeared normal throughout the experiment.

Immunohistochemical procedure

Under deep anesthesia, perfusion through the heart in the control, experimental, and treated rats was performed with 0.1 M phosphate buffer (PB) and 4% paraformaldehyde in 0.1 M PB (pH 7.4). After perfusion, the eyeballs were removed and postfixed in the same fixative for 4 hours, transferred into ethanol 70%, and processed following a

protocol for embedding in paraffin. Horizontal sections of eye cups through the optic disc were made at a thickness of 6 µm. The sections were mounted onto pretreated glass slides, deparaffinized in xylene and rehydrated in distilled water through the conventional ethanol scale, preincubated in citrate buffer (pH 6.0) in a pressure cooker, and treated with 0.06% H_2O_2 for 15 minutes. They were then incubated overnight with the primary monoclonal neuronal nuclei (NeuN) antibody (MAB377; Chemicon, Temecula, CA; dilution 1:500). The slides were rinsed in phosphate buffer and incubated with biotinylated anti-mouse IgG (1:200) for 1 hour and treated with the avidin-biotin peroxidase complex (Vectastain-ABC Kit, Vector Lab, Burlingame, CA) for 60 minutes and 3,3′ diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) as the peroxidase substrate for 5 minutes. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted with Entellan. As a control, one section from each animal was processed with the same protocol but with the omission of the primary antibody.

Number of neurons in the retinal ganglion layer

The number of NeuN-immunoreactive cells in the RGL was measured to evaluate the retinal damage using an Image Analysis System (Visilog, Noesis, France). The equipment used included a microscope (Elipse E400, Nikon, Tokyo, Japan) with a 20× objective lens, a digital color camera (Polaroid Corp, Waltham, MA), and image-processing and analysis software (version 5.2, Visilog). We measured the number of neurons in the RGL at 200× magnification in 6 fields in each eye in the control, experimental, and treated groups, at a total distance of ~1.2 mm either side of the center of the optic nerve. The values of the 6 measurements in each eye were averaged and expressed as the mean number of NeuN-immunoreactive neurons per mm² of retina.

Statistical analysis

The quantitative values obtained for each animal were expressed as the mean and the standard error of the mean (SEM). Statistical analysis was performed using the commercially available software SPSS Inc. (Statistical Program for Social Sciences, 13.0). Comparison between groups was made using either analysis of variance or the nonparametric Tukey test. Where significant differences were found, a multiple comparison test was carried out.

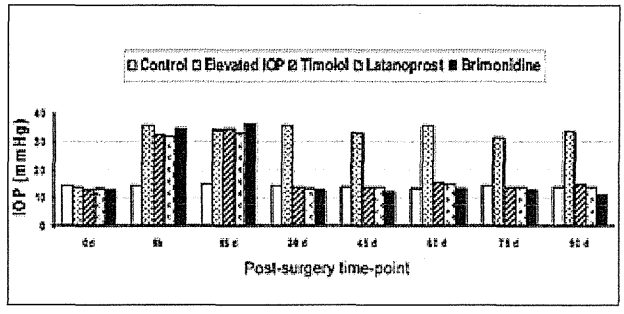

Fig. 1 - *Comparison of the intraocular pressure among control eyes, experimental eyes in which 3 episcleral veins were cauterized, and eyes treated with timolol, latanoprost, and brimonidine. Measurements were made every 2 weeks. Data are the mean ± SEM of the results in each group.*

RESULTS

Intraocular pressure

The average IOP in the control eyes was 14.85±0.65 mmHg (Figs. 1 and 3). Immediately after the surgical procedure, the mean IOP in the experimental eyes increased to 33.5±1.06 mmHg ($p<0.001$). Measurements made every 2 weeks over the next 3 months showed that the IOP values, represented in Figure 1, remained significantly elevated for the entire length of the experiment ($p<0.001$).

In the group of rats treated topically with timolol, the IOP was elevated immediately after the surgical procedure (32.27±0.98 mmHg) and 2 weeks later (34.91±1.12 mmHg) (Figs. 1 and 3), the point at which we started the treatment. After starting the treatment, measurements of the IOP taken every 2 weeks for 3 months showed a decrease to normal values (Fig. 1), with a mean value of 14.05±0.81 mmHg (Fig. 3). Likewise, in the rats treated topically with latanoprost, the IOP was elevated immediately after the procedure (32.22±0.99 mmHg) and 2 weeks later (32.89±0.37 mmHg). At this point we started the treatment and found that the measurements of the IOP taken every 2 weeks returned to normal values (Figs. 1 and 3), with a mean value of 14.11±0.72 mmHg (Fig. 3). Finally, in the group of rats treated topically with brimonidine, the IOP value was elevated immediately after the procedure (34.9±0.97 mmHg) and 2 weeks later (36.2±1.19 mmHg) (Figs. 1 and 3) when we started the treatment. The measurements of the IOP taken every 2 weeks for 3 months showed a decrease to normal values (Fig. 1), with a mean value of 12.34±0.63 mmHg (Fig. 3).

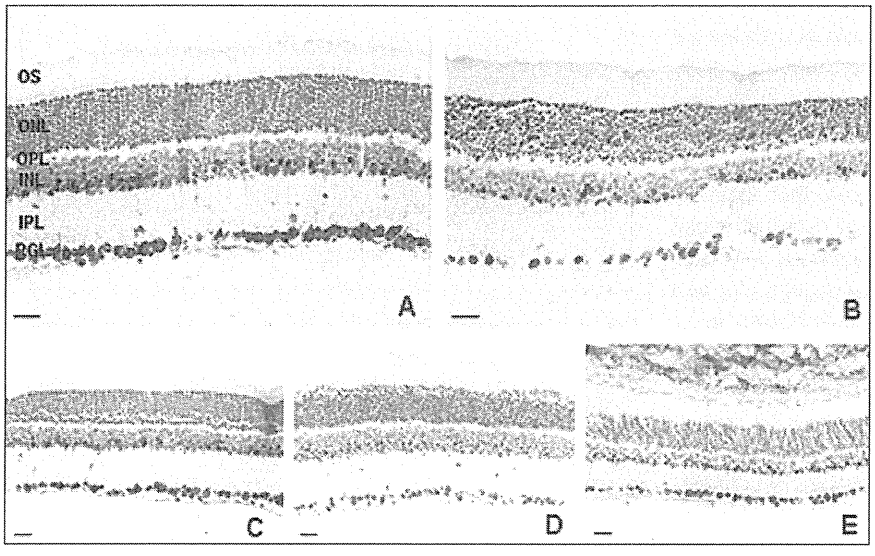

Fig. 2 - *Neuronal nuclei (NeuN) immunoreactivity in retinal sections from normal rat eyes, experimental rat eyes, and rat eyes treated with different antiglaucomatous drugs. Brown: NeuN label; blue: nuclear counterstain.* **(A)** *In normal retina, NeuN immunoreactive neurons are present in RGL and INL.* **(B)** *In glaucomatous retina, the number of NeuN-stained neurons in the RGL and INL is reduced at 2 weeks postinduction glaucoma.* **(C–E)** *Significant increase in the number of NeuN neurons was observed in both RGL and INL in the retina after timolol* **(C)**, *latanoprost* **(D)**, *and brimonidine* **(E)** *treatments. OS = outer segment; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; RGL = retinal ganglion layer. Scale bars = 16 µm.*

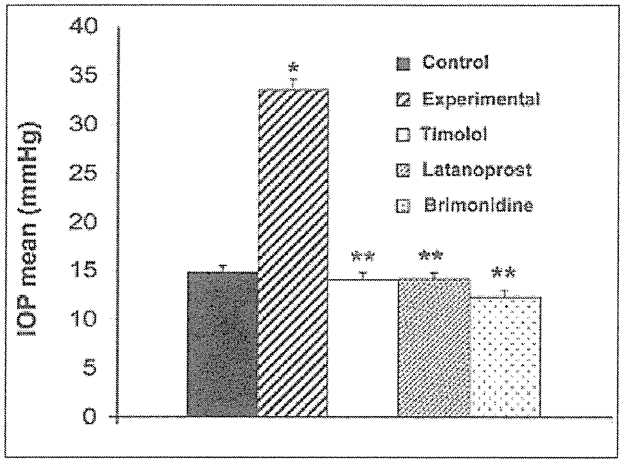

Fig. 3 - *Graph showing the mean ± SEM corresponding to the intraocular pressure (IOP). Each mean value corresponds to the values measured every 2 weeks for 3 months. A significant increase is seen in IOP in the experimental group submitted to the surgical procedure. After starting treatment with the hypotensive drugs timolol, latanoprost, and brimonidine, the IOP values returned to normal levels and remained stable throughout the treatment period (*p<0.001 experimental group compared to control group; **p<0.001 treated groups compared to experimental group).*

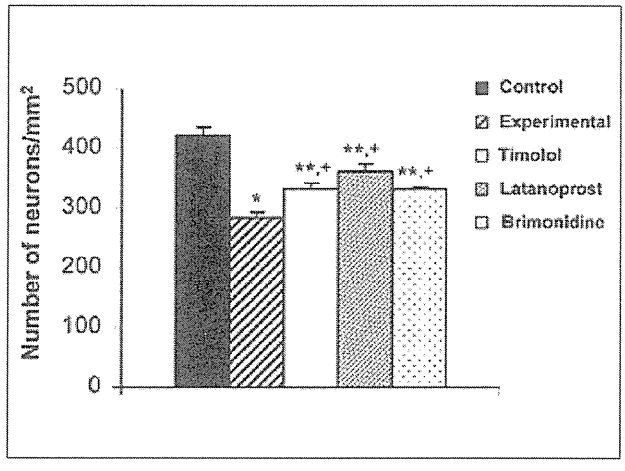

Fig. 4 - *Graph showing the mean ± SEM corresponding to the number of neurons/mm². A significant decrease is seen in the number of neurons/mm² in the experimental group submitted to the surgical procedure. However, the timolol-, latanoprost-, and brimonidine-treated animals observed an increase, but the number of neurons/mm² did not reach the same density as in normal eyes (*p<0.001 experimental group compared to control group; **p<0.001 treated groups compared to experimental group; +p<0.001 treated groups compared to control group).*

Number of neurons/mm² in the RGL

The number of NeuN-positive neurons in the RGL in control and experimental eyes was 423±11 cells/mm² and 283±10 cells/mm², respectively (Figs. 2 and 4). The chronic increase in IOP therefore caused a significant reduction in neurons compared with normal eyes (p<0.001), equivalent to a 33% cell loss (Fig. 5).

In relation to number of neurons/mm² in the RGL, after treatment with timolol, a 17% increase was seen in the density of cells immunoreactive for NeuN (331±10 cells/mm²) compared with experimental eyes (p<0.001) (Fig.

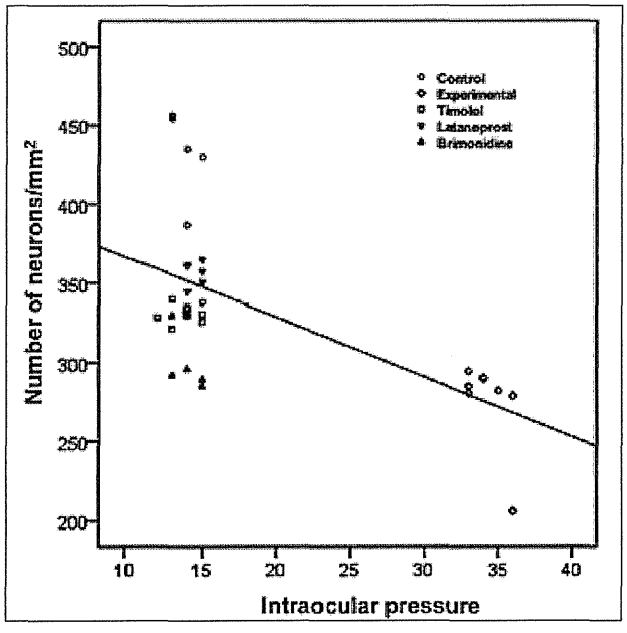

Fig. 5 - *Scatterplot showing relationship between intraocular pressure (IOP) and number of cells/mm² in the retinal ganglion layer (RGL). X-axis representing IOP and y-axis representing number of cells/mm² in the RGL.*

4). However, the number of neurons/mm² did not reach the same density as in normal eyes, with a 21.74% cell loss compared with normal eyes ($p<0.001$). On the other hand, after treatment with latanoprost, the mean number of neurons/mm² was 360±15 (Fig. 4), a 27% increase compared with experimental eyes ($p<0.001$), although, as with the timolol-treated rats, there was still a 15% loss in neuron immunoreactive cells compared with normal eyes ($p<0.001$). Finally, after treatment with brimonidine, the mean number of neurons/mm² was 333±3 (Fig. 4), a 27% increase compared with experimental eyes ($p<0.001$) (Fig. 4); nevertheless, there was still a 21.27% loss compared with the control group.

Comparison of the number of neurons/mm² in the RGL showed no significant differences among the 3 treatments.

DISCUSSION

Elevation of IOP has been implicated as the most critical risk factor in the generation of glaucomatous optic neuropathy; accordingly, the application of pressure-lowering drugs is of top priority in the treatment of this disease.

In the present study, the experimental procedure used was the cauterization of 3 episcleral veins to prevent normal outflow of the aqueous humor and produce an immediate, constant, and prolonged increase in the IOP (13, 22). Pang et al (23) concluded that rat IOP responds to compounds that reduce aqueous humor production in a similar way as in humans and, therefore, the rat is a useful study model for drug application.

We showed that the IOP was 1.25-fold higher in the cauterized eye compared to the control eye; these values remained constant in the experimental group for 3 months. Previous studies of glaucoma used many techniques to induce IOP elevation in rat eyes. These studies showed that most procedures were able to induce a significant elevation of IOP, a more than 1.5-fold increase compared to the control eyes (14, 16, 22, 24), which mostly lasted for 1–3 months (14, 24).

After starting treatment with the hypotensive drugs timolol, latanoprost, or brimonidine, the IOP values returned to normal levels and remained stable throughout the treatment period.

To assess the vulnerability of neurons in the RGL to the elevation of the IOP in our experimental model and determine the potential benefits of the hypotensive drugs timolol, latanoprost, and brimonidine, the neurons of RGL were labeled with a monoclonal antibody against the nuclear protein NeuN (25). NeuN is a DNA-binding protein that identifies most mature neuronal populations, and recent studies have used NeuN as a marker for RGCs in the RGL (18, 26-29). The immunoreactive number/mm² on either side of the center of the optic nerve was later counted. In the control animals with normal IOP, the average number of neurons/mm² in the RGL was 423±11. This number was considered to be 100% survival when compared to the experimental or treated groups.

Experimental animals with elevated IOP with no treatment had 283±10 neurons/mm², corresponding to 67% survival (i.e., a 33% cell loss) of neurons when compared to the control number. All the rat models reported by others showed that elevation of IOP causes ganglion cell or optic nerve fiber loss (13-20). WoldeMussie et al (30) noted a biphasic rate of ganglion cell loss, with a fast rate of 12% per week for the first 3 weeks of IOP elevation, followed by a slower rate of 2% per week for the remainder of the experimental period.

The mechanism of this loss is very likely apoptosis. In the early phase it may be pressure related, with evidence of neuronal cells undergoing apoptosis within 2 to 20 hours after being subjected to elevated IOP (31) or from 2 days to 2 weeks after the onset of glaucoma (32).

However, the exact mechanisms leading to neuronal loss have not been resolved, although they have been associated with optic nerve axoplasmic flow obstruction, depletion of the neurotrophic factors necessary for retinal ganglion cell survival, excess intraocular endothelin-1, retinal and optic nerve head (ONH) accumulation of nitric oxide and oxygen free radicals, and amino acid excitotoxicity and loss of intraneuronal calcium homeostasis at the ONH (33-35).

Application of each of the treatments used in our study showed that an important number of neurons were protected following timolol (331±11 neurons/mm^2; i.e., 78% survival), latanoprost (360±10 neurons/mm^2; i.e., 85% survival), or brimonidine treatment (333±3 neurons/mm^2; i.e., 79% survival). Comparison of these percentages with the survival found in experimental animals with no treatment (67%) showed that the rate of neuronal survival was from 11% to 18% greater than in the experimental group, depending on the particular treatment applied. The rate of survival was greater after the application of latanoprost, although the differences were not significant compared with the other 2 drugs used. These data show that, despite treatment, there is still a neuronal loss that probably occurs during the first 2 weeks, prior to starting therapy.

This would confirm the presence of neuronal loss immediately after induction of the glaucoma, during the initial weeks after the experimental IOP elevation. The results suggest a certain neuroprotective effect of the 3 drugs used. Timolol has a direct neuroprotective effect in experimental models of retinal injury against glutamate-induced neurotoxicity, suggesting that it has direct action on neuronal cells (36). In this same experimental model, we investigated the expression of nitric oxide synthase (NOS) isoforms -1 and -2 in the retina and ONH. Our results showed that rats treated with timolol showed reduced expression of NOS-1 in the retina and ONH, and NOS-2 was only detected in a few groups of astrocytes in the ONH, suggesting a possible neuroprotective effect of timolol in neurons exposed to excessive amounts of nitric oxide (37).

Regarding topical treatment with latanoprost, our results show that this prostaglandin also reduces neuronal loss in the RGL and, although it is generally accepted to reduce IOP by increasing pressure-independent outflow, the exact molecular mechanisms responsible for this are not fully understood. In ischemia-reperfusion injury, one researcher showed that the neuroprotective effect of latanoprost might be through the suppression of cyclooxygenase (COX-2) activity (38). According to previous reports, latanoprost may induce endogenous PGE_2 (39) in the retina. It has also been demonstrated that PGE_2 can protect neurons against excitotoxic and anoxic injury in the CNS (40). However, the real mechanism of the neuroprotective effect of latanoprost remains unknown, though latanoprost-induced PGE_2 may contribute to the effect of latanoprost on the retinal ganglion cells. Further studies are required to determine these mechanisms. Regarding brimonidine, several mechanisms underlying the neuroprotective activity of α_2-adrenergic agonists have been proposed. Brimonidine has been shown to increase neurotrophic factors (41) and the anti-apoptotic gene Bcl-2 (30) and thus may be neuroprotective by enhancing the survival of ganglion cells in this hostile environment. In addition, activation of presynaptic α_2-receptors results in inhibition of transmitter release (42). It is possible that brimonidine treatment attenuated the release of glutamate in the eyes with elevated IOP. An increase in vitreal glutamate has been implicated in excitoxicity of ganglion cells in glaucoma (43).

In conclusion, we have shown that this experimental model produces an immediate, constant, and prolonged increase in the IOP and causes a significant loss of neurons in the RGL. The topical administration of hypotensive drugs for 3 months, starting 2 weeks after the iatrogenic ocular hypertension, was sufficient to minimize the effects on the neurons in the RGL, noting a lower cell loss as compared with animals still having elevated IOP. Whether these drugs thus have a direct neuroprotective effect on the neurons or an indirect effect due to their hypotensive action is unknown.

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None of the authors has any proprietary interest in this report.

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Clinical Significance Following Breast Conservation Therapy with or without Irradiation in Breast Cancer Patients

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Background: We retrospectively examined the clinical outcome of irradiated versus non-irradiated groups of Japanese breast cancer patients according to their clinical and histopathological characteristics following breast-conserving therapy.
Methods: We retrospectively evaluated a total of 1197 Japanese female breast cancer patients (598 irradiated and 599 non-irradiated) who received breast-conserving therapy. The median age of the patients was 54 years (range: 24–99 years). We retrospectively examined the local recurrence-free survival rates in those with or without post-operative irradiation according to age, surgical margin status and histopathological characteristics including histological grade, estrogen receptor expression and HER2 status.
Results: Local recurrence-free survival rates in the irradiated group were significantly higher than those in the non-irradiated group, especially in surgical margin-positive [hazard ratio (HR): 0.334, 95% confidence interval: 0.14–0.79, $P = 0.001$], estrogen receptor-positive (HR: 0.249, 95% confidence interval: 0.11–0.54, $P < 0.001$), HER2-negative (HR: 0.382, 95% confidence interval: 0.21–0.69, $P = 0.001$) and non-triple-negative (HR: 0.382, 95% confidence interval: 0.21–0.69, $P = 0.001$) breast cancer patients.
Conclusion: The results indicated that irradiation after breast-conserving therapy is strongly recommended in Japanese breast cancer patients, especially those with surgically positive margins, estrogen receptor-positive tumors and HER2-negative invasive breast cancers.

Key words: breast cancer – conservation therapy – radiotherapy – ER – HER2

INTRODUCTION

Breast-conserving therapy (BCT) is considered the standard treatment for early breast cancer patients and the results of the survival analysis are equivalent to those reported following total mastectomy (1–3). Previous studies demonstrated that BCT plus local radiotherapy markedly reduced the subsequent risk of local recurrence in breast cancer patients (4–6). The results of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-06 randomized trial established the clinical effectiveness of radiation therapy in terms of the prevention of local breast cancer recurrence after lumpectomy in women with breast cancer associated with either the presence or absence of axillary lymph node metastasis (4). In addition, the results of the meta-analysis of the Early Breast Cancer Trialists' Collaborative Group revealed the need for radiotherapy following BCT based upon the fact that breast irradiation reduced the 5 year local recurrence rate (5). Therefore, adjuvant radiotherapy has become the golden standard for women with breast cancer after BCT. However it is also true that the rate of local recurrence after

BCT was approximately 5–10%. Therefore, some patients did not necessarily consent to receive radiotherapy after BCT and clinicians also did not strongly recommend the therapy in these cases (7). Histological tumor type, grade and molecular markers are currently considered the standard prognostic indicators. They have immensely contributed to the selection of the optimal treatment strategy including endocrine therapy, chemotherapy and targeted therapy in individual patients with breast cancer (8–10). Therefore, we retrospectively examined the outcomes in irradiation versus non-irradiation groups according to age, surgical margin, invasive or non-invasive status and histopathological characteristics including histological grade, estrogen receptor (ER) expression and human epidermal growth factor receptor 2 (HER2) status in women with breast cancer after BCT.

MATERIALS AND METHODS

Patients

We retrospectively evaluated 1197 Japanese female breast cancer patients (598 irradiated and 599 non-irradiated cases) who received BCT in the Nahanishi Clinic in Okinawa, Japan from June 1996 to December 2007. The protocol for the present study was approved by the ethics committee at the Nahanishi Clinic (NNCEC2012005). The median age of the patients was 54 years (range: 24–99 years); the irradiation group was 49 years (range: 24–86 years) and the non-irradiation group was 61 years (range: 30–99 years), respectively. Table 1 summarizes the characteristics of the patients examined. The median follow-up duration was 72.0 months.

Table 1. The characteristics of the patients of this study

Total number	1197
Irradiation status	
Irradiation	598
Non-irradiation	599
Surgical margin status	
Margin positive	250
Margin negative	947
Invasive or non-invasive status	
Invasive carcinoma	950
Non-invasive carcinoma	247
ER expression	
Positive	752
Negative	198
HER2 status	
Positive	95
Negative	855
Triple-negative	88
Non-triple-negative	872

The Criteria of Pre- and Post-operative Treatment

Breast-conserving surgery was performed in one single institution, Nahanishi Clinic, as a standardized sector resection with a 1cm margin of macroscopically normal tissue, and axillary dissection of levels I–III or sentinel lymph node biopsy (7,11). The indication of BCT is summarized as follows: Stages 0, I and II with a tumor size less than 3 cm (7,11). Adjuvant or neo-adjuvant chemoendocrine therapies were administered according to the established protocols (12,13).

The standard radiation dose in all conserved breasts has been 50 Gy to the whole breast, plus 10 Gy boost irradiation in surgical margin-positive breast cancer patients, margin positivity defined as cancerous lesions existing within 5 mm from the surgical margin (7). Irradiation of the whole breast was performed using two tangential megavoltage photon beam (high-energy X-ray of telecobalt) (1,7). A total dose of 50 or 60 Gy during a 5 or 6 weeks period, with a dose of 2 Gy per fraction, was delivered at the intersection of the central axes of the beams (1,7).

Histopathological Diagnoses

Surgical specimens were fixed in 10% neutral formaldehyde solution and cut into 5 mm thick slices, embedded in paraffin, cut into 4 lm thick sections and placed on glue-coated glass slides. We used the avidin–streptavidin immunoperoxidase method using the clone 6F11 antibody (Ventana, Tucson, AZ, USA) in an automated immunostainer (Benchmark System; Ventana) for ER staining after January 2002. From June 1996 to December 2001, ER was determined by a commercially available immunoassay kit (Abbott Diagnostic, North Chicago, IL, USA) and 16% and more was defined as positive. A standardized immunohistochemistry kit (HercepTest for Immunoenzymatic Staining; Dako, Copenhagen, Denmark) was used for HER2 staining. Histopathological evaluation was based on the World Health Organization histological classification of tumors of the breast and Rosen's Breast Pathology (14,15). ER was determined by nuclear staining graded from 0 to 8 using the Allred score, and ER positive was grade 3 or more (16). With regard to HER2 evaluation, membranous staining was graded as follows: score 0–1+, 2+ and 3+ (17). In cases in which the score was 2+, examination by fluorescence *in situ* hybridization (FISH) was used to calculate the gene copy ratio of HER2-to-CEP17 (PathVysion HER2 DNA Probe kit; Abbott, Chicago, IL, USA) (17). Positivity was defined as HER2:CEP17 signal ratio (FISH score) >2.2 (14).

Evaluation

We investigated the criteria of post-operative radiotherapy on local recurrence in irradiated and non-irradiated breast-conserving surgery groups. We retrospectively examined the local recurrence-free survival (RFS) rates in those with or without post-operative irradiation according to age, surgical

margin status, histopathological characteristics including ER expression and HER 2 status and adjuvant chemoendocrine status. The local RFS was defined as the time interval between the date of surgery and the date of disease recurrence in the same breast.

Statistical Methods

The distribution of time to local recurrence was estimated according to the Kaplan–Meier method and the distribution of the irradiated and non-irradiated groups were compared by means of the log-rank test (18,19). All analyses were performed with the use of statistical software (StatMate IV for Windows ATMS, Tokyo, Japan).

RESULTS

The Comparison of Local RFS Between Irradiated and Non-Irradiated Groups of Patients

Local RFS was worse in the non-irradiated group compared with that in the irradiated group [hazard ratio (HR): 0.394, 95% confidence interval (CI): 0.249–0.670, $P < 0.001$]. Five-year local RFS rates were 0.967 for the irradiated group and 0.935 for the non-irradiated group.

Surgical Margin Status

The local RFS in the irradiated group was significantly higher than that in the non-irradiated one in both surgical margin-negative (HR: 0.362, 95% CI: 0.15–0.82, $P = 0.015$) and positive patients (HR: 0.334, 95% CI: 0.14–0.79, $P = 0.001$). Five-year local RFS rates were 0.982 for the irradiated group and 0.945 for the non-irradiated group in surgical margin negative breast cancer patients. In surgical margin-positive breast cancer patients, 5-year local RFS rates were 0.935 for the irradiated group and 0.829 for the non-irradiated group (Fig. 1).

Age Groups

There was a statistically significant difference between the irradiation and non-irradiation only in the group of patients in their 50s (HR: 0.245, 95% CI: 0.06–0.51, $P = 0.002$). However, there were no statistically significant differences between the irradiated and non-irradiated groups in the patients in their 20–30s (HR: 2.236, 95% CI: 0.19–86.75, $P = 0.831$), patients in their 40s (HR: 0.921, 95% CI: 0.345–3.282, $P = 0.913$), patients in their 60s (HR: 0.279, 95% CI: 0.07–1.54, $P = 0.160$) and patients in their 70–80s (HR: 6.188, 95% CI: 0.15–1.13, $P = 0.087$).

Invasive and Non-Invasive Status

There were statistically significant differences between the irradiation and non-irradiation groups in invasive carcinoma (HR: 0, 95% CI: 0.06–0.18, $P < 0.001$). However, no statistically significant differences were detected between the irradiation and non-irradiation groups in non-invasive carcinoma ($P = 0.364$).

ER Status

The local RFS in the irradiated group was significantly higher than that in the non-irradiated group in ER-positive breast cancer patients (HR: 0.249, 95% CI: 0.11–0.54, $P < 0.001$). Five-year local RFS rates were 0.989 in the irradiated group and 0.939 in the non-irradiated group. However, there were no statistically significant differences between the irradiated and non-irradiated groups in ER negative breast cancer patients (HR: 0.586, 95% CI: 0.22–1.57, $P = 0.288$). Five-year local RFS rates were 0.913 in the irradiated group and 0.840 in the non-irradiated group (Fig. 2).

HER2 Status

Local RFS in the irradiated group was significantly higher than that in the non-irradiated one in HER2-negative breast cancer patients (HR: 0.382, 95% CI: 0.21–0.69, $P = 0.001$). Five-year local RFS rates were 0.980 for the irradiated group and 0.940 for the non-irradiated group, respectively. In HER2-positive breast cancer patients, there were no statistically significant differences between the irradiated and non-irradiated groups of patients (HR: 0.971, 95% CI: 0.22–4.24, $P = 0.969$). Five-year local RFS rates were 0.907 for

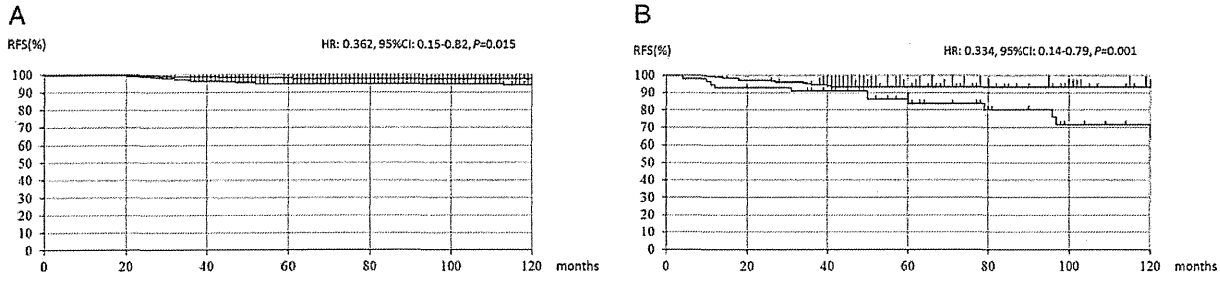

Figure 1. RFS of the (a) irradiation and (b) non-irradiation groups in (A) surgical margin negative and (B) surgical margin positive breast cancer patients.

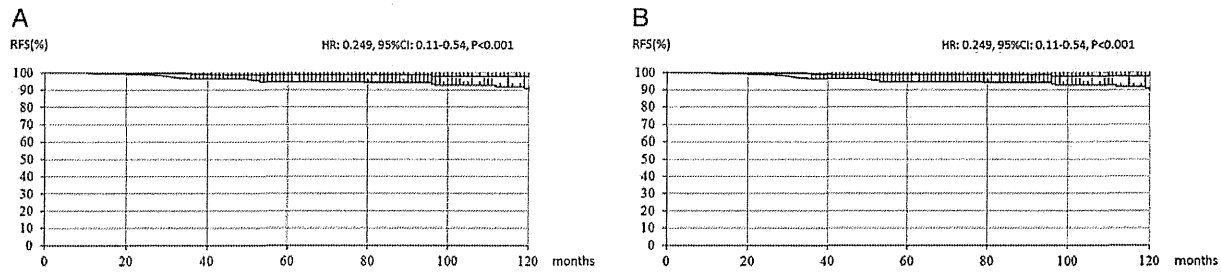

Figure 2. RFS of the (a) irradiation and (b) non-irradiation groups in (A) ER-positive and (B) ER-negative breast cancer patients.

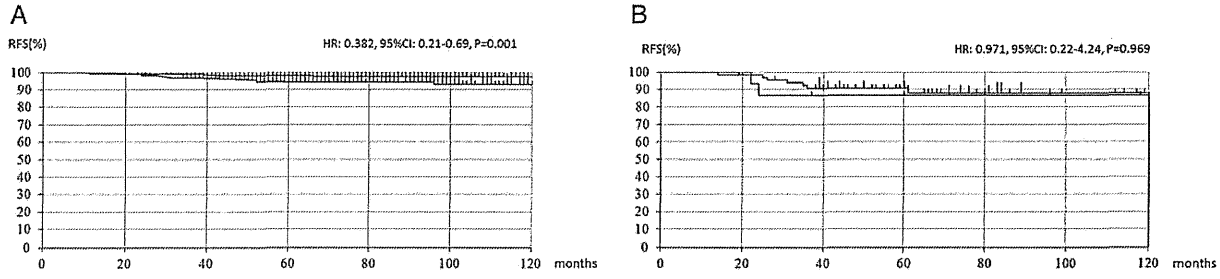

Figure 3. RFS of the (a) irradiation and (b) non-irradiation groups in (A) HER2-negative and (B) HER2-positive breast cancer patients.

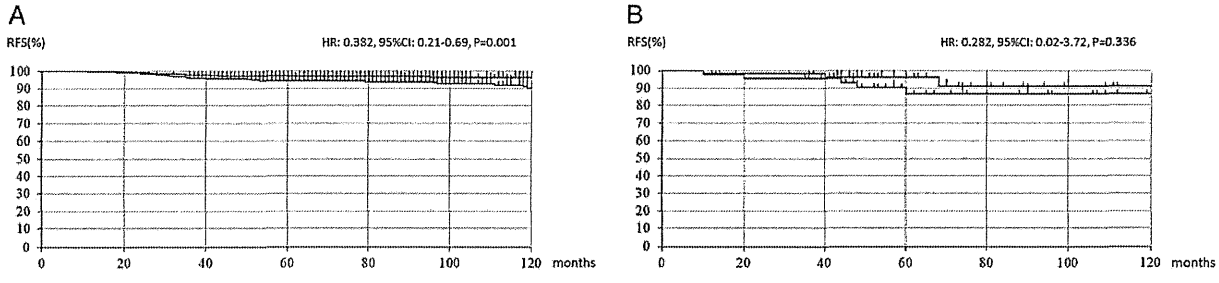

Figure 4. RFS of the (a) irradiation and (b) non-irradiation groups in (A) non-triple-negative and (B) triple-negative breast cancer patients.

the irradiated group and 0.819 for the non-irradiated group of patients, respectively (Fig. 3).

Triple-negative or Non-triple-negative Status

Local RFS in the irradiated group was significantly higher than that in the non-irradiated one in non-triple-negative breast cancer patients (HR: 0.382, 95% CI: 0.21–0.69, $P = 0.001$). Five-year local RFS rates of non-triple-negative breast cancer patients were 0.972 for the irradiated group and 0.942 for the non-irradiated group, respectively. There were, however, no statistically significant differences between the irradiated and non-irradiated groups of the triple-negative breast cancer patients (HR: 0.282, 95% CI: 0.02–3.72, $P = 0.336$). Five-year local RFS rates of triple-negative breast cancer patients were 0.963 of irradiated and 0.866 of non-irradiated groups of the patients, respectively (Fig. 4).

Adjuvant Chemoendocrine Therapy

Local RFS in the irradiation group was significantly higher than that in the non-irradiation group in both the adjuvant therapy group (HR: 0.577, 95% CI: 0.141–0.304, $P < 0.001$) and the non-adjuvant therapy group (HR: 0, 95% CI: 0.036–0.351, $P < 0.001$). Five-year local RFS rates were 0.957 for the irradiation group and 0.869 for the non-irradiation group in the adjuvant therapy group. Five-year local RFS rates were 0.875 for the irradiation group and 0.842 for the non-irradiation group in the non-adjuvant therapy group.

DISCUSSION

The results of previous studies demonstrated that BCT with radiotherapy certainly decreased the incidence of local recurrence in ipsilateral breast, when compared with BCT alone with statistical significance (3,20–23). These studies have

compared BCT with and without adjuvant radiotherapy and have consistently demonstrated ~70% substantial reduction in the risks of local recurrence with irradiation (20–23). The results of our present study also demonstrated that local RFS was worse in the non-irradiated group compared with that in the irradiated group with statistical significance. Therefore, many previous studies recommend routine breast irradiation after BCT (3,20–23). However, it is also true that some BCT cases are treated without subsequent or post-operative radiotherapy (7). In addition, all residual carcinoma cells are not necessarily radiation sensitive, and there are currently no established methods for determining the radiation responsiveness of individual patients (7). Previous studies demonstrated that many personal and biological factors including patients' age, surgical margin status or histological grade of the resected specimens could be independent predictors for local recurrences of breast cancer (2,7). Therefore, we examined the effects of radiotherapy following BCT according to the corresponding clinical and histopathological characteristics.

The highest rate of local recurrence (27%) following BCT was reported in the patients with extensively positive surgical margins, whereas the lowest one (7%) in those with either close or negative margins (23,24). An intermediate rate of local recurrence (14%) was reported among those with focally positive margins (23,24). Therefore, as expected, surgical margin status of the resected specimens is considered to be one of the most important factors related to the risk of local recurrence in individual patients. There are many definitions of positive margins of BCS in the world (25–27). We defined positive margins of BCS as the exposure of carcinoma cells <5 mm from the edge of the specimens according to the code of the Japan Breast Cancer Society (JBCS) (7). The definition of JBCS is longer than the definition of the other countries (7,25–27). Based on the results of our present study as well as those reported before, the patients with surgical margins positive for cancer after BCT are strongly recommended to be received post-operative irradiation. However, local RFS in the irradiated group was also significantly higher than that in the non-irradiated one in surgical margin negative patient in this study. Therefore, irradiation after BCT could prevent allochronic late recurrence in patients with surgical margins positive and negative for cancer. The results of our present study also demonstrated that local RFS in the irradiated groups was significantly higher than that in the non-irradiated ones in surgical margin-positive patients ($P = 0.001$). These results also reinforced the concept that all the breast cancer patients with surgical margins positive should receive irradiation following BCT.

Radiotherapy with tamoxifen was reported to significantly reduce the risks of ipsilateral breast cancer recurrence following BCT in patients with hormone receptor-positive breast cancers (6,20,23,24). The national Surgical Adjuvant Breast and Bowel Project B-21 examined tamoxifen, radiotherapy or combination of these therapies for the prevention of local recurrence of 1.0 cm or less invasive breast cancer following BCT (6). Eight-year cumulative local recurrence rates among the patients with ER-positive breast cancer were 16.8, 6.9 and 2.1%, respectively (6). In addition, Fyles et al. (24) randomized 769 women with early breast cancer to receiving breast irradiation plus tamoxifen (386 patients) or tamoxifen alone (383 patients). The 5-year local recurrence rate was 7.7% among those treated with tamoxifen alone compared with 0.6% in the group who received radiotherapy and tamoxifen (24). We also retrospectively examined the local RFS rates in those with or without post-operative irradiation in ER-positive breast cancer. The results of our present study also demonstrated that local RFS in the irradiated group was significantly higher than that in the non-irradiated one in ER-positive breast cancer patients (HR: 0.249, 95% CI: 0.11–0.54, $P < 0.001$). The results of our present study were similar to those of previously reported studies (6,20,23,24).

To the best of our knowledge, this is the first study to correlate the clinical effects of radiotherapy with ER/HER2 status and triple-negative or non-triple-negative status in breast cancer cases after BCT. Some previous studies investigated whether an approximation of breast cancer molecular subtypes prognosticated for local breast recurrence after BCT and radiotherapy or not (28,29). Arvold et al. (28) reported that the 5-year cumulative incidences of local recurrence were 0.8% for luminal A, 2.3% for luminal B, 1.1% for luminal HER2, 10.8% for HER2 and 6.7% for triple-negative, respectively. Five-year local RFS rate was reported to be 97.2% for 541 Asian breast cancer patients after BCT but found to be different among subtypes: 0.8% for luminal A, 1.4% for luminal B, 3.6% for HER2 and 12.7% for triple-negative breast cancers, respectively (29). The results of our present study did demonstrate that local RFSs in the irradiated group were significantly higher than that in the non-irradiated one in ER-positive, HER2-negative and non-triple-negative breast cancer cases. However, there were no statistically significant differences of RFS between the irradiated and non-irradiated groups in ER-negative, HER2-positive and triple-negative breast cancer cases. These results suggest that the potential clinical course or biological behavior of the breast cancer following BCT when surgical margins are negative is also dependent upon the biological characteristics of the corresponding breast carcinoma cells. In addition, a higher incidence of increased ratio of ER-positive and HER2-negative type were reported to be associated with poorly demarcated masses, whereas the presence of higher ratios of triple-negative and HER types were all associated with well-demarcated masses (8). Therefore, the effectiveness of irradiation after BCT is considered to depend partially upon these differentiation and/or infiltration pattern of breast carcinoma cells. On the other hand, there was possibility of selection bias for post-operative irradiation in HER2-positive or TNBC cases. For example, high HG, high proliferation or high lymphovascular invasion cases were latently classified into irradiation group. Therefore, it

would contribute to no statistical difference among patients with HER2 positive or TNBC, with or without radiation. As for age and non-invasive status, there were no statistically significant differences between the irradiated and non-irradiated groups in our present study. However, the results of previous studies did demonstrate the benefit of radiotherapy in patients younger than 50 years (30) and 60 years (7). In addition, the results of this study also demonstrated that local RFS in the irradiation group was significantly higher than that in the non-irradiation group in both the adjuvant therapy group and the non-adjuvant therapy group. The results of many previously reported studies demonstrated that radiotherapy after BCT significantly reduced the overall risks of local recurrence (31–33). Therefore, further investigations are required to clarify the correlation between the effects of irradiation and age or non-invasive status. Further investigations such as multicenter analysis are required to further refine the new irradiation criteria after BCT. This study may contribute to the new irradiation criteria based on biological characteristics of Japanese breast cancer patients.

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Conflict of interest statement

None declared.

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