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ORIGINAL ARTICLE

Repeat lumpectomy for ipsilateral breast tumor recurrence (IBTR) after breast-conserving surgery: the impact of radiotherapy on second IBTR

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Abstract
Objectives There are limited data on the outcomes of patients treated with repeat lumpectomy at the time of ipsilateral breast tumor recurrence (IBTR). Especially, the impact of radiotherapy (RT) on a second IBTR is unknown.
Methods We retrospectively analyzed 143 patients from 8 institutions in Japan who underwent repeat lumpectomy after IBTR. The risk factors of a second IBTR were assessed.
Results The median follow-up period was 4.8 years. The 5-year second IBTR-free survival rate was 80.7 %. There was a significant difference in the second IBTR-free survival rate according to RT ($p = 0.0003$, log-rank test). The 5-year second IBTR-free survival rates for patients who received RT after initial surgery, RT after salvage surgery, and no RT were 78.0, 93.5, and 52.7 %, respectively. Multivariate analysis revealed that RT was a significantly independent predictive factor of second IBTR-free survival.
Conclusion Repeat lumpectomy plus RT is a reasonable option in patients who did not undergo RT at the initial surgery. In contrast, caution is needed when RT is omitted in patients who have undergone repeat lumpectomy.

Keywords Breast cancer · Breast-conserving surgery · Ipsilateral breast tumor recurrence · Repeat lumpectomy

Introduction

Mastectomy has long been regarded as the standard of care for ipsilateral breast tumor recurrence (IBTR) after breast-conserving surgery [1], although many women with breast

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cancer recurrence previously treated with breast-conserving surgery desire repeat lumpectomies.

At present, there are limited data on the outcomes of patients treated with repeat lumpectomy at the time of IBTR [2–7]. Most of the available data on the outcomes of patients treated with repeat lumpectomy are those of patients treated with initial breast-conserving surgery followed by radiotherapy (RT) [2–6]. On the other hand, there is little information on the outcomes of repeat lumpectomy for patients treated with initial breast-conserving surgery without RT. Despite the robust benefits of RT for local control, a recent study suggested the underutilization of RT among patients treated with initial breast-conserving surgery [8]. Data from the Surveillance, Epidemiology, and End Results registry indicate that the omission of RT increased significantly from 1992 (15.5 %) to 2007 (25 %) [8]. Therefore, it is clinically useful to verify the risk of second IBTR according to RT (i.e., RT after initial surgery, RT after salvage surgery, or no RT).

This study investigated the risk factors of second IBTR after repeat lumpectomy using data from a multi-institutional series, focusing on RT.

Patients and methods

A total of 271 consecutive patients with histologically confirmed IBTR without distant metastases who underwent definitive surgery for IBTR between 1989 and 2008 were registered from 8 institutions in Japan. This retrospective study was approved by each institutional review board. Inclusion criteria were: (1) patients who underwent breast-conserving and axillary surgery (sentinel lymph node biopsy was only allowed if these nodes had no metastases); (2) patients in whom IBTR was confirmed histologically; (3) patients who underwent definitive surgery for IBTR before 2008. Exclusion criteria were the following: (1) synchronous (defined as occurring within 3 months) metastases; (2) bilateral breast cancer patients; (3) prior malignancy other than breast cancer; (4) patients with tumors located in the skin or muscle only, without associated parenchymal disease.

Of the 271 patients, as salvage surgery, mastectomy of the conserved breast was performed in 122 patients and repeat lumpectomy was performed in 149 patients. Of these 149 patients, 6 patients were excluded from this analysis for reasons as follows: unavailable data for radiotherapy for the ipsilateral breast ($n = 3$), and unavailable data for second IBTR ($n = 3$). Finally, 143 patients who underwent repeat lumpectomy were included in this analysis. Patients and tumor characteristics examined are listed in Table 1. Numbers of patients who received RT after the initial surgery, RT after salvage surgery, and no RT were 69, 55, and 19, respectively. One patient received RT both after the initial and salvage surgery, and this patient was included in the RT after salvage surgery group. A patient flowchart is shown in Fig. 1.

Family history was defined as positive when first-degree relatives had been diagnosed with breast cancer. For breast-conserving surgery, the margin was regarded as positive when an invasive or noninvasive component was present at the cut margin. Estrogen receptor (ER) status was

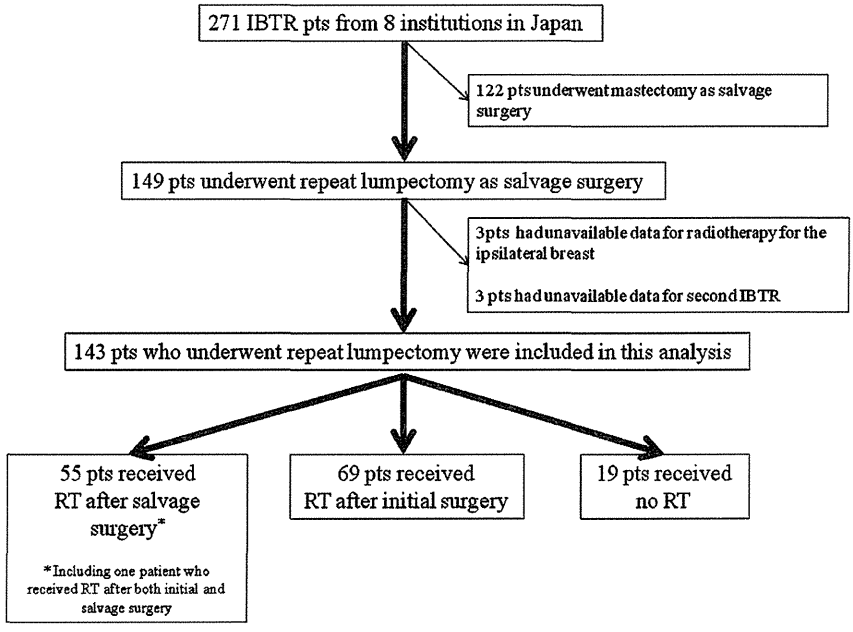

Fig. 1 Patient flowchart

Table 1 Patient characteristics ($n = 143$)

Characteristics	All ($n = 143$)	Patient groups according to RT			p value
	No. of patients (%)	After initial surgery ($n = 69$) No. of patients (%)	After salvage surgery[a] ($n = 55$) No. of patients (%)	No RT ($n = 19$) No. of patients (%)	
Age at initial diagnosis					
<40	37 (26)	19 (28)	15 (27)	3 (16)	0.5591
≥40	106 (74)	50 (73)	40 (73)	16 (84)	
Age at IBTR					
<40	19 (13)	8 (12)	8 (15)	3 (16)	0.8393
≥40	124 (87)	61 (88)	47 (86)	16 (84)	
Family history					
No	100 (70)	46 (67)	38 (69)	16 (84)	0.4701
Yes	14 (10)	9 (13)	5 (9)	0 (0)	
Unknown	29 (20)	14 (20)	12 (22)	3 (16)	
Time interval from initial surgery to IBTR (years)					
≤2	33 (23)	15 (22)	12 (22)	6 (32)	0.6401
>2	110 (77)	54 (78)	43 (78)	13 (68)	
Tumor location					
Same quadrant	96 (67)	45 (65)	38 (69)	13 (68)	0.5807
Different quadrant	39 (27)	18 (26)	15 (27)	6 (32)	
Unknown	8 (6)	6 (9)	2 (4)	0 (0)	
Tumor size of IBTR					
≤20 mm	124 (87)	63 (91)	47 (86)	14 (74)	0.0128
>20 mm	10 (7)	6 (9)	3 (6)	1 (5)	
Unknown	9 (6)	0 (0)	5 (9)	4 (21)	
Lymphovascular invasion of IBTR					
No	77 (54)	43 (62)	25 (46)	9 (47)	0.1629
Yes	44 (31)	20 (29)	17 (31)	7 (37)	
Unknown	22 (15)	6 (9)	13 (24)	3 (16)	
Histologic grade of IBTR					
1	33 (23)	17 (25)	9 (16)	7 (37)	0.1428
2	36 (25)	16 (23)	16 (29)	4 (21)	
3	44 (31)	26 (38)	13 (24)	5 (26)	
Unknown	30 (21)	10 (15)	17 (31)	3 (16)	
Margin of IBTR					
Negative	123 (86)	58 (84)	49 (89)	16 (84)	0.7901
Positive	10 (7)	5 (7)	4 (7)	1 (5)	
Unknown	10 (7)	6 (9)	2 (4)	2 (11)	
ER of IBTR					
Negative	50 (35)	29 (42)	12 (22)	9 (47)	0.1320
Positive	86 (60)	37 (54)	40 (73)	9 (47)	
Unknown	7 (5)	3 (4)	3 (6)	1 (5)	
HER2 of IBTR					
Negative	100 (70)	48 (70)	37 (67)	15 (79)	0.4939
Positive	25 (18)	14 (20)	8 (15)	3 (16)	
Unknown	18 (13)	7 (10)	10 (18)	1 (5)	
Ki-67 index of IBTR					
<20	68 (48)	31 (45)	24 (44)	13 (68)	0.4194
≥20	47 (33)	24 (35)	19 (35)	4 (21)	
Unknown	28 (20)	14 (20)	12 (22)	2 (11)	

Table 1 continued

Characteristics	All (n = 143)	Patient groups according to RT			p value
	No. of patients (%)	After initial surgery (n = 69) No. of patients (%)	After salvage surgery[a] (n = 55) No. of patients (%)	No RT (n = 19) No. of patients (%)	
Breast cancer subtype of IBTR					
Luminal-A	41 (29)	17 (25)	17 (31)	7 (37)	0.0787
Luminal-B	27 (19)	12 (17)	13 (24)	2 (11)	
Triple-negative	30 (21)	16 (23)	7 (13)	7 (37)	
HER2	17 (12)	12 (17)	3 (6)	2 (11)	
Unknown	28 (20)	12 (17)	15 (27)	1 (5)	
Hormone therapy after salvage surgery					
No	46 (32)	26 (38)	13 (24)	7 (37)	0.2372
Yes	95 (66)	41 (59)	42 (76)	12 (63)	
Unknown	2 (1)	2 (3)	0 (0)	0 (0)	
Chemotherapy after salvage surgery					
No	120 (84)	53 (77)	50 (91)	17 (90)	0.2177
Yes	21 (15)	15 (22)	4 (7)	2 (11)	
Unknown	2 (1)	1 (1)	1 (2)	0 (0)	
Trastuzumab after salvage surgery[b]					
No	19 (76)	8 (57)	8 (100)	3 (100)	0.0450
Yes	6 (24)	6 (43)	0 (0)	0 (0)	

IBTR ipsilateral breast tumor recurrence, *RT* radiotherapy, *ER* estrogen receptor

[a] Including one patient who received RT after both initial and IBTR surgery

[b] Including only patients with HER2-positive tumors at IBTR

determined by immunohistochemistry, and tumors with 10 % or more positively stained tumor cells were classified as positive for ER. HER2 status was considered positive if immunohistochemistry was 3+ or fluorescence in situ hybridization (her-2/*neu* to chromosome 17 ratio) was >2.0. Both ER and HER2 status was evaluated by each institution. Proliferation activity was assessed by immunostaining with the Ki-67 antibody (Dako). The Ki-67 index was centrally evaluated by one pathologist (N.A.), from whom all patient data were masked. The proportion of proliferating cells was determined by counting at least 500 tumor cells. Breast cancer subtypes were modified by the criteria recently recommended by the St. Gallen panelists [9]: triple-negative (ER- and HER2-negative), HER2 (HER2-positive and ER-negative), luminal-A (ER-positive, Ki-67-low, and HER2-negative), and luminal-B (ER-positive and Ki-67-high or HER2-positive or both). In this study, the cut-off value of the Ki-67 index was defined as 20 % (the median value of prior studies by Nishimura et al. [10]).

The association of RT with various clinicopathological factors was assessed using a Chi-square test.

Patients received a physical examination every 3–6 months for 5 years after salvage surgery and annually thereafter. Mammograms were performed annually after salvage surgery.

Second IBTR-free survival was calculated from the first IBTR to any local recurrence in the ipsilateral breast. Local recurrences were counted as events only when they were the first sites of failure or occurred concurrently with regional or distant metastasis. In the calculation of second IBTR-free survival, occurrences of regional or distant metastases, contralateral breast cancer, other second primary cancers, being alive without second IBTR, and deaths without evidence of recurrence were treated as censoring events.

Distant disease-free survival (DDFS) was defined as the period from the date of surgery for IBTR to the date of appearance of distant metastases.

Second IBTR-free survival and DDFS curves were calculated employing the Kaplan–Meier method. The log-rank test was used to evaluate the differences in second IBTR-free survival among the various patient subgroups. Multivariate analyses for second IBTR-free survival were performed using the Cox proportional hazards model. All of the statistical tests and p values were two-tailed, and p values of <0.05 were considered significant.

Results

Within a median follow-up period of 4.8 years (range 0.2–16.7 years), 29 of 143 patients (20.3 %) experienced a second IBTR. The 5-year second IBTR-free survival rates were 80.7 %.

Table 2 Five-year second IBTR-free survival rates according to various clinicopathological factors

Characteristics	5-year second IBTR-free survival (%)	*p* value
Age at initial diagnosis		
<40	71.4	0.1516
≥40	83.8	
Age at IBTR		
<40	59.6	0.0247
≥40	83.6	
Family history		
No	81.5	0.7406
Yes	83.9	
RT		
After initial surgery	78.0	0.0003
After salvage surgery[a]	93.5	
No RT	52.7	
Time interval from initial surgery to IBTR (years)		
≤2	68.2	0.0333
>2	84.0	
Tumor location		
Same quadrant	83.6	0.3807
Different quadrant	73.8	
Tumor size of IBTR (mm)		
≤20	82.6	0.7761
>20	80.0	
Lymphovascular invasion of IBTR		
No	84.2	0.3962
Yes	76.2	
Histologic grade of IBTR		
1	78.8	0.9602
2	85.3	
3	81.3	
Margin of IBTR		
Negative	81.7	0.0598
Positive	60.0	
ER of IBTR		
Negative	69.9	0.0268
Positive	86.0	
HER2 of IBTR		
Negative	80.2	0.4405
Positive	73.9	
Ki-67 index of IBTR		
<20	79.7	0.7725
≥20	79.6	
Breast cancer subtype of IBTR		
Luminal-A	81.9	0.1456
Luminal-B	87.7	
Triple-negative	65.6	
HER2	73.7	
Hormone therapy after salvage surgery		
No	67.8	0.0022
Yes	87.5	
Chemotherapy after salvage surgery		
No	83.9	0.0347
Yes	66.3	
Trastuzumab after salvage surgery[b]		
No	76.7	0.4940
Yes	66.7	

IBTR ipsilateral breast tumor recurrence, *RT* radiotherapy, *ER* estrogen receptor

[a] Including one patient who received RT after both initial and IBTR surgery

[b] Including only patients with HER2-positive tumors at IBTR

Patient characteristics according to RT are shown in Table 1. There were significant differences in the tumor size of IBTR and use of trastuzumab after salvage surgery according to RT ($p = 0.0128$ and 0.0450, respectively, Chi-square test).

The 5-year second IBTR-free survival rates according to the various clinicopathological parameters are shown in Table 2. There was a significant difference in the second IBTR-free survival rate according to RT ($p = 0.0003$, log-rank test). The 5-year second IBTR-free survival rates for patients who received RT after the initial surgery, RT after salvage surgery, and no RT were 78.0, 93.5, and 52.7 %, respectively (Fig. 2). Multivariate analysis including the age at IBTR, RT, time interval from initial surgery to IBTR, margin of IBTR, ER status of IBTR, hormone therapy after salvage surgery, and chemotherapy after salvage surgery showed that age at IBTR, RT, margin of IBTR, and hormone therapy after salvage surgery were significantly independent predictive factors of second IBTR-free survival ($p = 0.0026$, Table 3). Furthermore, to adjust the differences in patient characteristics between 3 groups according to RT, we added the tumor size of IBTR to this multivariate analysis, and significance persisted ($p = 0.0070$). Because all patients with HER2-positive tumors who received RT after salvage surgery or no RT did not receive trastuzumab after salvage surgery, odds calculation of the use of trastuzumab after salvage surgery could be unstable. Therefore, we could not add the use of trastuzumab after salvage surgery to this multivariate analysis.

We also analyzed the period from the date of initial surgery to the date of appearance of second IBTR according to RT. There was also a significant difference according to RT ($p = 0.0079$, log-rank test).

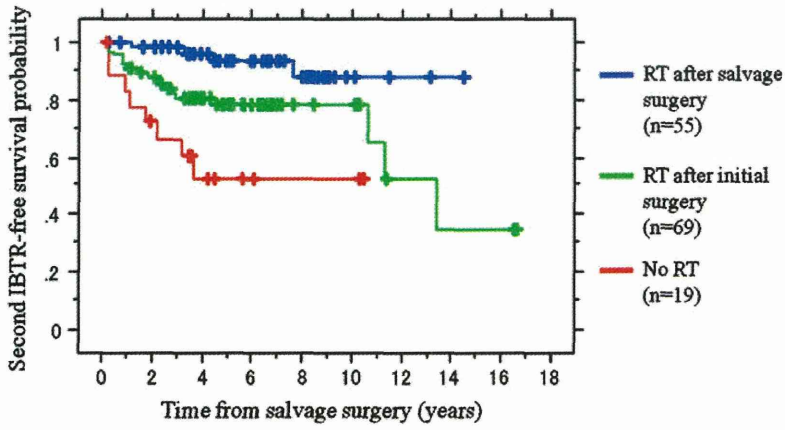

Fig. 2 Second IBTR-free survival rates of breast cancer patients according to RT

Table 3 Multivariate analyses of predictors for second IBTR after repeat lumpectomy

Characteristics	Variables	Hazard ratio	95 % CI	*p* value
Age at IBTR	≥40 vs. <40	3.788	1.374–10.417	0.0101
RT				0.0026
	After salvage surgery vs. after initial surgery	5.193	1.430–18.857	0.0123
	After salvage surgery vs. no RT	12.409	2.959–52.035	0.0006
Time interval from initial surgery to IBTR	>2 vs. ≤2 years	1.660	0.580–4.746	0.3446
Margin of IBTR	Negative vs. positive	3.984	1.229–12.821	0.0212
ER of IBTR	Positive vs. negative	1.326	0.424–4.149	0.6276
Hormone therapy after salvage surgery	Yes vs. no	3.479	1.147–10.546	0.0276
Chemotherapy after salvage surgery	No vs. yes	1.222	0.411–3.637	0.7186

IBTR ipsilateral breast tumor recurrence, *RT* radiotherapy, *ER* estrogen receptor, *CI* confidence interval

The 5-year DDFS rates after IBTR were 78.5 %. There were no differences in DDFS after IBTR according to RT ($p = 0.6241$, log-rank test). Five-year DDFS rates for patients who received RT after the initial surgery, RT after salvage surgery, and no RT were 76.4, 77.9, and 88.8 %, respectively.

Discussion

Our analyses revealed that the omission of RT after repeat lumpectomy was an independent risk factor of second IBTR after repeat lumpectomy. To date, little information exists regarding the impact of RT on the risk of second IBTR after repeat lumpectomy. One report from a single institute [7] showed no association of RT with second IBTR rates after repeat lumpectomy. The different findings may result from a small sample size ($n = 78$). Our results suggested that the omission of RT after repeat lumpectomy resulted in unacceptably high second IBTR rates in cases of RT absence after the initial breast-conserving surgery. Therefore, caution is needed when RT is omitted in patients who have undergone repeat lumpectomy. Although 5-year second IBTR-free survival for patients treated with RT after the initial breast-conserving surgery (78.0 %) was inferior to that for RT after salvage surgery (93.5 %) and unacceptable, RT after the initial breast-conserving surgery might also suppress the second IBTR, because it achieved apparently better outcomes than no RT (52.7 %). It is speculated that RT after initial breast-conserving surgery eradicated subclinical diseases left behind, at least to some degree.

One could assume that there were no differences in the periods from the date of initial surgery to the date of appearance of second IBTR according to RT because the time interval from initial surgery to IBTR might be shorter in patients who did not receive RT after initial surgery than in those who did. However, in this study, there was no difference in the time interval from initial surgery to IBTR according to RT. Furthermore, there was also a significant

difference in the period from the date of initial surgery to the date of appearance of second IBTR according to RT. Therefore, the assumption that there were no differences in the periods from the date of initial surgery to the date of appearance of second IBTR according to RT is not correct.

In this study, second-IBTR rate after repeat lumpectomy plus RT was acceptably low. However, our results do not indicate that RT after initial breast-conserving surgery can be omitted because RT after initial breast-conserving surgery not only substantially reduces the risk of recurrence but also moderately reduces the risk of death from breast cancer [11].

In our study, the age at IBTR, margin of IBTR, and hormone therapy after salvage surgery were also significantly independent predictive factors of second IBTR-free survival. Kurtz et al. [2] reported their experiences involving 50 patients who underwent repeat lumpectomy after IBTR, and reported that late recurrence with a negative surgical resection margin predicted more favorable local control after IBTR. This result was compatible with ours. Age and hormone therapy are both well-known risk factors of IBTR after initial breast-conserving surgery.

Recently, the breast cancer subtype has become known to be useful in estimating the risk of not only distant [12, 13] but also locoregional recurrences [14–16]. Our previous analysis suggested that the breast cancer subtype, as approximated by ER, HER2, and Ki-67 of IBTR, was associated with distant recurrence in patients with IBTR, which was reported elsewhere [17]. However, no association of the breast cancer subtype with second IBTR was observed in this study. To our knowledge, there has been no report regarding the role of the breast cancer subtype in second IBTR after repeat lumpectomy.

In conclusion, the omission of RT after repeat lumpectomy was an independent risk factor of second IBTR after repeat lumpectomy. Caution is needed when RT is omitted in patients who have undergone repeat lumpectomy. In contrast, repeat lumpectomy plus RT is a reasonable option in patients who did not undergo RT at the initial surgery.

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Luminal membrane expression of mesothelin is a prominent poor prognostic factor for gastric cancer

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BACKGROUND: Mesothelin is expressed in various types of malignant tumour, and we recently reported that expression of mesothelin was related to an unfavourable patient outcome in pancreatic ductal adenocarcinoma. In this study, we examined the clinicopathological significance of the mesothelin expression in gastric cancer, especially in terms of its association with the staining pattern.

METHODS: Tissue specimens from 110 gastric cancer patients were immunohistochemically examined. The staining proportion and intensity of mesothelin expression in tumour cells were analysed, and the localisation of mesothelin was classified into luminal membrane and/or cytoplasmic expression.

RESULTS: Mesothelin was positive in 49 cases, and the incidence of mesothelin expression was correlated with lymph-node metastasis. Furthermore, luminal membrane staining of mesothelin was identified in 16 cases, and the incidence of luminal membrane expression was also correlated with pT factor, pStage, lymphatic permeation, blood vessel permeation, recurrence, and poor patient outcome. Multivariate analysis showed that luminal membrane expression of mesothelin was an independent predictor of overall patient survival.

CONCLUSION: We described that the luminal membrane expression of mesothelin was a reliable prognostic factor in gastric cancer, suggesting the functional significance of membrane-localised mesothelin in the aggressive behaviour of gastric cancer cells.
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Mesothelin is a 40-kDa cell surface glycoprotein and is expressed on normal mesothelial cells lining the pleura, pericardium, and peritoneum (Chang *et al*, 1992; Chang and Pastan, 1996). Moreover, mesothelin is overexpressed in various types of malignant tumour, including malignant mesothelioma, ovarian cancer, and pancreatic cancer (Argani *et al*, 2001; Ordonez, 2003a, b; Hassan *et al*, 2005a; Einama *et al*, 2011). The full length of human *mesothelin* gene codes the primary product being a 71-kDa precursor protein. It can be physiologically cleaved by some furin-like proteases into a 40-kDa C-terminal fragment that remains membrane bound, and a 31-kDa N-terminal fragment, which is secreted into the blood (Chang and Pastan, 1996). The C-terminal 40-kDa fragment is named mesothelin and is attached to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor (Chang and Pastan, 1996; Hassan *et al*, 2004).

The biological functions of mesothelin are not clearly understood, although recent studies have suggested that overexpression of mesothelin increases cell proliferation and migration (Li *et al*, 2008). In ovarian cancers, diffuse mesothelin staining correlated significantly with prolonged survival in patients who had advanced-stage disease (Yen *et al*, 2006), and another report indicated that a higher mesothelin expression is associated with chemoresistance and shorter patient survival (Cheng *et al*, 2009). In pancreatic cancer, mesothelin expression was immunohistochemically observed in all cases, while its absence was noted in non-cancerous pancreatic ductal epithelium, with or without pancreatitis (Argani *et al*, 2001; Swierczynski *et al*, 2004; Hassan *et al*, 2005b; Einama *et al*, 2011). Furthermore, we recently explored that the expression of mesothelin was related to an unfavourable patient outcome in pancreatic ductal adenocarcinoma. However, in gastric cancer, which is one of the representative gastrointestinal cancers, mesothelin expression seems to correlate with prolonged patient survival (Baba *et al*, 2011); this is a paradoxical result for the other types of carcinomas. In this study, we investigated the immunohistochemical analysis of mesothelin in 110 primary gastric cancers, especially focussing in the localisation of mesothelin, that is, luminal membrane and/or cytoplasm, and its clinicopathological significance associated with patient's outcome.

PATIENTS AND METHODS

Patients' demography and tumour specimens

This study was performed with the approval of the Internal Review Board on ethical issues of Hokkaido University Hospital, Sapporo,

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Japan. The subjects of this study were 110 patients who underwent radical surgery for primary gastric cancer between 2002 and 2004 at the Department of General Surgery, Hokkaido University, Graduate School of Medicine, Sapporo, Japan. The clinicopathological characteristics of these cases are summarised in Supplementary Table 1.

Mean patient age was 62.1 years (±2.4 standard deviation (s.d.)). Seventy patients (63.6%) were men, and the remaining 40 (36.4%) were women. The location of the tumour was the upper third of the stomach in 38 (34.5%) patients and the middle and lower third in 72 (65.5%). Tumour stages comprising T factor, N factor, M factor, clinical stage were assigned according to the TNM classification of the Union Internationale Contre le Cancer (Sobin and Wittekind, 2002). Lymphatic permeation and blood vessel invasion were evaluated as either positive or negative. The median survival time of the patients was 54.8 months (±5.2 s.d.).

Formalin-fixed paraffin-embedded tissue blocks were prepared from patient's tumour specimens, and sections were cut and stained with haematoxylin and eosin (HE) for routine histopathological examination. All specimens were diagnosed as gastric adenocarcinomas, and lymphatic permeation and blood vessel invasion were evaluated using Elastica van Gieson staining and immunostaining with anti-podoplanin (D2-40) antibody, if necessary, as a routine operation for pathological diagnosis. A representative tissue block including metastatic lymph node was selected from each case to perform immunohistochemical studies.

Immunohistochemistry

Four-micrometre-thick sections were mounted on charged glass slides, deparaffinised, and rehydrated through a graded ethanol series. For antigen retrieval, Dako Target Retrieval Solution pH 9.0 (Catalogue number S2368) was used, and the slides were boiled in a pressure cooker (Pascal Pressure Cooker, Model: S2800; DAKO JAPAN, Tokyo, Japan) to a temperature of 125 °C for 3 min. Endogeneous peroxidase was blocked with 0.3% hydrogen peroxidase. The slides were incubated with a 1 : 50 dilution of a mouse monoclonal antibody to mesothelin (clone 5B2 diluted 1 : 50; Novocastra, Newcastle Upon Tyne, UK) at room temperature for 30 min and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako) for 30 min at room temperature. Specific antigen–antibody reactions were visualised with 0.2% diaminobenzine tetrahydrochloride and hydrogen peroxide. Slides were counterstained with haematoxylin for 10 min, then rinsed gently in reagent quality water.

Immunohistochemical evaluation

All assessments were made on the tumour region of the specimen (×400). Each slide was evaluated independently by two pathologists (TE, KT) who did not know the clinical outcomes.

Immunostaining for mesothelin was evaluated for both the proportion and staining intensity of tumour cells in each case. The proportion of mesothelin expression was assessed according to the percentage of mesothelin-positive cells as follows: +1, 1–10%; +2, 10–50%; and +3, >50%. The staining intensity of mesothelin was evaluated as weak (+1), moderate to strong (+2) in addition to the staining localisation in the luminal membrane or in cytoplasm. The final evaluation of mesothelin expression was assessed using the following scoring system according to the previous study for the pancreas cancer (Einama *et al*, 2011): 'mesothelin positive' was defined as greater than or equal to +4 of proportion score and/or +2 of intensity score, while 'mesothelin negative' was given when the total score was less than +3 except in the cases of proportion score +1 and intensity score +2 (Supplementary Figure 1).

Furthermore, among the 'mesothelin-positive' cases, the staining localisation of mesothelin was evaluated as luminal membrane and/or cytoplasm. In cases in which the entire circumference of the luminal membrane was explicitly stained even in partial throughout the section, 'luminal membrane positive' was given. When the luminal membrane was stained discontinuously and/or faintly, or in cases in which no membrane staining and only cytoplasmic staining was observed in any intensity level throughout the section, 'luminal membrane negative' was given (Figure 1; Supplementary Figure 1). Meanwhile, the mesothelin cytoplasmic expression was

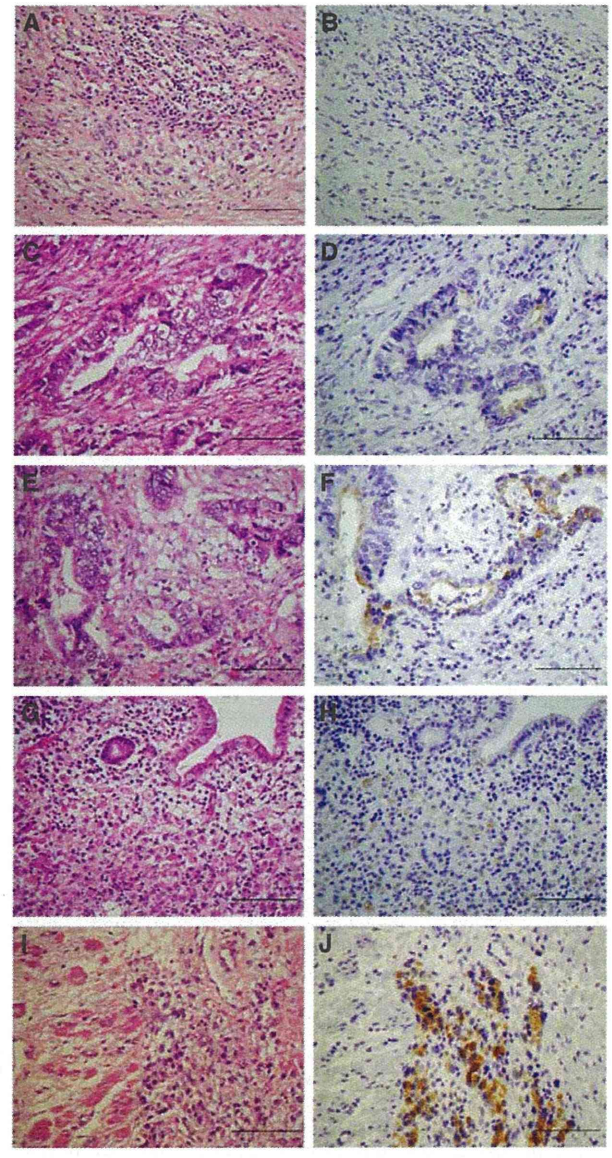

Figure 1 The expression variations of mesothelin and its cellular localisation in gastric cancer. (**A**, **C**, **E**, **G**, and **I**) HE stain. (**B**, **D**, **F**, **H**, and **J**) Immunohistochemical stain for mesothelin. (**A** and **B**) A case of 'mesothelin negative'. (**C** and **D**) A case of 'luminal membrane negative', although there was incomplete membrane staining in the cancer cells. (**E** and **F**) A case of 'luminal membrane positive'. The entire circumference staining of the cell membrane was stained. (**G** and **H**) A case of 'cytoplasmic positive' that represented the scant cytoplasmic staining of mesothelin. (**I** and **J**) A case of 'cytoplasmic positive' with granular staining in cancer cells. Scale bars: 100 μm.

evaluated as follows: in a case in which the cytoplasmic staining was clearly observed in the constituent cancer cells, including the cytoplasmic granular staining, we judged it as 'cytoplasmic positive' (Figure 1).

Statistical analysis

We used χ^2 test or Fisher's exact test to determine the correlation between mesothelin and clinicopathological data. Survival curves of patients were drawn by the Kaplan–Meier method. Differences in survival curves were analysed by the log-rank test. Prognostic implications of mesothelin expression and clinicopathological parameters were analysed by Cox univariate and multivariate proportional hazards models. All differences were considered significant at a *P*-value of <0.05. All statistical analyses were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Clinicopathological analysis for mesothelin expression

In the 110 gastric cancers, mesothelin expression was detected in 49 cases (44.5%), and the luminal membrane expression of

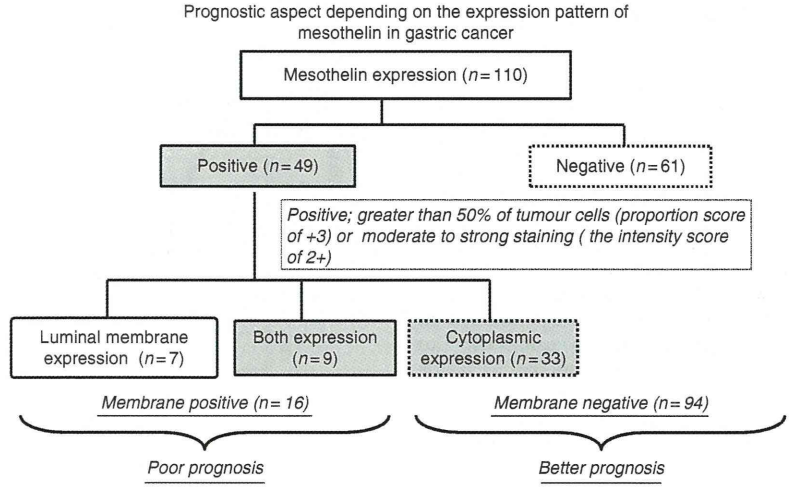

Figure 2 Flow chart of evaluation of mesothelin expression.

Table 1 Association between expression pattern of mesothelin and clinicopathological parameters

		Mesothelin expression			Luminal membrane expression			Cytoplasmic expression		
Parameter	**Total**	**Positive (*n*=49)**	**Negative (*n*=61)**	***P*-value**	**Positive (*n*=16)**	**Negative (*n*=94)**	***P*-value**	**Positive (*n*=42)**	**Negative (*n*=68)**	***P*-value**
1. *Histological classification*										
por2-sig	62	25	37	>0.99	8	54	0.60	22	40	0.56
Others	48	24	24		8	40		20	28	
2. *pT factor*										
pT1	62	23	39	0.085	3	59	0.0019	21	41	0.33
pT2–4	48	26	22		13	35		21	27	
3. *pN factor*										
Positive	37	22	15	0.028	11	26	0.0029	17	20	0.30
Negative	73	27	46		5	68		25	48	
4. *pStage*										
I, II	80	34	46	0.52	5	75	0.0002	35	48	0.66
III, IV	30	15	15		11	19		10	20	
5. *Lymphatic permeation*										
Positive	48	25	23	0.18	13	35	0.0019	20	28	0.56
Negative	62	24	38		3	59		22	40	
6. *Blood vessel permeation*										
Positive	41	21	20	0.32	11	30	0.0098	16	25	>0.99
Negative	69	28	41		5	64		26	43	
7. *Recurrence*										
Yes	26	14	12	0.37	11	15	<0.0001	9	17	0.82
No	84	35	49		5	79		33	51	