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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

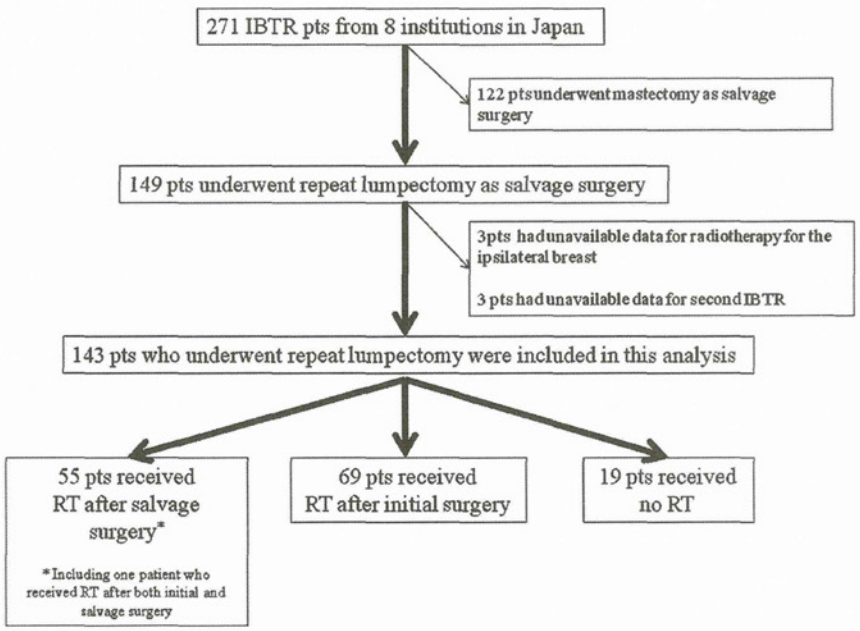

Fig. 1 Patient flowchart

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).

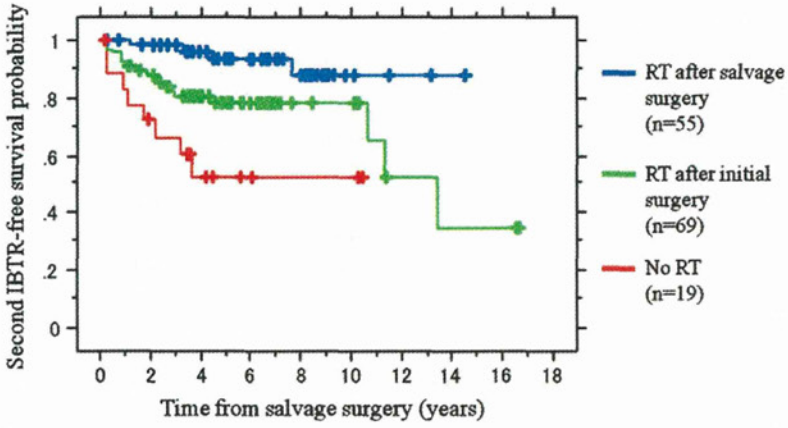

Fig. 2 Second IBTR-free survival rates of breast cancer patients according to RT

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

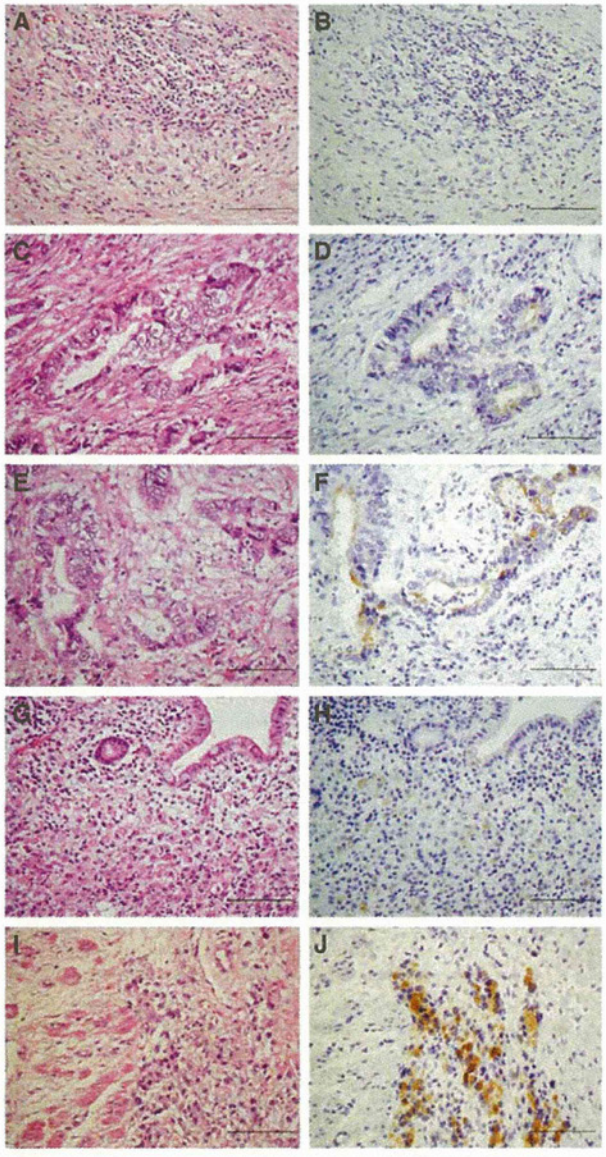

Figure 1 The expression variations of mesothelin and its cellular localisation in gastric cancer. (**A**, **C**, **E**, **G**, and **I**) HE stain. (**B**, **D**, **F**, **H**, and **J**) Immunohistochemical stain for mesothelin. (**A** and **B**) A case of 'mesothelin negative'. (**C** and **D**) A 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

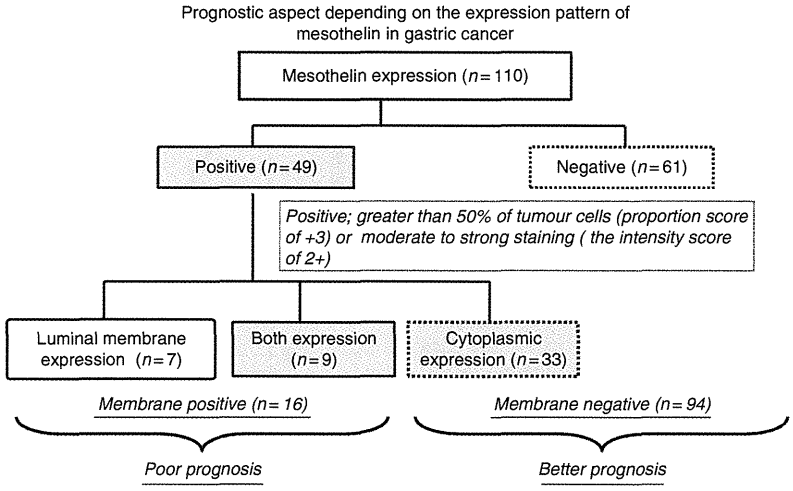

Figure 2 Flow chart of evaluation of mesothelin expression.

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	

mesothelin was observed in 16 cases, while the cytoplasmic expression was detected in 42 tumours, which included the 9 cases of 'positive for both luminal membrane and cytoplasm' (Figure 2). The detailed clinicopathological information of 16 cases with mesothelin luminal membrane expression was summarised in Supplementary Table 2. We never detected the mesothelin expression in the non-cancerous lesions (data not shown). The statistical analysis revealed that the incidence of mesothelin expression was only correlated with lymph-node metastasis ($P=0.028$), while the incidence of luminal membrane expression of mesothelin was correlated with pT factor ($P=0.0019$), lymph-node metastasis ($P=0.0029$), clinical stage ($P=0.0002$), lymphatic permeation ($P=0.0019$), blood vessel invasion ($P=0.0098$), and recurrence ($P<0.0001$). There were no significant correlations between mesothelin cytoplasmic expression and clinicopathological parameters (Table 1).

Survival analysis associated with mesothelin expression

The analysis for patients' overall survival denoted that the group of 'luminal membrane positive' for mesothelin indicated a significantly unfavourable outcome compared with the group of 'luminal membrane negative' ($P<0.001$). On the other hand, the pure mesothelin expression regardless of the localisation, and also 'cytoplasmic expression' were not correlated with the overall survival of the patients (Figure 3). To confirm the mesothelin expression as an independent prognostic factor, we performed the univariate analysis of the 110 gastric cancers using the Cox proportional hazards model, and obtained the result that pT factor, pN factor, clinical stage, lymphatic permeation, blood vessel invasion, and mesothelin luminal membrane expression were significantly correlated with the risk of cancer death (Table 2). Furthermore, to exclude the possible interference of any other factors, the multivariate analysis was performed including pT factor, pN factor, clinical stage, lymphatic permeation, blood vessel invasion, and mesothelin luminal membrane expression. Interestingly, the luminal membrane expression of mesothelin was an independent predictor of overall survival for gastric cancer patients as well as clinical stage and lymphatic permeation (Table 3).

Mesothelin expression in metastatic lymph nodes

As shown above, luminal membrane expression of mesothelin was correlated with lymphatic permeation and lymph-node metastasis; thus, we analysed the expression pattern of mesothelin in 35 out of 37 cases of lymph-node metastasis by immunohistochemistry, in which the tissue blocks of metastatic lymph node were available (Supplementary Figure 2). Interestingly, the incidence of luminal membrane positive including expression in both membrane and cytoplasm was increased in metastatic lymph nodes (51.4%; 18 out of 35) compared with primary lesions (31.4%; 11 out of 35). Moreover, in 4 cases out of 14 mesothelin-negative cases in primary lesion, luminal membrane expression of mesothelin was observed. These results support our idea that luminal membrane expression of mesothelin is associated with the malignant behaviour of tumour cells.

DISCUSSION

In this study, we demonstrated that the luminal membrane expression of mesothelin in gastric cancer was associated with unfavourable clinical outcome in patients after surgery. The univariate analysis indicated that the luminal membrane expression of mesothelin was also correlated with lymph-node metastasis, clinical stage, lymphatic permeation, blood vessel invasion, residual tumour, and recurrence, although a luminal membrane expression of mesothelin remained a statistically independent factor for favourable patient outcome after the multivariate analysis. Our result that total mesothelin expression including the case of exclusive cytoplasmic expression did not correlate with patients' prognosis will explain the discrepant previous report in which mesothelin expression correlates with prolonged patient survival in gastric cancer (Baba *et al*, 2011). We therefore emphasise that membrane-localised mesothelin might have an important role in the development of gastric cancer.

The full length of human *mesothelin* gene codes the primary product being a 71-kDa precursor protein. It can be

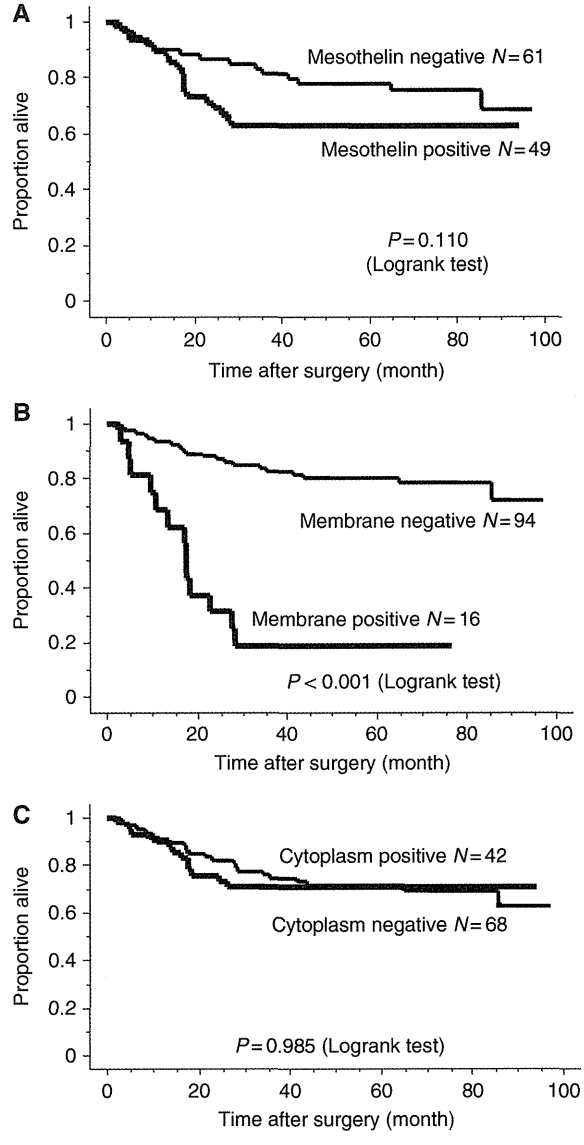

Figure 3 Overall survival for patients with gastric cancer after surgical therapy stratified by the status of mesothelin expression (**A**), mesothelin luminal membrane expression (**B**), and mesothelin cytoplasmic expression (**C**), respectively. The group of 'luminal membrane positive' represented a statistically significantly unfavourable outcome compared with the group of 'luminal membrane negative' (**B**: $P<0.001$). On the other hand, both total expression (**A**) and cytoplasmic expression of mesothelin (**C**) were not correlated with overall survival of the patients.

Table 2 Univariate analysis for clinicopathological parameters and mesothelin expression on overall survival of patients with gastric carcinoma

Factor	N	P	RR (95% CI)
1. *Histological classification*			
por2-sig	62	0.89	1
Others	48		0.954 (0.478–1.903)
2. *pT factor*			
pT1	62	<0.0001	1
pT2-4	48		13.354 (4.679–38.113)
3. *pN factor*			
Positive	73	<0.0001	1
Negative	37		9.301 (4.147–20.860)
4. *pStage*			
I, II	80	<0.0001	1
III, IV	30		18.837 (8.032–44.179)
5. *Lymphatic permeation*			
Positive	62	<0.0001	1
Negative	48		18.529 (5.637–60.534)
6. *Blood vessel permeation*			
Positive	69	<0.0001	1
Negative	41		11.493 (4.722–27.971)
7. *Mesothelin expression*			
No	61	<0.0001	1
Yes	49		1.749 (0.874–3.500)
8. *Luminal membrane expression*			
No	94	<0.0001	1
Yes	16		7.205 (3.489–14.877)
9. *Cytoplasmic expression*			
No	68	0.98	1
Yes	42		1.007 (0.493–2.055)

Abbreviation: CI = confidence interval. RR indicates relative risk/hazard ratio.

Table 3 Multivariate analysis for clinicopathological parameters and mesothelin expression on overall survival of patients with gastric carcinoma

Factor	P	RR (95% CI)
1. *pT factor*		
pT1 *vs* pT2–4	0.35	2.497 (0.374–16.660)
2. *pN factor*		
Positive *vs* Negative	0.060	3.532 (0.946–13.181)
3. *pStage*		
I, II *vs* III, IV	0.0003	12.336 (2.533–60.069)
4. *Lymphatic permeation*		
Positive *vs* Negative	0.0043	11.996 (2.180–65.996)
5. *Blood vessel permeation*		
Positive *vs* Negative	0.29	2.091 (0.533–8.195)
6. *Luminal membrane expression*		
No *vs* Yes	0.0073	2.969 (1.341–6.573)

Abbreviation: CI = confidence interval. RR indicates relative risk/hazard ratio.

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 referred to as mesothelin, which is attached to the cell membrane by a GPI anchor (Chang and Pastan, 1996; Hassan *et al*, 2004). The 5B2 anti-mesothelin antibody (Novocastra Laboratory Vision BioSystems, Boston, MA, USA), which we employed here for IHC, can detect the 71-kDa precursor protein and also the 40-kDa C-terminal fragment (Inami *et al*, 2008); therefore, we could not decide which form of mesothelin has a pivotal role in malignant behaviour of gastric cancer cells. Recent studies reported that mesothelin is not only associated with increased cell proliferation and with the migration of pancreatic cancer cells *in vitro* (Bharadwaj *et al*, 2008; Li *et al*, 2008), but also contributes to tumour progression *in vivo* (Li *et al*, 2008). Mesothelin inhibits paclitaxel-induced apoptosis through concomitant activation of phosphoinositide-3-kinase (PI3K) signalling in the regulation of Bcl-2 family expression (Chang *et al*, 2009), and induces the activation of signal transducer and activator of transcription (Stat) 3, which leads to increased expression of cyclin E and makes pancreatic cancer cells proliferate faster (Bharadwaj *et al*, 2008). In addition, mesothelin-activated nuclear factor-kappaB (NF-κB) induces elevated interleukin (IL)-6 expression, which acts as a growth factor to support pancreatic cancer cell survival/proliferation through a novel auto/paracrine IL-6/soluble IL-6R trans-signalling (Bharadwaj *et al*, 2011a, b). Our study provided a new aspect that luminal membrane expression of mesothelin is associated with the malignant behaviour of tumour cells, such as depth of tumour invasion and vascular invasion, although it remains necessary to clarify the biological function of the 71-kDa mesothelin precursor and/or 40-kDa mesothelin protein in *in-vitro* and *in-vivo* studies, including the processing system by furin-like proteases.

In terms of discovering the clinicopathological parameters for gastric cancer, there are many previous studies demonstrating the prognostic significance of various molecules, such as epidermal growth factor receptor and c-erB-2 (HER-2). These molecules also could be of unique significance as the indicators of eligibility to specific molecular targeting therapies, because most of them are located in the cell membrane as the useful targets for the molecular targeted drugs such as antibody drugs. We believe that the immunohistochemical evaluation for luminal membrane expression of mesothelin in gastric cancer would be of clinical benefit not only as a prognostic factor but also as a predictive factor for the eligibility to mesothelin-targeting therapies in the future (Hassan *et al*, 2004, 2007a, b, c, 2010; Hassan and Ho, 2008; Li *et al*, 2008; Inami *et al*, 2009).

In conclusion, we demonstrated the clinicopathological significance of the luminal membrane expression of mesothelin in gastric cancer as an independent prognostic factor, although additional studies to increase the number of the cases for luminal membrane expression ($n = 16$) might be required for further confirmation. The immunohistochemical examination of mesothelin expression in surgically resected tumour specimens should be clinically useful for prognostication and for decision making about further treatment procedures after surgical therapy in patients with gastric cancer.

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ORIGINAL ARTICLE

A rare point mutation in the Ras oncogene in hepatocellular carcinoma

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Abstract
Purpose The Ras gene is one of the oncogenes most frequently detected in human cancers, and codes for three proteins (K-, N-, and H-Ras). The aim of this study was to examine the mutations in codons 12, 13 and 61 of the three Ras genes in cases of human hepatocellular carcinoma (HCC).
Methods Paired samples of HCC and corresponding non-malignant liver tissue were collected from 61 patients who underwent hepatectomy. A dot-blot analysis was used to analyze the products of the polymerase chain reaction (PCR) amplification of codons 12, 13, and 61 of K-, N- and H-Ras for mutations.
Results Only one mutation (K-Ras codon 13; Gly to Asp) was detected among the 61 patients. Interestingly, this patient had a medical history of surgery for both gastric cancer and right lung cancer. No mutations were found in codons 12 and 61 of K-Ras or codons 12, 13 and 61 of the N-Ras and H-Ras genes in any of the HCCs or corresponding non-malignant tissues.
Conclusions These findings indicated that the activation of Ras proto-oncogenes by mutations in codons 12, 13, and 61 does not play a major role in hepatocellular carcinogenesis.

Keywords Ras · Mutation · Hepatocellular carcinoma · Sorafenib

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Abbreviations

Asp	Asparagine
Glu	Glutamate
Gly	Glycine
HCC	Hepatocellular carcinoma
Lys	Lysine
PCR	Polymerase chain reaction
TTP	Time to progression
Val	Valine

Introduction

Hepatocellular carcinoma (HCC) is a global health problem, accounting for more than 80 % of all primary liver cancers, and is one of the most common malignancies worldwide [1]. Most patients with HCC also present with concomitant cirrhosis, which is the major clinical risk factor for hepatic cancer, and results from alcoholism or infection with the hepatitis B or hepatitis C virus. Primary liver malignancies (95 % of which are HCC) are the third and fifth leading causes of cancer death among males and females, respectively, in Japan [2]. Both liver resection and liver transplantation are potentially curative treatments for HCC [3–5]. Although other treatment options, including percutaneous radiofrequency ablation or chemolipiodolization are also available, there is no standard systemic therapy for advanced cases.

Sorafenib (BAY 43-9006, Nexavar) is a novel oral kinase inhibitor that targets multiple tyrosine kinases in vivo and in vitro, and is widely used for HCC [6]. The main targets of sorafenib are the receptor tyrosine kinase pathways which are frequently deregulated in cancer, such as the Ras pathway. The Ras pathway represents a dominant signaling network promoting cell proliferation and

survival. The binding of different growth factors (e.g. epidermal growth factor: EGF) to their receptors (e.g. epidermal growth factor receptor: EGFR) induces the activation of Ras, which in turn activates c-raf, MEK and ERK. Phosphorylated ERK in the nucleus activates transcription factors that regulate the expression of genes involved in cell proliferation and survival.

A phase II trial involving 137 patients with advanced HCC showed that sorafenib induced partial responses in less than 5 % of patients, but the observed median survival of 9.2 months with a median time to progression of 5.5 months was classified as evidence of potential clinical benefit, since the expected median survival of these patients is 6 months [7]. Consequently, a large phase III clinical trial (SHARP) was conducted in 602 patients with advanced HCC. The results showed a 31 % decrease in the risk of death, with a median survival of 10.6 months in the sorafenib arm versus 7.9 months for placebo [8]. In addition, sorafenib showed a significant benefit in terms of the time to progression (TTP) as assessed by independent radiological review, with a median TTP of 5.5 months for the sorafenib and 2.8 months for the placebo arm.

Because Ras is one of the targets of sorafenib, it is important to determine whether mutations in the Ras gene result in the activation of the Ras/MAPK pathway in human HCCs. However, the relationship between Ras mutations and human HCC has not been fully evaluated. The present study was designed to investigate K-, N- and H-Ras (*KRAS, NRAS, HRAS*) somatic mutations in human HCC.

Materials and methods

Patients and tumor samples

Tumor tissue samples were obtained from 61 Japanese patients who underwent surgical resection for HCC during the period between December 1989 and April 1992 in the Department of Surgery and Science, Kyushu University Hospital, Fukuoka, Japan. Surgically resected tissue samples were frozen at −80 °C immediately after resection and were stored until use in this study. Written informed consent was obtained from all patients examined, and the current study was approved by the Kyushu University ethics committee.

DNA preparation and detection of Ras point mutations

High molecular weight DNA was isolated from frozen tumor samples, as described elsewhere [9]. Selective amplification of the Ras gene sequence was done using a PCR technique. The nucleotide sequences of the primers used are listed in Table 1. The PCR was performed at 96 °C to denature the DNA (1 min), at 55 °C (*NRAS*), 57 °C (*KRAS*), 62 °C (*HRAS*) to anneal the primer (30 s), and at 72 °C to synthesize DNA (10 s to 1 min) using Taq DNA polymerase for 35–40 cycles in a DNA thermal cycler (Perkin-Elmer-Cetus). Amplified DNA samples were spotted onto nylon membranes (Hybond N+) for the hybridization analysis. All of the DNA isolated from the 61 tumor samples and the corresponding non-malignant liver tissues were screened for activated point mutations in codons 12, 13, and 61 of all three Ras genes using an oligonucleotide specific for the different sequences. The filters were prehybridized for 1 h at 55 °C in solution A (3.0 M tetramethylammonium chloride, 50 mM Tris-HCI, 2 HIMEDTA, 0.1 % SDS, 5× Denhardt's solution, 100 fg/ml denatured herring sperm DNA), and hybridized for 1 h at 55 °C in the same solution with 5 pmol ^{32}P-labeled probe. These filters were washed twice in 0.3 M NaCl, 0.02 M NaH2PO4, 2 mM EDTA and 0.1 % SDS at room temperature for 5 min, and in solution A without Denhardt's solution and herring sperm DNA, once for 5 min at room temperature and twice for 10 min at 60 °C. These filters were then exposed to Kodak XAR5 film. Human cancer cell lines carrying Ras genes mutations were used as positive controls. The colon cancer cell lines: SW620 (*KRAS* codon 12 GTT:Val), LSI80 (*KRAS* codon 12 GAT:Asp), and LOVO (*KRAS* codon 13 GAC:Asp) were obtained from the Japanese Cancer Research Resources Bank, and KMS4 (*KRAS*s codon 12 TGT:Cys) was provided by Dr. Sugio (Institution?).

Table 1 Ras gene primers used in this study

Gene/codon	Length (bp)	Sequence	
KRAS/12, 13	108	Forward	GACTGAATATAAACTTGTGG
		Reverse	CTATTGTTGGATCATATTCG
KRAS/61	128	Forward	TTCCTACAGGAAGCAAGTAG
		Reverse	CACAAAGAAAGCCCTCCCCA
HRAS/12, 13	63	Forward	GACGGAATATAAGCTGGTGG
		Reverse	TGGATGGTCAGCGCACTCTT
HRAS/61	73	Forward	AGACGTGCCTGTTGGACATC
		Reverse	CGCATGTACTGGTCCCGCAT
NRAS/12, 13	109	Forward	GACTGAGTACAAACTGGTGG
		Reverse	CTCTATGGTGGGATCATATT
NRAS/61	103	Forward	GGTGAAACCTGTTTGTTGGA
		Reverse	ATACACAGAGGAAGCCTTCG

bp base pairs

Results

The age of the 61 patients ranged from 43 to 79 years (average, 64.1 years), and 46 were males and 15 were

females. The positive rate of hepatitis surface B antigen was 12.9 %, and the positive rate of anti-hepatitis C virus antibody was 72.7 %. The mean tumor size was 4.47 cm.

One of the 61 HCCs (1.6 %) carried a point mutation, which was a G to A transition at codon 13 of the *KRAS* gene (Fig. 1). DNA extracted from the corresponding non-malignant liver tissue had the normal codon, suggesting that mutational activation of K-ras was involved in the malignant transformation in this case. This patient was positive for anti-hepatitis C virus antibodies, and was classified to have Child-Pugh A disease. The diameter of this patient's tumor was 12 cm, and the tumor was composed of well to moderately differentiated hepatocellular carcinoma. Interestingly, this patient had undergone surgery for gastric cancer 18 years before and lung cancer 12 years before the surgery for HCC.

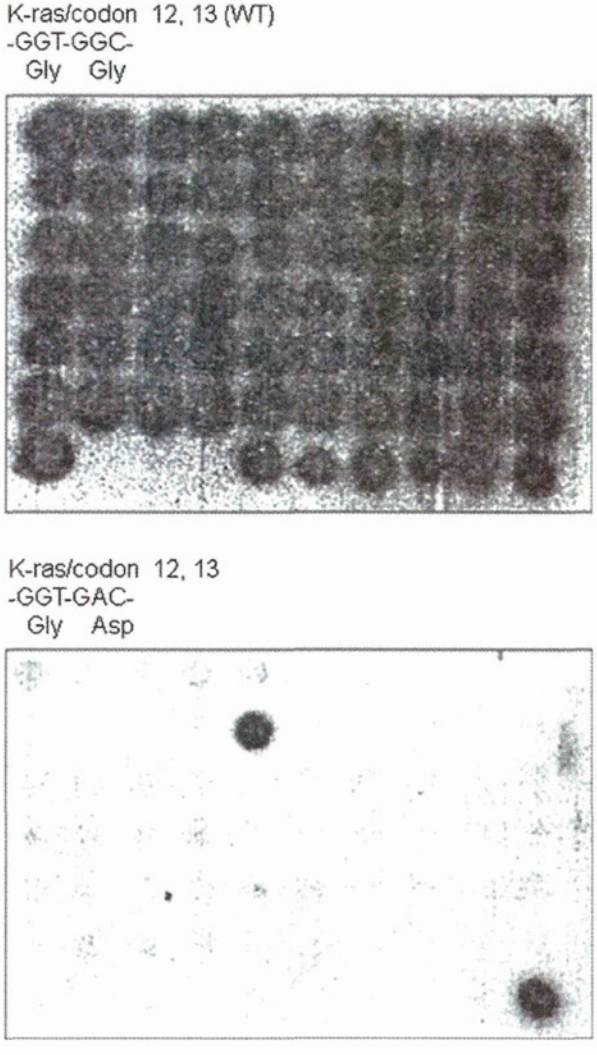

Fig. 1 Detection of a *KRAS* gene mutation in a patient with hepatocellular carcinoma. PCR-amplified DNA from 61 tumor samples was dotted onto nylon membranes and hybridized to a ^{32}P-labeled oligonucleotide probe. *WT* wild type *KRAS*

No mutational activation was found in codons 12 and 61 of *KRAS* or codons 12, 13 and 61 of the *NRAS* and *HRAS* genes in any of the HCCs or corresponding non-malignant tissue samples.

Discussion

This study examined 61 HCC tissues and their corresponding non-malignant liver tissues for a somatic mutation in codons 12, 13, and 61 of the *KRAS*, *HRAS*, or *NRAS* genes, which are known hot spots in various malignancies. However, the study showed the only one of the 61 HCCs (1.6 %) had a somatic mutation in codon 13 of the *KRAS* gene, indicating that Ras gene mutations do not appear to be related to the pathogenesis of most HCCs.

There have been several reports with small sample sizes regarding Ras gene mutations in HCC (Table 2). Most have reported that somatic mutations of the Ras gene in HCCs are uncommon, similar to the current study. Tsuda et al. [10] found only two tumors with Ras point mutations in surgically resected specimens from 30 HCC patients. In their patients, codon 12 of *KRAS* was altered from GGT, coding for Gly, to GTT, coding for Val in one case, and codon 61 of *NRAS* was altered from CAA, coding for Glu, to AAA, coding for Lys, in the other case. Tada et al. analyzed the mutations of the three Ras genes in 23 primary hepatic malignant tumors (12 hepatocellular carcinomas, nine cholangiocarcinomas, and two hepatoblastomas). Point mutations in *KRAS* codon 12 or *KRAS* codon 61 were found in 6 of the 9 cholangiocarcinomas. In contrast, there were no point mutations in any of 12 HCCs or two hepatoblastomas in codons 12, 13, or 61 of the Ras genes. The authors concluded that Ras gene mutations are not related to the pathogenesis of HCC, but play an important role in pathogenesis of cholangiocarcinoma.

Sorafenib is the first molecule with specific targets involved in the pathogenesis of HCC that has become available for routine clinical use. It is an orally applicable

Table 2 Reported Ras gene mutations in HCC patients

Author [references]	No. of patients	Ras gene mutation		
		KRAS	*NRAS*	*HRAS*
Tsuda et al. [10]	30	1 (codon 12)	1 (codon 61)	0
Tada et al. [14]	12	0	0	0
Ogata et al. [15]	19			2
Challen et al. [16]	19	1 (codon 61)	3 (codon 61)	0
Leon et al. [17]	12	1 (codon 61)	0	0
This study	61	1 (codon 13)	0	0

multi-kinase inhibitor that acts by blocking tumor cell proliferation and angiogenesis through the inhibition of serine/threonine kinases [11]. Sorafenib can increase survival by up to 3 months in patients with advanced HCC and acceptable liver function [8]. On the other hand, severe side effects have been reported with sorafenib, including hand-foot skin reactions or liver dysfunction [7, 8]. Therefore, it is important to identify prognostic markers and to establish the proper selection criteria for using sorafenib. Mutations of the Ras genes in cases of HCCs were systemically evaluated in this study because the Ras signaling pathway is the main target of sorafenib. The results indicated that mutational activation of Ras genes is uncommon in the pathogenesis of HCCs. Caraglia et al. [12] reported that the presence of phosphorylated ERK activity in peripheral blood mononuclear cells is valuable for predicting the response to sorafenib therapy in HCC patients. An in vitro study confirmed that phosphorylated ERK was a potential biomarker predicting the sensitivity of HCC to sorafenib [13]. Therefore, a mutation in the RAF/MEK/ERK pathway may be involved in the drug resistance to sorafenib, rather than a Ras mutation.

In summary, only one of 61 HCCs (1.6 %) in the present study carried a point mutation, which was a G to A transition in codon 13 of the *KRAS* gene. No mutational activation was found in codons 12 and 61 of *KRAS* or in codons 12, 13 and 61 of the *NRAS* or *HRAS* genes in any of the HCCs or corresponding non-malignant tissue samples. These findings suggested that Ras gene mutations are not related to the pathogenesis of most HCCs. The signaling pathways downstream of Ras should be examined to identify markers to predict a response to sorafenib.

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Conflict of interest None of the authors has any conflict of interest.

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Tumor-Associated Macrophage Promotes Tumor Progression via STAT3 Signaling in Hepatocellular Carcinoma

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Key Words
Hepatocellular carcinoma · STAT3 · Macrophage

Abstract
Objective: Signal transducer and activator of transcription 3 (STAT3) is activated in hepatocellular carcinoma (HCC), and tumor-associated macrophage plays an important role in tumor progression. Therefore, we examined STAT3 activation, cytokine expression and infiltration of tumor-associated macrophages in resected HCCs as well as the alteration of cell growth and migration by cytokine stimulation in HCC cell lines. ***Methods:*** Immunohistochemical staining of phosphorylated STAT3 (pSTAT3), CD163, interleukin (IL)-6, Ki-67 and Bcl-XL was performed for 101 cases of resected HCC, and correlations between pSTAT3 staining and clinicopathological findings were analyzed. In HCC cell lines (PLC/PRF/5 and Huh7), cell proliferation and migration by IL-6 stimulation and S3I-201 (STAT3 inhibitor) treatment were analyzed. ***Results:*** In HCC specimens, the pSTAT3-positive group showed high levels of α-fetoprotein ($p = 0.0276$), large tumor size ($p = 0.0092$), frequent intrahepatic metastasis ($p = 0.0214$), high Ki-67 ($p = 0.0002$) and Bcl-XL ($p = 0.0001$), poor prognosis ($p = 0.0234$), and high recurrence rate ($p = 0.0003$). CD163-positive cells were frequently observed in the pSTAT3-positive group ($p = 0.0013$). In two HCC cell lines, IL-6 stimulation promoted cell proliferation and migration via the STAT3 phosphorylation, and S3I-201 inhibited this activation. ***Conclusions:*** STAT3 activation was correlated with aggressive behavior of HCC and may be mediated via tumor-associated macrophage. We expect that STAT3 signaling and tumor-associated macrophages can be attractive therapeutic targets in HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer in the world [1]. Although surgical therapies for HCC have progressed and outcomes of HCC have improved, HCC still often recurs after surgery [2, 3]. Sorafenib, one of the molecular targeted therapies, was reported to show activity against unresectable HCCs;

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however, its survival advantage is only 3.7 months [4]. New therapeutic targets are required to improve the survival of patients with HCC.

Signal transducer and activator of transcription 3 (STAT3) is an important molecule in tumor progression [5]. STAT3 activation occurs via phosphorylation and dimerization of tyrosine residue (Tyr705), leading to nuclear entry, DNA binding and gene transcription. STAT3 was regarded as a critical transcription activator for cell cycle- or cell survival-related genes. Bcl-XL is an antiapoptotic protein transcribed by STAT3 activation [6]. Some cytokines such as interleukin (IL)-6 or IL-10 activate STAT3 signaling via their receptors [7]. Constitutive activation of STAT3 has been demonstrated to contribute to tumorigenesis in breast cancer [8], colon cancer [9], lung cancer [10], pancreatic cancer [11], prostate cancer [12], and melanoma [13]. In human HCC, STAT3 phosphorylation was also detected and related to tumor progression [14], angiogenesis [15] and tumorigenesis [16]. The tumor microenvironment is closely associated with the growth of tumor cells, and tumor-associated macrophages play an important role in tumor progression [17]. Macrophages are major inflammatory cells that infiltrate tumors; several studies have shown that high infiltration of tumor-associated macrophages was associated with tumor progression and metastasis [17–20] and predicts poor prognosis in patients with HCC [21]. Tumor-associated macrophages activate STAT3 in ovarian cancer [22] and glioblastoma [23]. However, the correlation between tumor-associated macrophages and STAT3 activation of HCC tumor cells is unknown. Therefore, we examined STAT3 activation, cytokine expression and infiltration of tumor-associated macrophages in resected HCCs and analyzed their association with clinicopathological findings. Alterations in cell growth and migration by cytokine stimulation and STAT3 inhibitor were also analyzed in HCC cell lines.

Materials and Methods

Patients and Samples

One hundred and one available paraffin-embedded specimens from patients with HCC who underwent hepatectomy between January 1997 and December 2001 in our institute were selected by reviewing their pathology data. Any patients undergoing previous or noncurative surgery were excluded. After the surgery, monthly measurement of the serum α-fetoprotein (AFP) level was performed. In addition, ultrasonography and dynamic CT were performed every 3 months. The postoperative survival period or recurrence was entered into the database immediately when a patient died or if recurrence was strongly suspected on diagnostic imaging such as CT or magnetic resonance imaging.

This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of Kyushu University Hospital (grant No. 21-117). Informed consent was obtained from each patient included in the study.

Immunohistochemistry

Sections of resected specimens were fixed in 10% buffered formalin, embedded in paraffin and stained by Envision+ system and DAB kit (Dako, Glostrup, Denmark). Immunohistochemical stains were performed with antibodies of phosphorylated STAT3 (pSTAT3; Tyr 705; D3A7, 1:50; Cell Signaling Technology), CD163 (10D6, 1:200; Novocastra), IL-6 (rabbit polyclonal, 1:1,000; Abcam), Ki-67 (MIB-1, 1:200; Dako), and Bcl-XL (rabbit polyclonal, 1:200; Santa Cruz Biotechnology, Santa Cruz, Calif., US). Sections were pretreated before being incubated with primary antibodies in a microwave oven at 99°C for 20 min for pSTAT3, CD163, IL-6 and Bcl-XL or in a pressure cooker for 25 min for Ki-67.

Each slide was stained in serial sections and examined by two pathologists (Y.M. and S.A.). In nuclear staining of pSTAT3 and Ki-67 and in cytoplasm staining of Bcl-XL, the percent positive cells was estimated by count of 1,000 tumor cells in most staining areas (hot spots). Staining of CD163, a marker of tumor-associated macrophages [19, 22–25], and IL-6 was evaluated by estimating the total counts of cytoplasm or membrane at 3 high-power fields. The mean of nuclear pSTAT3-positive cells in HCCs was 10.7% (range 0–82.0), and pSTAT3 stain was classified into a positive (≥10.7% of tumor cell nuclei) and a negative group (<10.7% of tumor nuclei). Furthermore, in the cases of the pSTAT3-positive group (n = 36), the CD163-positive cells were counted separately in areas of pSTAT3-positive and pSTAT3-negative HCC cells.

For double staining of IL-6 and CD163, HCC specimens were boiled in 10 mM citrate buffer (pH 6.0) for 20 min and incubated with IL-6 primary antibody (1:1,000) at room temperature for 15 min. The sections were washed three times and incubated with anti-rabbit horseradish peroxidase-conjugated polymer at room temperature for 45 min; IL-6 was visualized by DAB kit. Next, the sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min, incubated with CD163 primary antibody (1:200) for 90 min and incubated with anti-mouse alkaline phosphatase-conjugated polymer at room temperature for 45 min. CD163 of the sections was visualized by New Fuchsin Substrate kit (Nichirei, Tokyo, Japan).

Cell Culture

Human HCC cell lines PLC/PRF/5 and Huh7 were obtained from Riken Bioresource Center, Tsukuba, Japan, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 or 10% fetal bovine serum (FBS). PLC/PRF/5 and Huh7 cells were maintained in DMEM containing 1% FBS for 24 h prior to IL-6 (Peprotech, Rocky Hill, N.J., USA) stimulation. All in vitro experiments were done in triplicate.

Immunoblotting

Cellular proteins were solubilized in lysis buffer containing protease inhibitor and phosphatase inhibitor 30 min after stimulation with IL-6 (20 μg/ml). Equal amounts of protein were separated by SDS-PAGE and then transferred to the polyvinylidene fluoride membrane. Following blocking in Tris buffer containing 2% BSA, the membrane was stained with 1:1,000 dilution of anti-STAT3 (Cell Signaling Technology, Danvers, Mass., USA) and anti-pSTAT3 (Cell Signaling Technology) antibodies, then