Table V. Sub-analysis among three groups according to the intracellular expression pattern of mesothelin.

Parameter	Total (n=44)	Luminal membrane positive (n=32)	Cytoplasmic expression (n=12)	P-value	Total (n=49)	Luminal membrane positive (n=32)	Negative expression (n=17)	P-value	Total (n=29)	Cytoplasmic expression (n=12)	Negative expression (n=17)	P-value
Histopahological grade												
1 or 2	39	28	11	1.000	43	28	15	1.000	26	11	15	1.000
3	5	4	1		6	4	2		3	1	2	
pT-factor												
pT1-2	23	19	4	0.179	28	19	9	0.765	13	4	9	0.452
pT3-4	21	13	8		21	13	8		16	8	8	
pN-factor												
Negative	18	16	2	0.083	23	16	7	0.764	9	2	7	0.234
Positive	26	16	10		26	16	10		20	10	10	
pStage												
I-IIB	37	26	11	0.653	39	26	13	0.722	24	11	13	0.370
III-IV	7	6	1		10	6	4		5	1	4	
Lymphatic permeation												
Negative	14	12	2	0.282	21	12	9	0.370	11	2	9	0.064
Positive	30	20	10		28	20	8		18	10	8	
Blood vessel permeation												
Negative	16	11	5	0.732	21	11	10	0.134	15	5	10	0.462
Positive	28	21	7		28	21	7		14	7	7	
Perineural invasion												
Negative	3	3	0	0.551	9	3	6	**0.049**	6	0	6	**0.028**
Positive	41	29	12		40	29	11		23	12	11	
Resection margin												
pR0	30	24	6	0.152	32	24	8	0.065	14	6	8	1.000
pR1	14	8	6		17	8	9		15	6	9	
Recurrence												
No	11	6	5	0.139	13	6	7	0.172	12	5	7	1.000
Yes	33	26	7		36	26	10		17	7	10	
Liver metastasis												
No	30	20	10	0.282	36	20	16	**0.020**	26	10	16	0.553
Yes	14	12	2		13	12	1		3	2	1	
Local recurrence												
No	34	25	9	1.000	37	25	12	0.729	21	9	12	1.000
Yes	10	7	3		12	7	5		8	3	5	
Peritoneal metastasis												
No	34	22	12	**0.041**	37	22	15	0.175	27	12	15	0.498
Yes	10	10	0		12	10	2		2	0	2	

human *mesothelin* gene encodes a 71-kDa precursor protein that is proteolytically cleaved by some furin-like proteases into an *N*-terminal secreted form and a *C*-terminal fragment, the 40-kDa mesothelin, which is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein (6,13,15). Many researchers have investigated the role of the mesothelin expression in tumor biology and demonstrated the importance of mesothelin expression for tumor progression *in vitro* (14,24-26) and *in vivo* (27,28); however, the clinicopathological significance of the membrane localization of mesothelin has not been clarified. The 5B2 anti-mesothelin antibody, which we employed here for IHC, can detect both the 71-kDa precursor protein and the 40-kDa *C*-terminal fragment, but not the 30-kDa *N*-terminal fragment. According to the reported molecular processing mechanism of mesothelin and specificity of antibody, luminal membrane staining probably indicates the 40-kDa membrane-bound form of mesothelin, while cytoplasmic staining would mean the 71-kDa precursor form of mesothelin. Our results support the idea that the 40-kDa membrane-bound form of mesothelin is an active form and promotes the aggressive features including increased cell motility, invasion or migration capabilities and growth of metastatic tumors (24,25,29).

The fact that 'cytoplasmic expression' of mesothelin paradoxically resulted in better OS than mesothelin with 'mesothelin negative' took us by surprise (Fig. 5B). The RFS rate at 3 years (58 and 40%, respectively) and OS at 5 years (51 and 20%, respectively) were demonstrably better in 'cytoplasmic expression' compared to 'mesothelin negative', although the final RFS and OS were not statistically significant (RFS, P=0.06; OS, P=0.10). As indicated above, the majority of mesothelin in cytoplasm must be the 71-kDa precursor form and might behave like a dominant negative form of mesothelin as a tumor suppressor. The conflicting results in some previous reports in which mesothelin expression was correlated with prolonged patient survival in gastric cancer (18) and in ovarian serous carcinoma (30), may be explained by confusing the luminal membrane and cytoplasmic expression of mesothelin. Isolation of 'mesothelin negative' might give us another disease entity, mesothelin-independent EHBDCA. The tumor cells in such a type of EHBDCA would obtain invasive ability without the association of mesothelin; therefore, this could indicate an alternative gene expression profiling. In fact, additional sub-analysis for clinicopathological parameters among the three groups showed interesting results. Frequent perineural invasion was observed in 'mesothelin negative' rather than in mesothelin positive cases even in luminal membrane or cytoplasm (P=0.049 and 0.028, respectively), while liver metastasis was abundantly found in 'luminal membrane positive' (Table V). Such conflicting results may suggest the distinct oncogenic process between mesothelin-associated and mesothelin-independent EHBDCA.

In terms of discovering the clinicopathological parameters, there are many previous studies demonstrating the prognostic significance of various molecules, such as epidermal growth factor receptor (EFGR) and c-erbB-2 (HER-2) in colorectal, breast and lung cancer (31). There are some other case reports describing a series of promising results targeting EGFR in patients with advanced biliary tract cancer (32-34); however, identification of useful prognostic markers for EHBDCA still needs investigation. In addition, lack of effective adjuvant therapy against advanced EHBDCA requires establishing new therapeutic methods based on reliable molecular targeting markers; thus, mesothelin could be one of the potential targets for cancer molecular targeting therapy. Recombinant anti-mesothelin immunotoxin SS1P (CAT-5001) and a high affinity chimeric anti-mesothelin monoclonal antibody MORAb-009 recently entered phase II clinical trials (35,36). To evaluate the therapeutic effect of such antibody-based medicine, pathological verification of membranous expression of the target molecule must be performed, because antibody-based drugs can usually access the molecules located on the cell membrane. We believe that luminal membrane expression of mesothelin in EHBDCA would be of clinical benefit not only as a prognostic factor but also as a predictive factor for the eligibility to mesothelin-targeting therapies (13,14,27,37,38).

In conclusion, we demonstrated the clinicopathological significance of the mesothelin expression as an independent prognostic factor. Moreover, identification of luminal membrane or cytoplasmic expression of mesothelin could be a reliable prognostic factor for EHBDCA and might offer a novel therapeutic strategy for patients with EHBDCA, including immunotherapy using peptide vaccine or monoclonal antibody therapy.

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ORIGINAL ARTICLE

A rare point mutation in the Ras oncogene in hepatocellular carcinoma

Akinobu Taketomi · Ken Shirabe · Jun Muto · Shohei Yoshiya · Takashi Motomura · Yohei Mano · Tohru Ikegami · Tomoharu Yoshizumi · Kenji Sugio · Yoshihiko Maehara

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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

A. Taketomi (✉) · K. Shirabe · J. Muto · S. Yoshiya · T. Motomura · Y. Mano · T. Ikegami · T. Yoshizumi · K. Sugio · Y. Maehara
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Japan
e-mail: taketomi@med.hokudai.ac.jp; taketomi@surg2.med.kyushu-u.ac.jp

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

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

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 (*KRASs* codon 12 TGT:Cys) was provided by Dr. Sugio (Institution?).

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.

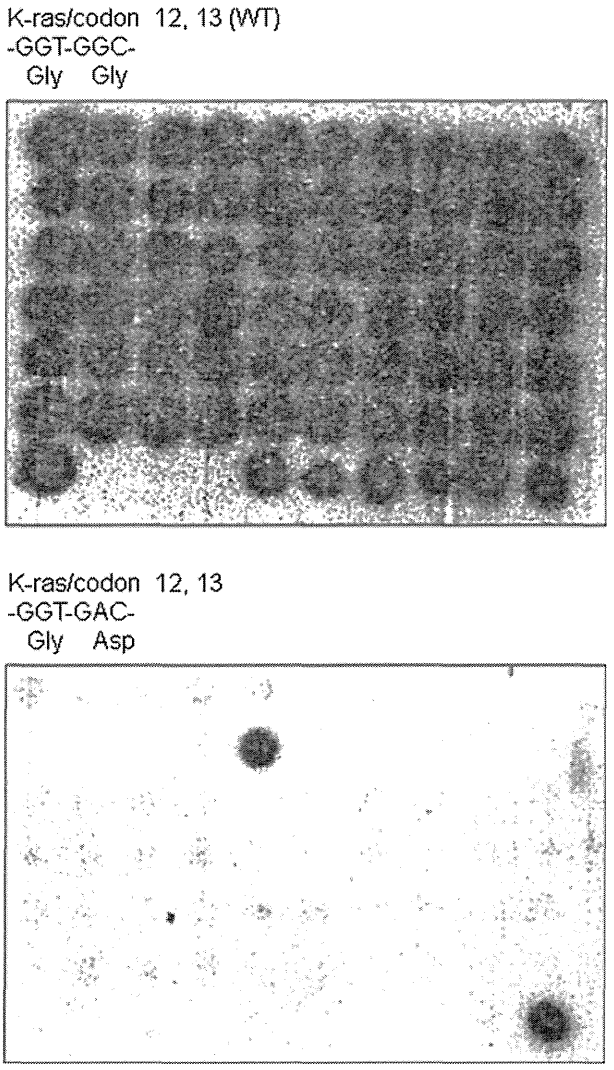

Fig. 1 Detection of a *KRAS* gene mutation in a patient with hepatocellular carcinoma. PCR-amplified DNA from 61 tumor samples was dotted onto nylon membranes and hybridized to a ^{32}P-labeled oligonucleotide probe. *WT* wild type *KRAS*

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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RESEARCH Open Access

Analysis of the risk factors for early death due to disease recurrence or progression within 1 year after hepatectomy in patients with hepatocellular carcinoma

Toshiya Kamiyama[1*], Kazuaki Nakanishi[2], Hideki Yokoo[1], Hirofumi Kamachi[2], Munenori Tahara[1], Tatsuhiko Kakisaka[1], Yosuke Tsuruga[1], Satoru Todo[2] and Akinobu Taketomi[1]

Abstract

Background: Liver resection for hepatocellular carcinoma (HCC) has the highest local controllability among all local treatments and results in a good survival rate. However, the recurrence rates of HCC continue to remain high even after curative hepatectomy Moreover, it has been reported that some patients with HCC have an early death due to recurrence. We analyzed the preoperative risk factors for early cancer death.

Methods: Between 1997 and 2009, 521 consecutive patients who underwent hepatectomy for HCC at our center were assigned to group ED (death due to HCC recurrence or progression within 1 year after hepatectomy) and group NED (alive over 1 year after hepatectomy). Risk factors for early cancer death were analyzed.

Results: Group ED included 48 patients, and group NED included 473 patients. The cause of death included cancer progression (150; 78.1%), operation-related (1; 0.5%), hepatic failure (15; 7.8%), and other (26; 13.5%). Between the ED and NED groups, there were significant differences in albumin levels, Child-Pugh classifications, anatomical resections, curability, tumor numbers, tumor sizes, macroscopic vascular invasion (portal vein and hepatic vein), alpha-fetoprotein (AFP) levels, AFP-L3 levels, protein induced by vitamin K absence or antagonism factor II (PIVKA-II) levels, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, and distant metastasis by univariate analysis. Multivariate analysis identified specific risk factors, such as AFP level > 1,000 ng/ml, tumor number ≥ 4, tumor size ≥ 5 cm, poor differentiation, and portal vein invasion. With respect to the preoperative risk factors such as AFP level, tumor number, and tumor size, 3 (1.1%) of 280 patients with no risk factors, 12 (7.8%) of 153 patients with 1 risk factor, 24 (32.9%) of 73 patients with 2 factors, and 9 (60.0%) of 15 patients with 3 risk factors died within 1 year of hepatectomy ($p < 0.0001$).

Conclusions: Hepatectomy should be judiciously selected for patients with AFP level > 1,000 ng/ml, tumor number ≥ 4, and tumor size ≥ 5 cm, because patients with these preoperative risk factors tend to die within 1 year after hepatectomy; these patients might be better treated with other therapy.

Keywords: Hepatocellular carcinoma, Hepatectomy, Early death

* Correspondence: t-kamiya@med.hokudai.ac.jp
[1]The Department of General Surgery, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan
Full list of author information is available at the end of the article

Background

Liver resection for the treatment of hepatocellular carcinoma (HCC) has the highest controllability among all local treatments and results in a good survival rate [1,2]. However, recurrence rates remain high and are the main cause of early death even after curative hepatectomy [3]. Moreover, it has been reported that some patients with HCC have an early death due to recurrence [4]. In the remnant liver after hepatectomy, tumor recurrence is recognized as intrahepatic metastasis caused by dissemination of cells in the portal vein or metachronous multicentric hepatocarcinogenesis [5]. The risk factors for early recurrence are reported to be related to tumor cell dissemination due to tumor characteristics such as vascular invasion [6,7] and intrahepatic metastasis [8]. Though the two algorithms that were proposed from the Barcelona Clinic Liver Cancer (BCLC) classification [9] and Japanese guideline [10] recommend that multiple HCCs be treated by transcatheter arterial chemoembolization with lipiodol (TACE) or sorafenib, hepatectomy beyond these algorithms was actually performed in the clinical scene. However, the risk factors for early death due to HCC recurrence or progression within 1 year after hepatectomy have not been clearly evaluated [11].

On the other hand, the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial [12] recently reported the effectiveness of sorafenib in the treatment of advanced HCC. In this report, median overall survival was 10.7 months in the sorafenib group and 7.9 months in the placebo group. If patients have an early death within 1 year due to recurrence after hepatectomy, there might be no benefit of hepatectomy compared to sorafenib. Therefore, the risk factors for early death within 1 year after hepatectomy due to HCC recurrence or progression should be evaluated, and the appropriateness of hepatectomy for patients with advanced HCC should be investigated.

To identify the risk factors related to early death after hepatectomy, we analyzed the outcomes of 521 consecutive patients who underwent primary hepatectomy for HCC at our center.

Methods

Patients

Between January 1997 and May 2009, 521 consecutive patients underwent hepatectomy for HCC at our center. These patients were followed for at least 1 year, and then assigned to group ED (death due to HCC recurrence or progression within 1 year after hepatectomy) or group NED (alive over 1 year after hepatectomy). The resulting ED group included 48 (9.2%) patients, and the resulting NED group included 465 (89.3%) patients. Of all 521 patients, 8 (1.5%) patients who died of liver failure, other causes, and postoperative complications within 1 year after hepatectomy were excluded from group ED and NED. The mean age of 513 patients of group ED and NED was 61.3 years, with a range of 18–87 years. Of the 513 patients, 427 (83.2%) were male and 86 (16.8%) were female, 221 (43.1%) were hepatitis B virus surface antigen-positive, 189 (36.8%) were hepatitis C virus antibody-positive, and 175 (34.1%) had cirrhosis. At least 2 weeks before hepatectomy, imaging studies were performed and preoperative serum alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3), and protein induced by vitamin K absence or antagonism factor II (PIVKA-II) levels were simultaneously measured using standard methods. Among the 513 patients, 499 (97.3%) were categorized as Child-Pugh class A (Table 1). The patients were followed up for a median of 84.2 months (range, 12.5–165.0 months). This study was approved by the Institutional Review Board of the Hokkaido University, School of Advanced Medicine.

Hepatectomy

Anatomical resection is defined as a resection in which lesion(s) are completely removed anatomically on the basis of Couinaud's classification (segmentectomy, sectionectomy, and hemihepatectomy or extended hemihepatectomy) in patients with sufficient functional reserve. Non-anatomical partial resection was performed as a limited resection or tumor enucleation. When R0 resections were performed, the resection surface was found to be histologically free of HCC. Indocyanine green retention rates at 15 min (ICGR15) were measured to evaluate liver function reserve, regardless of the presence or absence of cirrhosis.

HCC recurrence

Every 3 months for the first 2 years after hepatectomy, the patients underwent follow-up evaluations comprising liver function tests, measurements of tumor markers AFP and PIVKA-II, ultrasonography (US), and dynamic computed tomography (CT). After 2 years, routine CT was performed only once every 4 months. If recurrence was suspected, CT and magnetic resonance imaging (MRI) were performed; if necessary, CT during angiography and bone scintigraphy were also performed. This enabled precise diagnoses of the site, number, size, and invasiveness of the recurrent lesions.

Statistical analysis

Patient survival (PS) rates were determined via the Kaplan-Meier method. Univariate analysis was performed; then multivariate analysis and logistic regression were performed only on significant variables. Statistical analyses (StatView 5.0 for Windows: SAS Institute Inc., Cary, NC) were performed using standard tests (X^2, t-test) where appropriate. Significance was defined as $p < 0.05$.

Table 1 Univariate analysis of the risk factors of death from cancer progression within 1 year after hepatectomy

		Group ED ($n=48$)	Group NED ($n=465$)	*p*-value
Sex	Male	40	387	
	Female	8	78	0.9849
Age	<60	24	211	
	60 ≤	24	254	0.5405
HBV	+	26	195	
	-	22	270	0.1033
HCV	+	14	175	
	-	34	290	0.2469
Albumin	<4	33	214	
(g/dl)	4 ≤	15	251	0.0027
Total bilirubin	<0.8	32	283	
(mg/dl)	0.8 ≤	16	182	0.4314
ICGR15 (%)	<15	27	250	
	15 ≤	21	215	0.7421
Child-Pugh	A	42	457	
	B	6	8	<0.0001
AFP (ng/ml)	≤200	15	355	
	200<, ≤1,000	5	37	
	1,000<	28	73	<0.0001
AFP-L3 (%)	≤15	23	334	
	15% < 40<	5	49	
	40<	20	83	0.0002
PIVKA-II (mAU/ml)	≤100	10	258	
	100<, ≤1,000	8	93	
	1,000<	30	114	<0.0001
Liver cirrhosis	Present	17	158	
	Absent	31	307	0.8414
Curability	R0 R1	40	443	
	R2	8	22	0.0008
Anatomical resection	Yes	42	326	
	No	6	139	0.0108
Tumor number	1	16	321	
	2, 3	11	113	
	4 ≤	21	31	<0.0001
Tumor size	≤2 cm	4	64	
	2-5 cm	6	254	
	5 cm ≤	38	147	<0.0001
Macroscopic vascular invasion	Absent	28	440	
(portal vein, hepatic vein)	Present	20	25	<0.0001
Differentiation	Well	0	50	
	Moderate	19	308	
	Poor	29	92	
	Necrosis	0	15	<0.0001
Microscopic portal vein invasion	vp0	10	369	
	vp1	13	60	
	vp2	7	15	
	vp3	12	15	
	vp4	6	6	<0.0001
Microscopic hepatic vein invasion	vv0	28	439	
	vv1	8	12	
	vv2	9	10	
	vv3	3	4	<0.0001
Distant metastasis	Absent	43	459	
	Present	5	6	<0.0001

HCC: hepatocellular carcinoma.
NED: alive 1 year after hepatectomy.
ED: death due to HCC recurrence or progression within 1 year after hepatectomy.
HBV: hepatitis B virus s antigen.
HCV: anti-hepatitis C virus antibody.
ICGR15: indocyanin green retention rate at 15 min.
AFP: alpha-fetoprotein.
AFP-L3: *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein.
PIVKA-II: protein induced by vitamin K absence or antagonism factor II.
vp0: no tumor thrombus in the portal vein.
vp1: tumor thrombus distal to the second branches of the portal vein.
vp2: tumor thrombus in the second branches of the portal vein.
vp3: tumor thrombus in the first branch of the portal vein.
vp4: tumor thrombus extension to the trunk or the opposite side branch of the portal vein.
vv0: no tumor thrombus in the hepatic vein.
vv1: tumor thrombus in a branch of the hepatic vein.
vv2: tumor thrombus in the right, middle, or left hepatic vein trunk or the short hepatic vein.
vv3: tumor thrombus to the inferior vena cava.

Results

Causes of death

PS rates ($n=521$) at 1, 2, 5, and 10 years were 90.5%, 84.6%, 69.0%, and 54.2%, respectively (Figure 1), with 192 deaths (36.9%). The causes of death, whether within 1 year post-hepatectomy or later, included HCC recurrence or progression ($n=150$; 78.1%), liver failure ($n=15$; 7.8%), other causes ($n=26$; 13.5%), and postoperative complications ($n=1$; 0.5%). Of the 150 patients who died of HCC recurrence or progression, 48 (32.0%) died within 1 year after hepatectomy (Figure 2). The patients who died of liver failure ($n=4$), other causes ($n=3$), and postoperative complications ($n=1$) within 1 year after hepatectomy were excluded from group ED and NED.

Clinicopathological characteristics and operative variables

Patient characteristics and perioperative outcomes are shown in Table 1. Between the ED and NED groups,

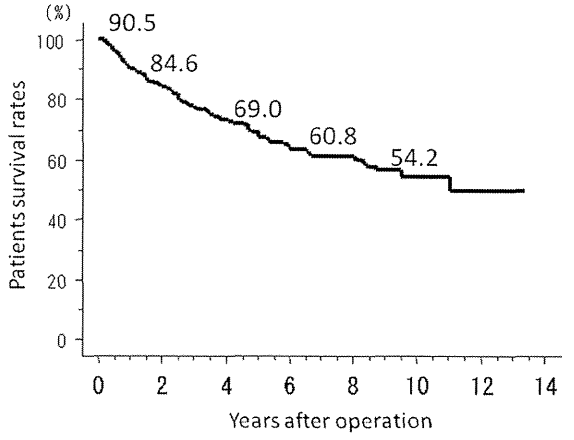

Figure 1 Survivals of all 521 patients at 1, 2, 5, 7, and 10 years were 90.5%, 84.6%, 69.0%, 60.8%, and 54.2%, respectively.

there were significant differences in albumin levels, Child-Pugh classifications, anatomical resections, curability, tumor numbers, tumor sizes, macroscopic vascular invasion (portal vein and hepatic vein), AFP levels, AFP-L3 levels, PIVKA-II levels, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, and distant metastasis. Tumor-related factors are also shown in Table 1. When the risk factors that were identified as significant by univariate analysis were included in a multivariate analysis via logistic regression, it was found that AFP level, tumor number, tumor size, differentiation, and microscopic portal vein invasion were independent risk factors for early death due to HCC recurrence or progression within 1 year after hepatectomy (Table 2).

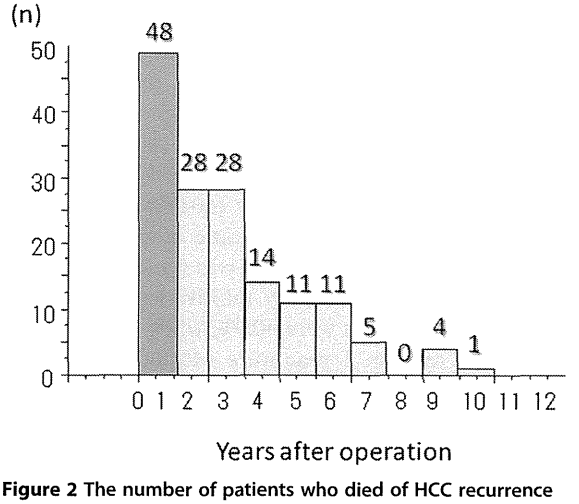

Figure 2 The number of patients who died of HCC recurrence or progression after hepatectomy. Of the 150 patients who died of HCC recurrence or progression, 48 patients (32.0%) died within 1 year after hepatectomy.

Table 2 Logistic regression analysis based on univariate analysis of the risk factors of death from cancer progression within 1 year after hepatectomy

Risk factor	*p*	Risk ratio	95% CI
AFP(ng/ml):>1,000(vs. ≤ 200)	0.0079	4.098	1.447-11.628
Tumor number 4 ≤ (vs. 1)	0.0208	3.535	1.206-10.361
Tumor size (cm) 5 ≤ (vs. 2–5)	0.0295	3.687	1.139-11.936
Differentiation poor (vs. moderately)	0.0179	2.8	1.194-6.565
vp1(vs. vp0)	0.0037	5.02	1.691-14.909
vp2(vs. vp0)	0.0034	8.507	2.029-35.667

AFP: alpha-fetoprotein.
vp0: no tumor thrombus in the portal vein.
vp1: tumor thrombus distal to the second branches of the portal vein.
vp2: tumor thrombus in the second branches of the portal vein.

Risk factors for early death

Independent, preoperatively evaluable risk factors for early death were identified by multivariate analysis as AFP > 1,000 ng/ml, tumor number ≥ 4, and tumor size ≥ 5 cm. The patients of group ED and NED ($n = 513$) were categorized into three levels of risk: risk 0 if they had no risk factors ($n = 276$), risk 1 if they had any one risk factor ($n = 151$), risk 2 if they had any two risk factors ($n = 71$), and risk 3 if they had all three risk factors ($n = 15$). In risk 0, 3 patients (1.1%), in risk 1, 12 patients (7.9%), in risk 2, and 24 patients (33.8%); in risk 3, 9 patients (60.0%) died within 1 year after hepatectomy ($p < 0.0001$) (Table 3). PS rates for risk 0, risk 1, risk 2, and risk 3 at 1 year were 98.9%, 91.7%, 66.1%, and 40.0%, respectively (Figure 3). Multivariate analysis showed that the risk ratio of risk 1 vs. risk 0 was 7.856, that of risk 2 vs. risk 0 was 46.468, and that of risk 3 vs. risk 0 was 136.5 (Table 3).

Discussion

When the patients were categorized by the number of independent, preoperatively evaluable risk factors, the

Table 3 Logistic regression analysis of three risk levels of death from cancer progression within 1 year after hepatectomy

	No. of patients	No. of ED (%)	Risk ratio	95% CI
Risk 0	276	3 (1.1)	1	
Risk 1	151	12 (7.9)	7.856	2.181-28.302
Risk 2	71	24 (33.8)	46.468	13.452-160.514
Risk 3	15	9 (60.0)	136.5	29.354-634.752

HCC: hepatocellular carcinoma.
ED: death due to HCC recurrence or progression within 1 year after hepatectomy.
Risk 0: they had no risk factors.
Risk 1: they had any 1 risk factor.
Risk 2: they had any 2 risk factors.
Risk 3: they had all 3 risk factors.
Risk factors: AFP > 1,000 ng/ml, tumor number ≥ 4, and tumor size ≥ 5 cm.

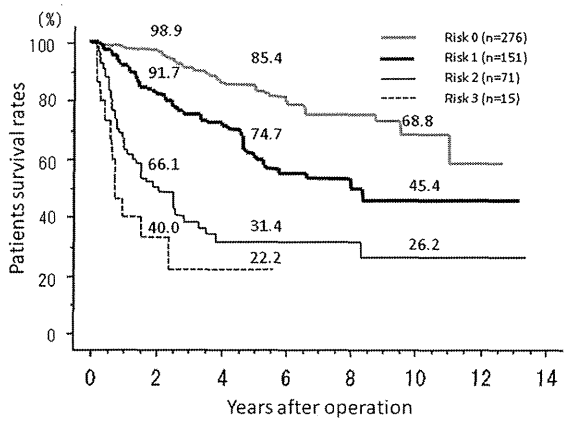

Figure 3 PS rates for risk 0, risk 1, risk 2, and risk 3 at 1 year were 98.9%, 91.7%, 66.1%, and 40.0%, respectively. PS rates for risk 0, risk 1, risk 2, and risk 3 at 1 year were 85.4%, 61.5%, 31.4%, and 22.2%, respectively.

early death rate within 1 year was 60.0% for patients with three risk factors: AFP > 1,000 ng/ml, tumor number ≥ 4, and tumor size ≥ 5 cm, while the early death rate was 1.1% for patients with no risk factors. Therefore, the appropriateness of hepatectomy for HCC should be carefully examined for patients who have large and multiple HCC with high AFP levels; these patients might be better treated with other therapeutic options, such as TACE or sorafenib.

Early recurrence is the main cause of early death within 1 year after hepatectomy. The risk factors for early recurrence are reported to be related to tumor cell dissemination due to tumor characteristics such as vascular invasion [6,7] and intrahepatic metastasis [8]. Because these factors are diagnosed only by postoperative pathological examination, preoperatively evaluable factors are necessary to decide the appropriateness of hepatectomy in advanced HCC. Among preoperative risk factors, an HCC tumor larger than 5 cm is reported to be an important indicator of a high risk of recurrence after resection [13] and has a higher incidence of intrahepatic metastasis and portal venous invasion [14,15]. Therefore, it is believed that an HCC tumor larger than 5 cm has high malignant potential. In this study and another report [11], tumor size ≥ 5 cm reflected this high malignant potential and was selected as an independent risk factor for early death due to HCC recurrence or progression within 1 year after hepatectomy.

Multivariate analysis also shows that tumor number is an important predictor of recurrence. Lai et al. [16] reported that the presence of multiple nodules was the most powerful predictor of both long-term survival and tumor recurrence. Because multiple HCC originates from disseminated cancer cells and not from multicentric carcinogenesis, multiple HCC is a more aggressive phenotype than solitary HCC. Yang reported that, after resection of solitary large HCC, the clinical and pathological characteristics and outcome are similar to those of small HCC, but are significantly better than those of nodular HCC (node number ≥ 2) [17]. It has also been reported that the expression levels of some human genes closely related to invasion and metastasis were significantly lower in solitary large HCC than in nodular HCC [17,18]. They proposed solitary large HCC as a specific subtype, less malignant than nodular HCC. Moreover, in multiple HCC, it was speculated that latent tumors, intrahepatic micrometastases that might be subsequently found to produce early recurrent tumors, could already be present in the remnant liver at the time of surgery. Therefore, tumor number ≥ 4 was selected in the current study as a significant factor predicting early death after hepatectomy.

In our study, multivariate analysis showed that an AFP level over 1,000 ng/ml was an independent factor related to early death. Previous reports have shown that AFP is an independent predictor of prognosis [19], even in patients who had undergone hepatectomy [20]. High levels of AFP in fully developed HCC or in the serum of the host are associated with more aggressive behavior and increased anaplasis [21]. On the other hand, it is well known that AFP levels may increase in some patients with acute and chronic hepatitis without HCC [22,23] and that elevation of AFP levels correlates with inflammation caused by background diseases and hepatocyte regeneration [24]. However, because the elevation of AFP levels by hepatitis or regeneration is usually not so high, only 200 ng/ml [25], AFP levels over 1,000 ng/ml might specifically indicate tumor malignancy. Yamanaka et al. [26] also reported that the serum AFP value per tumor diameter was the most significant risk factor for early death within 1 year after resection in patients with stage II–III HCC by multivariate analysis.

Given these preoperatively evaluable risk factors, the probability of early death after hepatectomy can be estimated by the number of risk factors. In risk 0, 3 patients (1.1%), in risk 1, 12 patients (7.9%), in risk 2, 24 patients (33.8%), and in risk 3, 9 patients (60.0%) died within 1 year after hepatectomy. The risk ratio of risk 1 vs. risk 0 was 7.856, that of risk 2 vs. risk 0 was 46.468, and that of risk 3 vs. risk 0 was 136.5 by multivariate analysis. PS rates for risk 3 at 1 year were 40.0%, while in the SHARP trial, survival rates at 1 year were 44% in the sorafenib group [12]. Moreover, Takayasu et al. reported that the survival rate at 1 year of patients with ≥ 4 tumors, ≥5.1 cm in diameter was 74% [27]. In this way, because the surgical outcome of patients with all three risk factors was worse than that of the patients treated with sorafenib or TACE, these patients might be better treated with other therapeutic options than hepatectomy for the first

line treatment. However, selected patients with risk 1 and 2 who might be beyond BCLC and Japanese algorithms should not be excluded from hepatectomy because of their good outcome: 91.7%, 66.1% at 1 year of PS.

On the other hand, in this study, macroscopic vascular invasion (portal and hepatic veins) was not indicated by multivariate analysis as an independent risk factor related to early death. It has been reported that the prognosis of patients with portal vein tumor thrombus (PVTT) in the main trunk or first branch is very poor; the median survival period of patients with portal thrombosis is only 2.7 months without appropriate treatment [28]. However, recently reported patients showed long-term survival rates when hepatectomy was combined with pre- or postoperative treatment. We reported the efficacy of a combination of hepatectomy and preoperative radiotherapy for PVTT in the main trunk or first branch. The 1-, 3-, and 5-year survival rates in hepatectomized patients with preoperative radiotherapy for PVTT were 100%, 53.3%, and 40.0%, respectively [29]. Minagawa [30] reported that the survival rate of patients with PVTT, including those who underwent hepatic resection with preoperative transcatheter arterial chemoembolization, was 42% at 5 years. Nagano [31] reported that 15 patients with HCC with PVTT were treated with FU arterial infusion and interferon therapy (FAIT) and surgery, and that all the patients (100%) survived over 1 year; without FAIT and surgery, 10 patients (67%) died within 1 year. Therefore, even if patients have HCC with macroscopic vascular invasion, particularly PVTT in the main trunk or first branch, hepatectomy is not contraindicated in these patients when combined with pre- or postoperative treatment. In the patients with risk 0, 1, of 45 patients 19 had macroscopic vascular invasion. Of these 19 patients, only 5 (26.3%) died within 1 year after hepatectomy. In the 26 patients with risk 2, 3, 15 patients (57.7%) died within 1 year after hepatectomy. Concerning Child-Pugh B cirrhosis, the high-risk patients could be also indentified. From these data, though macroscopic vascular invasion and Child-Pugh B cirrhosis were poor prognostic factors, the patients who had these factors did not always die in 1 year after hepatectomy. Using our risk levels, the patients with extremely poor prognosis could be identified from the patients who had poor prognostic factors such as macroscopic vascular invasion or Child-Pugh B. Therefore, concerning risk levels, risk 0 to 3 was very important and useful for predicting the prognosis of patients with HCC who underwent hepatectomy.

Conclusions

In conclusion, the appropriateness of hepatectomy in the treatment of HCC should be carefully considered when patients have the following preoperative risk factors: AFP > 1,000 ng/ml, tumor number ≥ 4, and/or tumor size ≥ 5 cm; these patients might be better treated with other therapeutic options, i.e., sorafenib and TACE. However, even if patients have HCC with PVTT in the main trunk or first branch, hepatectomy is not contraindicated when combined with pre- or postoperative treatment.

Abbreviations

HCC: Hepatocellular carcinoma; PS: Patient survival; ICGR15: Indocyanine green retention rate at 15 min; AFP: Alpha-fetoprotein; AFP-L3: *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein-L3 fraction; PIVKA-II: Protein induced by vitamin K absence or antagonism factor II; US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; TACE: Transcatheter arterial chemoembolization.

Competing interests

The authors declare that they have no competing interests.

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Author details

[1]The Department of General Surgery, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan. [2]The Department of Transplantation Surgery, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo060–8638, Japan.

Authors' contributions

TK designed the research; TK, KN, and HY acquired of the data; TK, KN, HY, HK, TK, YT, ST, and AT analyzed the data; TK wrote the paper. All authors read and approved the final manuscript.

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肝炎等克服緊急対策研究事業

ゲノムワイド関連解析を用いた革新的な肝移植後肝炎ウイルス再感染予防・治療法の確立に関する研究

平成23年度～平成25年度　総合研究報告書（2/2冊）

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

T Einama[1,2], S Homma[1], H Kamachi[1], F Kawamata[1], K Takahashi[3], N Takahashi[1], M Taniguchi[2], T Kamiyama[1], H Furukawa[2], Y Matsuno[4], S Tanaka[3], H Nishihara[*,5], A Taketomi[1] and S Todo[1]

[1]Department of General Surgery, Graduate School of Medicine, Hokkaido University, Sapporo, Japan; [2]Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University, Asahikawa, Japan; [3]Department of Cancer Pathology, Hokkaido University School of Medicine, Sapporo, Japan; [4]Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan; [5]Department of Translational Pathology, Hokkaido University, Graduate School of Medicine, Kita-Ku, Kita 14, Nishi 7, Sapporo 060-8638, Japan

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,

*Correspondence: Dr H Nishihara; E-mail: hnishihara@s5.dion.ne.jp
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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

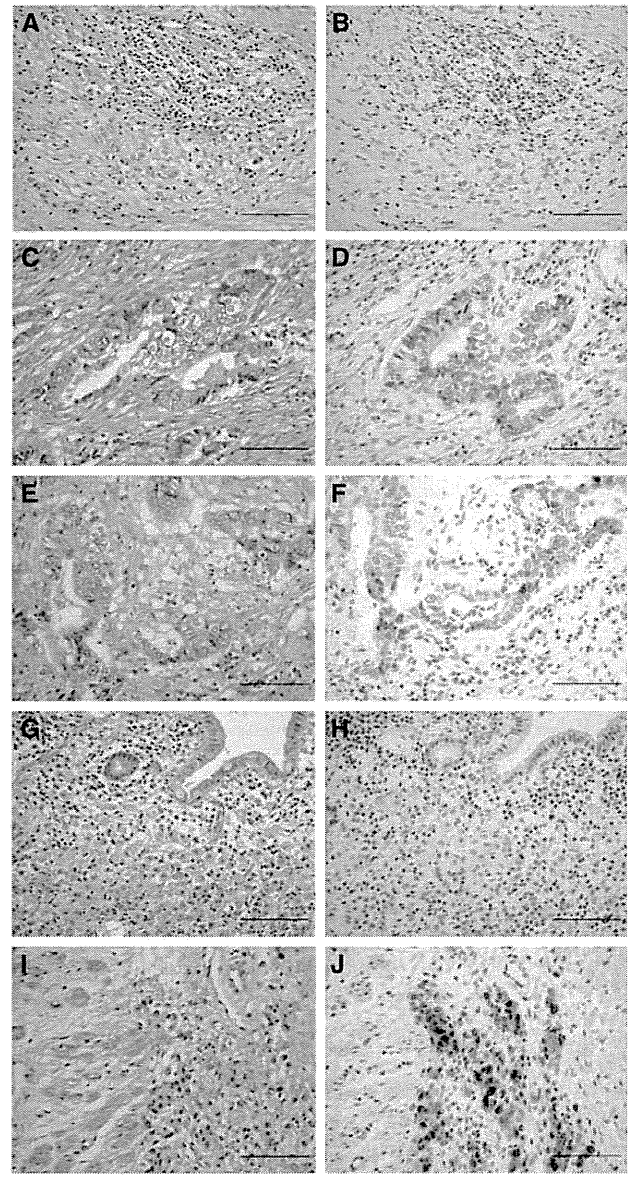

Figure 1 The expression variations of mesothelin and its cellular localisation in gastric cancer. (**A**, **C**, **E**, **G**, and **I**) HE stain. (**B**, **D**, **F**, **H**, and **J**) Immunohistochemical stain for mesothelin. (**A** and **B**) A case of 'mesothelin negative'. (**C** and **D**) A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

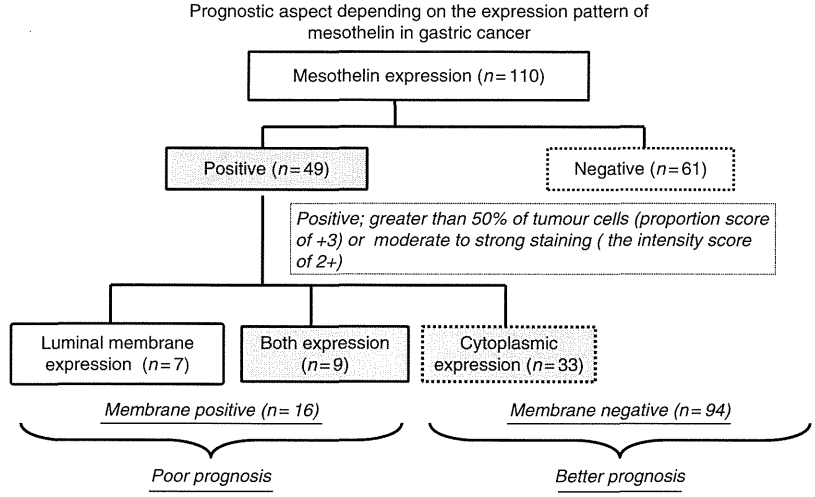

Figure 2 Flow chart of evaluation of mesothelin expression.

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.

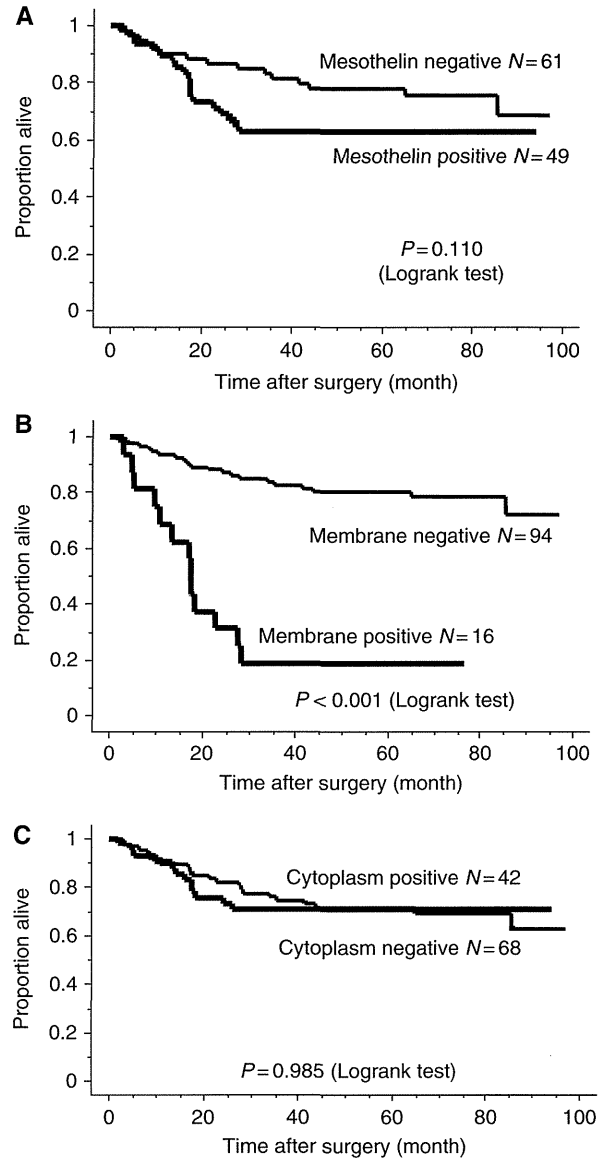

Figure 3 Overall survival for patients with gastric cancer after surgical therapy stratified by the status of mesothelin expression (**A**), mesothelin luminal membrane expression (**B**), and mesothelin cytoplasmic expression (**C**), respectively. The group of 'luminal membrane positive' represented 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.

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