identity genes overexpressed in metastatic tumor, the metastatic lesion was used as a tracer, and the primary tumor as a driver. Tracer and driver cDNA libraries were synthesized from 1 μg of total cellular RNA and the driver library was amplified by polymerase chain reaction (PCR) using a 5′ biotinylated primer. After hybridization of the tracer and driver libraries, hybrids were removed using streptavidin and the unbound fraction was amplified by PCR. The PCR product was ligated into a plasmid vector and then transformed into *Escherichia coli.*

Real-Time Quantitative Reverse Transcriptase–PCR

mRNA expression levels were determined by the real-time detection of PCR product from first-strand cDNA by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. The reaction mixture for real-time quantitative PCR contained 1 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of each primer, and synthesized cDNA. PCR amplifications were performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using a 96-well microplate and optical caps. Serial dilutions of the PCR products were used to generate a standard curve from which the amount of copy number of each gene in the sample was obtained. Quantitative reverse transcriptase-PCRs (RT-PCRs) were performed in triplicate for each sample and the mean value was determined. The calculated expression level of each gene was normalized to porphobilinogen deaminase (PBGD) and then expressed as a ratio of tumor to normal tissue. For the metastatic lesions, we used a ratio of the metastatic tumor to normal colon mucosa adjacent to the primary tumor of the same patient. We used the following primers for amplification: *CLN2* sense 5′-tccagcccctatgtcaccac-3′ and antisense 5′-agccacgggt-tacatcaaag-3′, generating a 424-base pair (bp) fragment; *CLN1* sense 5′-tgggggacaacatcaaggtgttt -3′ and antisense 5′-caggcggtcctgtgtgtacagg-3′, generating a 391-bp fragment; cathepsin (Cat)B sense 5′-actggggt-gacaatggcttct-3′ and antisense 5′-gtttggccaatccagtc-cttc-3′, generating a 234-bp fragment; CatD sense 5′-ttcagggcgagtacatgatcc-3′ and antisense 5′-ccctgtt-gttgtcacggtcaa-3′, generating a 249-bp fragment; CatH sense 5′-agaaccggcatctactccagtact-3′ and antisense 5′-gttgggggtcccagtggatctc-3′, generating a 335-bp fragment; CatL sense 5′-tgtggggcccatttctgttgcta-3′ and antisense 5′-catgcgccatccccagtcaagt-3′, generating a 326-bp fragment; PBGD sense 5′-tgctgtgggccaggggcctt-3′ and antisense 5′-atcttcatgctgggcagggaca-3′, generating a 283-bp fragment. Primers were designed to cross exon–intron boundaries to prevent amplification from contaminated chromosomal DNA. We verified that the PCR product was not detected when chromosomal DNA was used as a template.

Recombinant Protein Expression and Antibody Preparation

The coding sequence corresponding to amino acids 19–536 of CLN2 was amplified by RT-PCR using total cellular RNA from normal colon mucosa. The PCR product was digested with *Bgl* II and *Sal* I, which cut at restriction sites generated by the primers for amplification, and then ligated into a pQE80 plasmid (Qiagen). The bacterially expressed protein was purified using Ni-NTA agarose (Qiagen) and eluted with 200 mM imidazole. The protein was used to immunize rabbits to raise a polyclonal antibody.

Immunoblotting

Proteins were precipitated with tricholoacetic acid from tissue homogenates in guanidine isothiocyanate solution. The proteins were resolved on 10% sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nylon membrane. After transfer the membrane was incubated first with a rabbit polyclonal antibody raised against recombinant CLN2 at a dilution of 1:500 and then with an antirabbit immunoglobulin (Ig) G alkaline phosphatase conjugated secondary antibody (AP304A; Chemicon International, Temecula, CA) at a dilution of 1:10,000 followed by incubation with CDP-star (Roche Diagnostics, Tokyo, Japan) and detection with an Epipro image analyzer (Aisin, Aichi, Japan). For the detection of beta-actin, we used a monoclonal anti-beta-actin antibody (A5441; Sigma-Aldrich, Tokyo, Japan) at a dilution of 1:10,000 and an antimouse IgG alkaline phosphatase conjugated secondary antibody (AP124A; Chemicon) at a dilution of 1:10,000.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks of colorectal carcinomas were used for immunohistochemistry. After deparaffinization, rehydration, and heat-induced antigen retrieval, sections were incubated with a rabbit polyclonal antibody raised against a recombinant CLN2 protein, then treated with a biotinylated antirabbit IgG secondary antibody.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). StatView 5.0 software (SAS Institute Inc., Cary, NC) was used for statistical analysis. The Student *t*-test was used to assess differences in mRNA levels. *P*-values $< .05$ were considered significant.

TABLE 2
Comparison of mRNA Expression Levels between Primary Tumors and Liver Metastases in All Patients

	CLN2	CLN1	CatB	CatD	CatH	CatL
Primary*						
No.†	124	124	115	106	120	105
Mean	1.50	2.31	1.79	1.08	2.90	1.48
SEM	0.18	0.21	0.12	0.11	0.26	0.16
Metastasis‡						
No.†	29	27	26	26	27	25
Mean	2.70	4.60	3.45	3.16	3.03	2.77
SEM	0.58	1.14	0.37	0.98	0.50	0.53
Significance§ (*P*)	.012	.0012	< .0001	.0001	.839	.0029

CLN2: ceroid lipofuscinosis, neuronal 2; CLN1: ceroid lipofuscinosis, neuronal 1; CatB: cathepsin B; CatD: cathepsin D; CatH: cathepsin H; CatL: cathepsin L; SEM: standard error of the mean.
mRNA level of:
* Primary tumor is expressed as a ratio of tumor to adjacent normal tissue.
† Number of patients were not equal because of insufficient sample quantity.
‡ Metastatic lesion is expressed as a ratio of metastatic tumor to the normal colon mucosa adjacent to the primary tumor of the same patient.
§ Student *t* test for unpaired data.

TABLE 3
Comparison of mRNA Expression Levels of Lysosomal Enzymes between Primary and Metastatic Lesions in the Same Patient

	CLN2	CLN1	CatB	CatD	CatH	CatL
No. of patients*	29	27	26	26	27	25
Primary†						
Mean	1.93	1.79	1.85	1.24	2.68	1.24
SEM	0.61	0.20	0.20	0.31	0.62	0.27
Metastasis‡						
Mean	2.70	4.60	3.45	3.16	3.03	2.77
SEM	0.58	1.14	0.37	0.98	0.50	0.53
Significance§ (*P*)	.257	.0187	.0009	.0435	.530	.0218

CLN2: ceroid lipofuscinosis, neuronal 2; CLN1: ceroid lipofuscinosis, neuronal 1; CatB: cathepsin B; CatD: cathepsin D; CatH: cathepsin H; CatL: cathepsin L; SEM: standard error of the mean.
mRNA level of:
* Number of patients were not equal because of insufficient sample quantity.
† Primary tumor is expressed as a ratio of tumor to adjacent normal tissue.
‡ Metastatic lesion is expressed as a ratio of metastatic tumor to the normal colon mucosa adjacent to the primary tumor of the same patient.
§ Student *t* test for paired data.

RESULTS

Subtraction Cloning of Colorectal Carcinoma Metastasis

To identify genes that are overexpressed in colorectal carcinoma metastases, subtraction cloning was performed using the metastatic lesion as a tracer and the paired primary tumor as a driver. We then isolated a clone encoding CLN2. Real-time quantitative RT-PCR using the cDNAs used for the subtraction demonstrated a 20.9-fold increase in the metastatic lesion than in the primary tumor. After the confirmation of overexpression of *CLN2* in liver metastases in a small number of paired primary and metastasis samples, we then characterized the gene's expression along with other lysosomal enzymes.

Comparison of Lysosomal Enzyme Levels between Liver Metastases and Primary Tumors

Using real-time quantitative RT-PCR we examined the mRNA level of *CLN2*, along with that of other lysosomal enzymes, *CLN1*, and cathepsins B, D, and H. Due to insufficient sample quantities, the CatB, CatD, CatH, and CatL mRNA levels of primary tumors were not determined in 9, 18, 4, and 19 patients, respectively, and the *CLN1*, CatB, CatD, CatH, and CatL mRNA levels of metastatic lesions were not determined in 2, 3, 3, 2, and 4 patients, respectively. Table 2 shows the levels of mRNA expression of *CLN2* and other lysosomal enzymes in the liver metastases and primary tumors of all the examined patients. Apart from CatH, the mRNA levels of all the examined lysosomal enzymes were found to be significantly higher in the liver metastases than in the primary tumors. Among the lysosomal enzymes there were correlations between *CLN2* and *CLN1* (correlation coefficient [r] = 0.457; $P < .0001$) and between CatB and CatL ($r = 0.479$; $P < .0001$). Table 3 shows the comparison of mRNA levels in paired samples from the primary and liver metastasis of the same patients with synchronous ($n = 20$) or metachronous (TNM Stage II, $n = 5$ patients; Stage III, $n = 4$ patients) liver metastases. The mRNA levels of *CLN1* and cathepsins B, D, and L were significantly higher in the liver metastases than in the primary tumors. The percentage of patients with a metastasis to primary tumor ratio of greater than 2 were, in descending order: CatB, 57.7% (15 of 26 patients); CatL, 56.0% (14 of 25 patients); CatD, 50.0% (13 of 26 patients); *CLN1*, 48.2% (13 of 27 patients); *CLN2*, 31.0% (9 of 29 patients); and CatH, 18.5% (5 of 27 patients).

mRNA Levels of Lysosomal Enzymes in Primary Tumors

Table 4 shows the tumor to normal tissue ratios of mRNA levels of *CLN2* and other lysosomal enzymes in primary tumors. When the mRNA levels of lysosomal enzymes were compared between Stage I and Stage II tumors versus Stage III and Stage IV tumors, of those enzymes examined the mRNA levels of *CLN2* and cathepsin D in Stages III and IV tumors were significantly higher ($P = .015$ and $P = 0.031$, respectively) than those in Stages I and II tumors. When the mRNA levels of the lysosomal enzymes were compared between primary tumors with or without synchronous

TABLE 4
Expression of Lysosomal Enzymes in Primary Tumors

	CLN2	CLN1	CatB	CatD	CatH	CatL
TNM						
I and II						
No.*	63	63	59	56	61	56
Mean	1.06	2.05	1.80	0.85	3.11	1.47
SEM	0.09	0.27	0.18	0.10	0.39	0.25
III and IV						
No.*	61	61	56	50	59	49
Mean	1.95	2.58	1.79	1.34	2.69	1.49
SEM	0.35	0.33	0.17	0.21	0.34	0.20
Significance† (*P*)	.015	.212	.981	.031	.429	.969
TNM						
I-III						
No.*	96	96	89	80	94	80
Mean	1.22	2.33	1.80	0.98	2.87	1.53
SEM	0.10	0.24	0.15	0.10	0.28	0.20
IV						
No.*	28	28	26	26	26	25
Mean	2.44	2.25	1.77	1.40	3.03	1.32
SEM	0.71	0.46	0.23	0.33	0.66	0.30
Significance† (*P*)	.005	.872	.922	.108	.801	.600
Depth of tumor invasion						
pT1 and pT2						
No.*	32	32	30	27	31	27
Mean	1.07	1.81	1.82	1.08	3.04	1.42
SEM	0.12	0.29	0.30	0.18	0.65	0.39
pT3 and pT4						
No.*	92	92	85	79	89	78
Mean	1.65	2.49	1.78	1.08	2.85	1.50
SEM	0.24	0.27	0.13	0.14	0.28	0.18
Significance† (*P*)	.163	.161	.908	.992	.754	.825

CLN2: ceroid lipofuscinosis, neuronal 2; CLN1: ceroid lipofuscinosis, neuronal 1; CatB: cathepsin B; CatD: cathepsin D; CatH: cathepsin H; CatL: cathepsin L; SEM: standard error of the mean.
* Number of patients were not equal because of insufficient sample quantity.
† mRNA level of the primary tumor is expressed as a ratio of tumor to adjacent normal tissue. mRNA levels were compared using the Student *t* test for unpaired data.

liver metastases (Stage IV vs. Stages I, II, and III) only the mRNA levels of *CLN2* were found to be significantly higher ($P = .0049$) in tumors with synchronous liver metastasis than those without (Table 4). With regard to the depth of invasion of the primary tumor (pT1 and pT2 vs. pT3 and pT4), the mean values for *CLN1* and *CLN2* in pT3 and pT4 tumors were higher than those of pT1 and pT2, but the differences were not significant. There were no correlations noted between the mRNA levels of lysosomal enzymes with other clinicopathologic features including age, gender, tumor size, location, lymphatic invasion, venous invasion, and histology. When the mRNA expression levels of lysosomal enzymes were compared in pT3 tumors, the CatD mRNA level in Stages III and IV tumors was significantly higher than those in Stage II tumors ($P = .022$) and the *CLN2* level was significantly higher in tumors with synchronous liver metastases than in those without synchronous distant metastases ($P = .027$). Multivariate logistic regression analysis was performed to evaluate the relative importance of each risk factor for the occurrence of liver metastases. Factors included in the analysis were: age, gender, level of lysosomal enzymes (*CLN2*, *CLN1*, and cathepsins B, D, H, and L), venous invasion (negative vs.positive), lymphatic invasion (negative vs. positive), depth of invasion, tumor size, histology (well and moderately differentiated vs. poorly differentiated). The following factors were identified as significant, (*P* value and odds ratio [95% confidence interval (95% CI)]): depth of invasion, .0038, 5.89 (1.77–19.58); *CLN2*, .0023, 1.90 (1.26–2.86); and size, .0066, 1.03 (1.01–1.06). The *P*-values for the other factors were: age, .92; gender, .49; *CLN1*, .055; CatB, .61; CatD, .12; CatH, .79; CatL, .89; venous invasion, .55; lymphatic invasion, .99; and histology, .57. Multivariate logistic regression analysis was repeated on pT3 tumors, except for depth of invasion, and the following factors were found to be significant: *CLN2*, .012, 1.72 (1.13–2.64); and *CLN1*, .045, 0.75 (0.57–0.99). The *P*-values for the other factors were: age, .72; gender, .58; CatB, .61; CatD, .12; CatH, .96; CatL, .87; venous invasion, .35; lymphatic invasion, .99; and histology, .99.

mRNA Levels of Lysosomal Enzymes and Patient Survival

Patients were divided into 2 groups according to the median value of the ratio of tumor to adjacent normal mucosa, using the following threshold: *CLN2*, 1.03; *CLN1*, 1.67; CatB, 1.42; CatD, 0.75; CatH, 1.99; and CatL, 0.89. Using the Kaplan–Meier method, the prognosis of patients then was analyzed according to the level of *CLN2* ($n = 124$ patients), *CLN1* ($n = 124$ patients), CatB ($n = 115$ patients), CatD ($n = 106$ patients), CatH ($n = 120$ patients), and CatL ($n = 105$ patients). The analysis showed that there was no significant difference between the 2 groups for any lysosomal enzyme except *CLN2* (log-rank [Mantel–Cox] test, $P = .014$). When the prognosis of patients with pT3 tumors was analyzed according to the levels of *CLN2* ($n = 88$ patients), *CLN1* ($n = 88$ patients), CatB ($n = 80$ patients), CatD ($n = 75$ patients), CatH ($n = 84$ patients), and CatL ($n = 74$ patients) using the same method, once again the only difference between the two groups was seen with *CLN2* ($P = .026$). When the prognosis was compared in each clinical stage according to the level of expression, no difference was seen in any of the lysosomal enzymes.

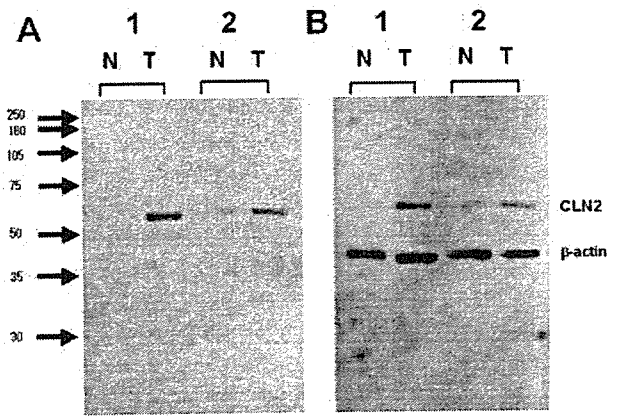

FIGURE 1. Characterization of antibody against ceroid lipofuscinosis, neuronal 2 (CLN2) by immunoblotting. (A) Expression of CLN2 was investigated using a polyclonal antibody raised against a recombinant CLN2 protein in paired tissue from the tumor (T) and adjacent normal mucosa (N) of individual patients. (B) Immediately after the detection of CLN2, the membrane was reprobed with an anti-beta–actin antibody. Molecular weight markers (in kilodaltons [kD]) are indicated by arrows on the left of the figure.

Antibody Preparation, Immunoblotting, and Immunostaining

We raised a polyclonal antibody against a recombinant CLN2 protein expressed in *E. coli*. Immunoblotting using this antibody and paired samples of tumor and adjacent normal mucosa showed a single, approximately 60-kilodalton (kD) species overexpressed in the tumor samples (Fig. 1). Using this antibody, we performed immunohistochemical staining in 20 cases of the primary colorectal carcinoma, and one case each of metastatic focus in the lymph node and in the liver. Essentially, CLN2 was detected in all of the lesions examined and positive reactivity was confined to the cytoplasm. Mucus-containing cells in the carcinoma and the normal epithelial cells remained unstained. Positive staining was observed in nearly all carcinoma cells regardless of the histologic structure (i.e., tubular, papillary, or cribriform patterns). However, carcinoma cells invading the muscular layer or subserosal tissue, the so-called 'invasive front,' showed a higher intensity of positive staining. Figure 2A shows positive cytoplasmic immunoreactivity in the primary tumor and very weak staining in the overlaying normal epithelial cells. Figure 2B shows intense immunoreactivity at the 'invasive front' of the carcinoma. Intense immunoreactivity was also observed in the tumor cells showing vascular invasion and in the infiltrating carcinoma cells in the subserosa (Fig. 2C) and in the metastatic carcinoma cells in the lymph node (Fig. 2D). In addition, immunostaining of the metastatic lesion of the liver showed strong immunoreactivity in metastatic cells and weaker expression in adjacent nonneoplastic hepatocytes (Fig. 2E).

DISCUSSION

CLN2 is a lysosomal enzyme with the combined enzymatic activity of tripeptidyl peptidase I (TPP I) and of an endopeptidase.[29] TPP I removes tripeptides from the N-terminus of peptides and it has been suggested that TPP I activity may play a role in the degradation of collagen before further degradation by dipeptidyl peptidase II.[30,31] Although various candidates for substrates of CLN2 have been reported, including subunit c of ATP synthase[32] and neuromedin B,[33] the natural substrate of CLN2 has not been identified to date. To our knowledge to date, many studies have implicated lysosomal proteases, the cathepsins, as being involved in cancer progression and metastasis.[1–3,6–18] Therefore, to elucidate the role of CLN2 in colorectal carcinoma progression and metastasis, we studied the expression level of *CLN2* along with that of cathepsins and compared their expression patterns in the same sample. Because several studies have revealed an implication of the proteins involved in NCL in tumor growth, we also studied the expression level of another causative gene of NCL, *CLN1*, in the same sample. *CLN1* encodes a lysosomal enzyme, palmitoyl-protein thioesterase, which removes fatty acids from fatty-acylated cysteine residues in proteins.[22]

Comparison of *CLN2* levels between liver metastases and primary tumors showed a significantly higher level in 29 liver metastases compared with 124 primary colon carcinomas. However, a pairwise comparison of the metastatic lesion and primary tumor within the same patient was not significant. Therefore, although we isolated a clone encoding CLN2 as a gene that is overexpressed in metastases by subtraction, we conclude that, among the lysosomal enzymes, *CLN2* expression is not specific to the metastatic lesions of colorectal carcinoma. Conversely, we found that mRNA levels of *CLN1* are significantly higher in the metastatic lesion than in the primary tumor in both samples obtained from the same patient and also within samples overall; this was also seen with cathepsins B, D, and L. It has been reported that *CLN1* overexpression in neuroblastoma cells protects against apoptosis.[27] Furthermore, it has been shown that an inhibitor of CLN1 killed cultured tumor cells and also enhanced the killing of tumor cells by chemotherapeutic drugs.[28] Therefore, the data presented in the current study indicate that CLN1 might be a molecular target for preventing the growth of metastatic cells of colorectal carcinoma.

To our knowledge to date, many studies have investigated the correlation between levels of lysosmal proteases and clinical features of colorectal carcinoma

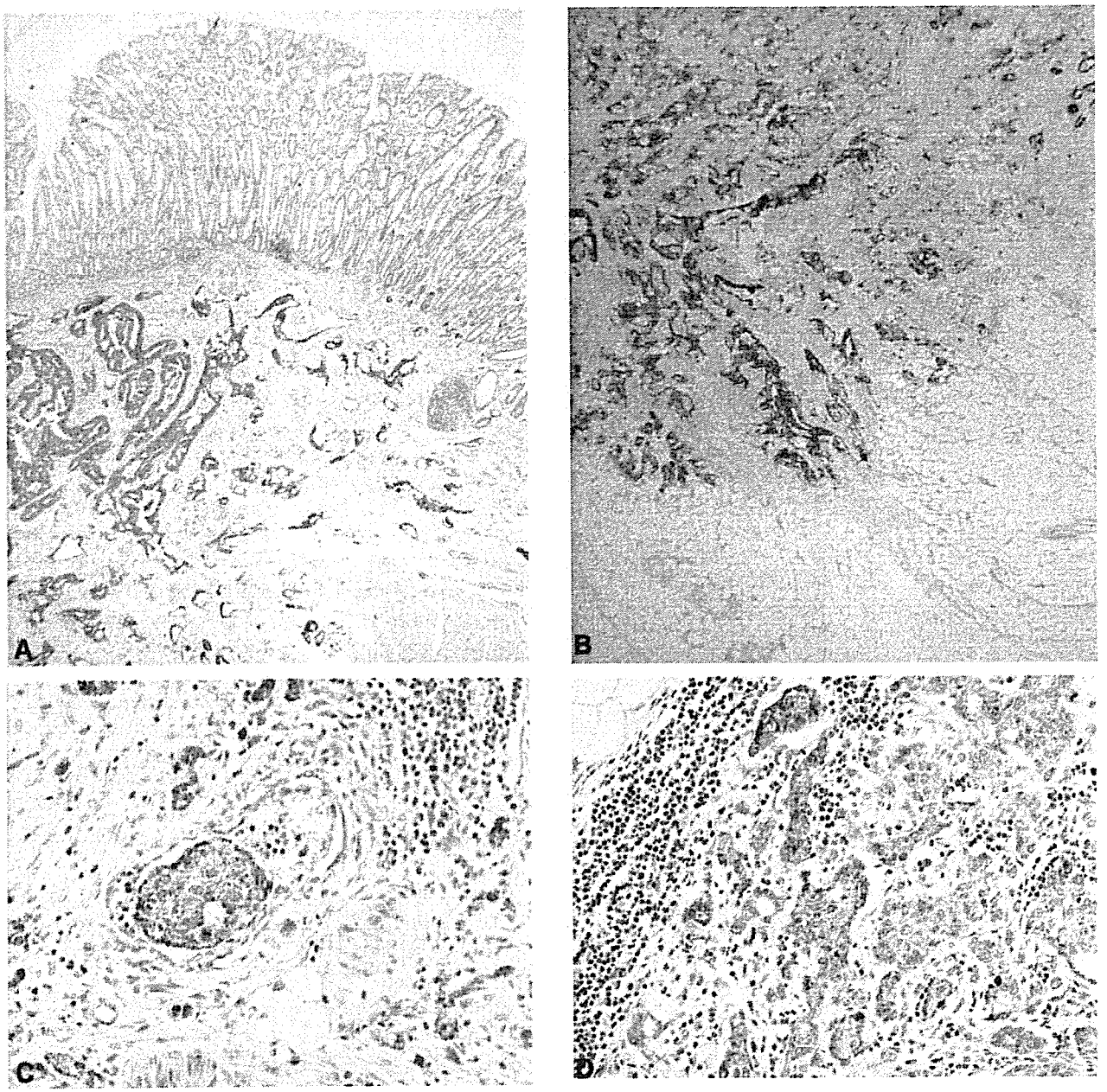

FIGURE 2. Immunostaining of ceroid lipofuscinosis, neuronal 2 (CLN2) in colorectal carcinomas. (A) Positive cytoplasmic staining was observed in the primary tumor with far weaker staining noted in adjacent normal epithelial cells. High expression of the CLN2 protein was demonstrated (B) at the invasive front of the tumor and in carcinoma cells demonstrating vascular invasion, as well as in (C) the infiltrating carcinoma cells in the subserosa. Positive staining of CLN2 was observed in metastatic carcinoma cells in (D) the lymph node and in (E) the liver. In liver metastasis, weaker but positive cytoplasmic staining also was observed in the nonneoplastic hepatocytes. Original magnification ×4 (A,B); ×40 (C); ×20 (D).

by various methods, including examination of enzyme activity,[5,6,9,10,14,17] antigen,[10,12,16,17] and mRNA[7,13] in tissue homogenates and immunostaining[8,13,15,16,18] of the enzyme. The outcomes, among studies using different methods and also among those using the same method, have been conflicting. In addition, to our knowledge no more than 3 lysosomal proteases have ever been compared in the same sample. For example, with respect to the occurrence of distant metastases in colorectal carcinoma, significantly higher antigen levels of CatB and CatL have been found in the tissue homogenate of tumors with liver metastases than in

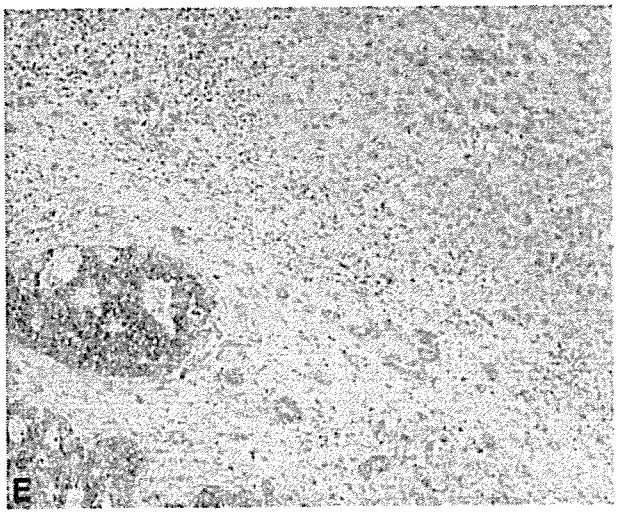

FIGURE 2. *(Continued)*

those without.[12] In addition, an immunostaining study demonstrated a significantly higher percentage of CatB-positive cases in a group of patients with liver metastases than in a group without.[13] In other studies, by contrast, significantly lower protein levels of CatB and CatL have been found in Dukes D stage tumors than in Dukes A and B stages.[11] Furthermore, whereas the tumor-to-normal ratios of CatB and CatL activities were not found to be increased in Dukes D stage, these ratios were significantly increased in other stages.[10] To our knowledge, the mRNA level of cathepsin has not been examined in a large set of colorectal carcinoma samples at various clinical stages. Studies to date have shown that the mRNA level of CatB has higher expression in early-stage (Dukes A and B) tumors compared with late-stage (Dukes C and D) tumors,[7] or has the highest expression level in Dukes A stage tumors.[13] In the current study, among the lysosomal enzymes examined, expression levels of *CLN2* and CatD were associated with advanced clinical stage, but only the *CLN2* level was associated with tumors with distant metastases. Further analysis in pT3 tumors, for a comparison at the same depth of invasion to eliminate this factor, also showed that only the *CLN2* level was associated with distant metastases. These results suggest that, compared with other lysosomal enzymes, *CLN2* is a good indicator of liver metastasis in colorectal carcinoma and that it might be involved in the actual metastatic process.

Analysis of an association between patient prognosis and the level of *CLN2* mRNA expression in tumors at the same clinical stage showed no significant difference. This result, together with our finding that *CLN2* expression was associated with the progression of clinical stage, indicates that *CLN2* expression is not an independent marker for patient survival.

We further analyzed the role of CLN2 by raising a polyclonal antibody against a bacterially expressed recombinant CLN2 protein and using it for immunohistochemistry. Immunoblotting using this antibody mainly recognized a single species of protein (of approximately 60 kD) highly expressed in the tumor. CLN2 is synthesized as a precursor protein and autodigested to produce a mature active enzyme of approximately 46 kD.[34] Therefore, the band recognized using this antibody is not a processed active protein and may correspond to the full-length or a partially processed precursor protein. Immunostaining using this antibody demonstrated diffuse positive staining in the adenocarcinoma cells, regardless of the histologic structure. However, more intense immunoreactivity was observed at the 'invasive front' of the primary tumor, in metastatic foci in the lymph node, and in the liver. Therefore, these expression patterns may suggest that CLN2 protein plays a role as a kind of matrix protease in invasion and in subsequent distant metastasis. In the case of liver with metastasis, normal hepatocytes adjacent to the tumor were diffusely stained. Using real-time quantitative RT-PCR, we also detected moderately elevated expression in several samples of normal hepatocytes adjacent to the tumor (data not shown). Although to our knowledge the precise mechanism underlying this elevation of expression is not known, the possibility that the enzyme might be induced by the metastatic tumor cells cannot be ruled out.

The results of the current study have demonstrated that *CLN2*, which is one of the causative genes for the neurodegenerative disorder NCL and encodes tripeptidyl-peptidase I (TPP I), was overexpressed in colorectal carcinomas in the late stage of tumor progression and especially in primary tumors with synchronous metastases. In addition, we also found that another causative gene of NCL, *CLN1*, was significantly overexpressed in metastatic lesions of colorectal carcinoma. Although to our knowledge, the precise roles of CLN2 and CLN1 in cell viability and growth have not been elucidated to date, these findings indicate that the expression of these lysosomal enzymes is a good indicator for tumor progression and metastasis, and that these genes may be potential molecular targets.

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Short Time to Recurrence After Hepatic Resection Correlates with Poor Prognosis in Colorectal Hepatic Metastasis

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Background: Early recurrence is a major problem after hepatic resection of colorectal hepatic metastasis (CHM). Our aim was to investigate the relationship between time to recurrence after CHM resection and overall survival.
Methods: A retrospective analysis was performed for 101 consecutive patients who underwent hepatic resection for CHM and have been followed more than 5 years.
Results: Among 101 patients, 82 (81%) had a recurrence. Overall survival of patients with recurrence within 6 months after CHM resection was significantly worse than that of patients with recurrence after more than 6 months ($P < 0.01$). Overall survival was poorer when time to recurrence was shorter. One of the reasons for poor prognosis of patients with recurrence within 6 months was that only a few patients could undergo a second resection for recurrence after CHM resection. Histological type, including poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor, bilobar metastases, microscopic positive surgical margin and carcinoembryonic antigen (CEA) above 15 ng/ml had predictive value for decreased recurrence-free survival after CHM resection.
Conclusion: Short time to recurrence after CHM resection correlates with a poor prognosis. Histological type of poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor might be a predictor for early recurrence after CHM resection.

Key words: colorectal cancer – hepatic metastasis – resection – recurrence

INTRODUCTION

Hepatic resection is currently the only potentially curative treatment for colorectal hepatic metastasis (CHM) (1–6). However, frequent recurrence is a major problem after surgery, with 80–85% of patients experiencing a recurrence (2,3,6). Thus, reduction of recurrence is necessary to improve prognosis after CHM resection.

A correlation between a short time to recurrence after resection of the primary tumor and poor prognosis after resection of recurrence has been demonstrated in colorectal cancer (2,5), breast cancer (7), hepatocellular carcinoma (8) and renal cell carcinoma (9). In CHM, however, the correlation between time to recurrence after resection for CHM and prognosis is still obscure. The relation between time to recurrence after resection and prognosis is complicated in CHM because many recurrences after CHM resection can be resected, and resection sometimes contributes to long-term survival (10–12).

This study was conducted to determine the correlation between time to recurrence after CHM resection and prognosis by scrutinizing recurrence after CHM resection, which may suggest the best timing for adjuvant chemotherapy and elucidate whether time to recurrence can be a surrogate endpoint for adjuvant study in resectable CHM. We also compared clinicopathological factors and time to recurrence to find out preoperative predictive factors for early recurrence.

PATIENTS AND METHODS

Patient Population

A total of 101 patients who had undergone hepatic resection for CHM at the National Cancer Center Hospital East between September 1992 and January 2000 and have been followed precisely for more than 5 years were examined retrospectively. The patients consisted of 56 (55%) men and 45 (45%) women, ranging in age from 23 to 78 years (mean, 60 years). None of the patients had received adjuvant chemotherapy after primary colorectal resection.

The criteria for hepatectomy were as follows: metastatic lesions were confined to the liver and all lesions could be

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resected using oncologic principles while preserving liver function. Extended lobectomy plus partial resections were considered as the upper limit of hepatectomy that could be performed safely, and trisegmentectomy was applied only when the volume of the residual liver was deemed to be abundant. Neither the number of metastatic tumors nor tumor size, in themselves, excluded patients from hepatectomy.

No patient received adjuvant therapy after CHM resection.

Surgical Procedure

After laparotomy, a careful search was performed for local recurrences, extrahepatic metastases and peritoneal dissemination in the abdominal cavity. Any suspicious lesions were examined by biopsy. If the regional lymph nodes (hepatoduodenal or peripancreatic lymph nodes) were positive, dissection of the regional lymph nodes was performed. Intraoperative bimanual liver palpation and ultrasonography were performed to confirm tumor location and size of the lesions in all patients; all resections were ultrasound-guided procedures. Hepatic resection was performed with tumor-free resection margins using the forceps fracture method under inflow occlusion (Pringle's maneuver).

Clinical Follow-up

After hepatic resection, patients were closely followed up with diagnostic imaging (chest X-ray and abdominal CT every 3 months, measurement of serum carcinoembryonic antigen (CEA) levels every month and annual colonoscopy to detect tumor recurrence) up to 5years. After 5 years patients were followed up every 6 months or annually.

Morphologic Investigations

The resected colorectal specimens and hepatic specimens were fixed in 10% phosphate-buffered formalin and cut at intervals of 5 mm and 10 mm, respectively, and then embedded in paraffin. Serial sections of 3 μm thickness were stained with hematoxylin and eosin for morphologic examination. Histological diagnosis was performed according to the World Health Organization intestinal tumor classification (13).

Statistical Analysis

The chi-square test and student *t*-test were used to compare data (Dukes' stage, primary location, positive regional lymph node, size of tumor, number of tumors, synchronous/metachronous, tumor distribution and ratio of recurrence) between subgroups based on time to recurrence. Mann–Whitney's *U*-test was used to compare preoperative serum CEA level between subgroups. Analyses of survival were performed using the Kaplan–Meier method (14), and differences between the curves were tested using the log-rank test. The log-rank test was also used to examine the significance of associations between survival curves and CEA cutoff values of 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 and 200 ng/ml.

Factors related to survival were analyzed with the Cox proportional hazards regression model (15). A P-value of <0.05 was considered statistically significant.

RESULTS

Surgical Resections

Partial resection was performed on 47 patients, subsegmentectomy on 9, segmentectomy on 25, lobectomy on 11, extended lobectomy on 6 and trisegmentectomy on 3 according to Couinaud's anatomical classification (16). A microscopic positive surgical margin was observed in 14 patients. There was no perioperative mortality. Twenty-one complications were observed: 7 cases of biliary leak; 6 cases of intra-abdominal abscess; 4 cases of wound infection; and 1 case each of liver failure, ileus, lung abscess and urinary tract infection.

Survival After CHM Resection

The overall 5-year Kaplan–Meier survival rate after hepatic resection for CHM was 42%, with a median survival of

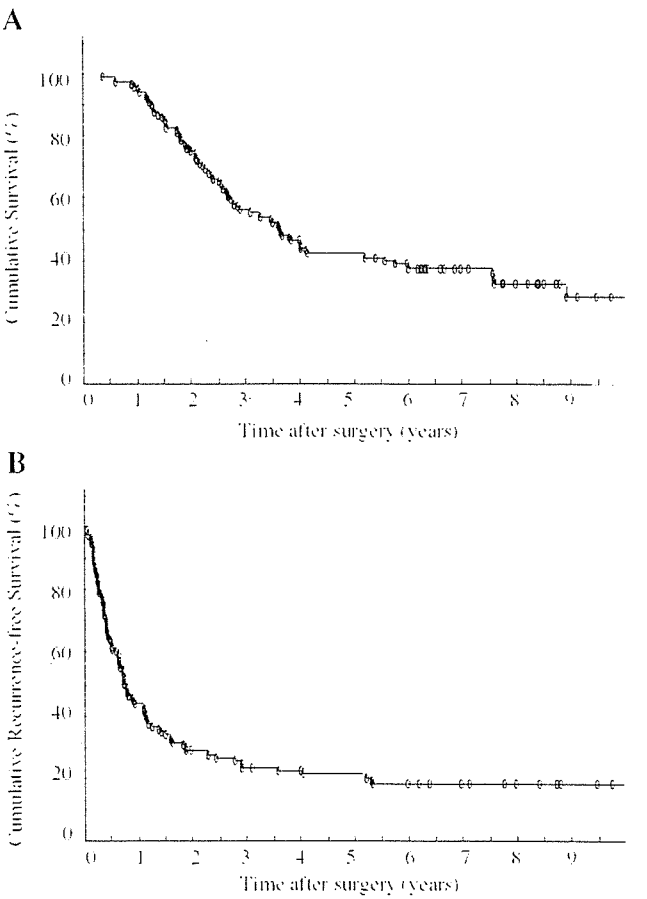

Figure 1. Cumulative survival (A) and recurrence-free survival curves (B) for 101 patients with resected colorectal hepatic metastasis

34 months (Fig. 1A). Recurrence-free 1-, 3- and 5-year survival rates were 43, 23 and 21%, with a median recurrence-free survival of 9 months (Fig. 1B). The median follow-up duration of survivors was 87 months.

Recurrences After CHM Resection (Fig.2)

Among the 101 patients who underwent CHM resection, 82 (81%) developed recurrences. Locations of recurrences were as follows: liver in 36 patients, lung in 17, both liver and lung in 9, lymph node in 6, peritoneum and local recurrence in 4 each, brain and adrenal gland in 2 each, and ovary and bone in 1 each. Thirty-seven recurrences (45%) occurred within 6 months after hepatic resection and 72 recurrences (88%) occurred within 2 years. The ratio of hepatic recurrences to total recurrences was significantly higher in 1st–12th month than that after 12th month from CHM resection ($P = 0.01$). The ratio of pulmonary recurrence and that of recurrence in organs other than the liver and lung were significantly higher after 24th month ($P < 0.05$) and in 13th–24th month ($P < 0.05$) from CHM resection, respectively, than those in the other period. Of the 82 patients with recurrence after hepatic resection 36 received re-resection. Re-resection could be performed in only 10 of 24 patients (42%) whose recurrence occurred in the liver or lung within 6 months after hepatic resection, whereas re-resection could be performed in 22 of 29 patients (76%) whose recurrence occurred in the liver or lung more than 6 months later ($P = 0.01$). Of the remaining 46 patients, 33 received systemic chemotherapy, 7 received hepatic arterial infusion, 2 received radiation therapy and 4 received best supportive care.

Clinicopathological Features According to Time to Recurrence

Table 1 summarizes the primary and metastatic tumor characteristics. Patients were classified into three subgroups according to time to recurrence after hepatic resection as follows: no recurrence, recurrence within 6 months and recurrence after more than 6 months. There were no significant differences in primary tumor characteristics between the three subgroups. All patients in the no recurrence group had a primary tumor that was classified as a well- or moderately differentiated carcinoma.

In terms of characteristics of the metastatic tumor, the number of tumors was significantly less ($P < 0.01$) and unilobar distribution was seen significantly more frequently ($P < 0.01$) in the no recurrence group compared with the other subgroups.

Survival According to Time to Recurrence

Kaplan–Meier curves for overall survival after CHM resection according to time to recurrence in patients who developed recurrences are shown in Fig. 3A. Patients were divided into four subgroups according to time to recurrence after hepatic resection as follows: within 6 months, 7th–12th month, 13th–24th month and after 24th month. Overall survival of

Resection n=101

Recurrence n=82

Time to recurrence	n	%	Location			
			Liver (resected case)	Lung	Liver + Lung	Others
6 months	37	45.1	19 (8)	5 (2)	6 (0)	7 (1)
7–12 months	20	24.4	11 (7)	3 (2)	2 (1)	4 (1)
13–24 months	15	18.3	3 (3)	3 (2)	1 (0)	8 (1)
25 months	10	12.2	3 (3)	6 (5)		1 (0)

Figure 2. Locations of recurrence according to time to recurrence after resection of colorectal hepatic metastasis. The number of resected cases for the recurrence is shown in parentheses.

Table 1. Clinicopathological findings of 101 patients with colorectal hepatic metastases according to time to recurrence

Variable	No recurrence (19)	Recurrence within 6 months (37)	Recurrence after more than 6 months (45)	*P*-value*
Primary colorectal tumor				
TNM Classification				0.63
I	1	1	2	
II	4	11	6	
III	10	12	21	
IV	4	13	16	
Location				0.85
Rectum	4	7	17	
Colon	15	30	28	
Number of positive lymph nodes (mean ± SD)	1.3 ± 2.1	2.3 ± 3.8	1.4 ± 1.7	0.29
Histological type of adenocarcinoma				
Well- or moderately differentiated	19	33	42	
Poorly differentiated signet ring cell or mucinous	0	4	3	
Hepatic metastases				
Maximum size of tumor (mean ± SD, cm)	4.5 ± 3.1	3.6 ± 2.1	4.3 ± 3.3	0.26.
Number of tumors (mean ± SD)	1.3 ± 0.6	2.5 ± 1.6	1.9 ± 1.4	<0.01
Preoperative CEA level (mean ± SD, ng/ml)	264.0 ± 818.0	41.3 ± 53.8	220.7 ± 879.7	0.25
Synchronous/metachronous				0.94
Synchronous	7	14	18	
Metachronous	12	23	27	
Distribution of metastases				<0.01
Unilobar	18	20	29	
Bilobar	1	17	16	

SD, standard deviation; CEA, carcinoembryonic antigen.
*Difference between patients with no recurrence and those with recurrence within 6 months.

patients with recurrence within 6 months after resection was significantly worse than that of patients with recurrence in 7th–12th month ($P = 0.04$), that of patients with recurrence in 13th–24th month ($P < 0.01$) and that of patients with recurrence after 24th month ($P < 0.01$). Overall 5-year survival rate in patients who developed recurrence within 6 months after hepatic resection was only 10% with a median survival of 26 months. Overall survival was poorer when time to recurrence was shorter.

Figure 3B shows overall survival after recurrence according to time to recurrence. Overall survival after recurrence of patients with recurrence within 6 months after resection was still worse than that of patients with recurrence in 13th–24th month ($P < 0.04$) and that of patients with recurrence after 24th month ($P < 0.03$). Overall survival after recurrence of patients with recurrence in 7th–12th month after resection seemed to be better than that of patients with recurrence within 6 months, but the difference was not significant ($P = 0.14$). Survival after recurrence tended to be poorer when time to recurrence was shorter. Overall survival after recurrence of patients with recurrence within 6 months after resection was significantly worse than that of patients with recurrence in more than 6 months ($P < 0.01$).

Correlation Between Clinicopathological Factors and Recurrence-free Survival

To find prognostic factors for recurrence-free survival after CHM resection, correlations between clinicopathological factors and recurrence-free survival were analyzed (Table 2). Histological type of tumor, including poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor ($P < 0.01$) (Fig. 4), two or more hepatic tumors ($P < 0.01$), bilobar distribution ($P < 0.01$), microscopic positive surgical margin ($P = 0.03$) and CEA level before hepatic resection above 15 ng/ml ($P = 0.04$) were significantly associated with poor recurrence-free survival.

We examined the independent predictive value of the aforementioned factors in recurrence-free survival. Data were analyzed using a Cox regression model (Table 3). Histological type of poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor [$P < 0.01$;

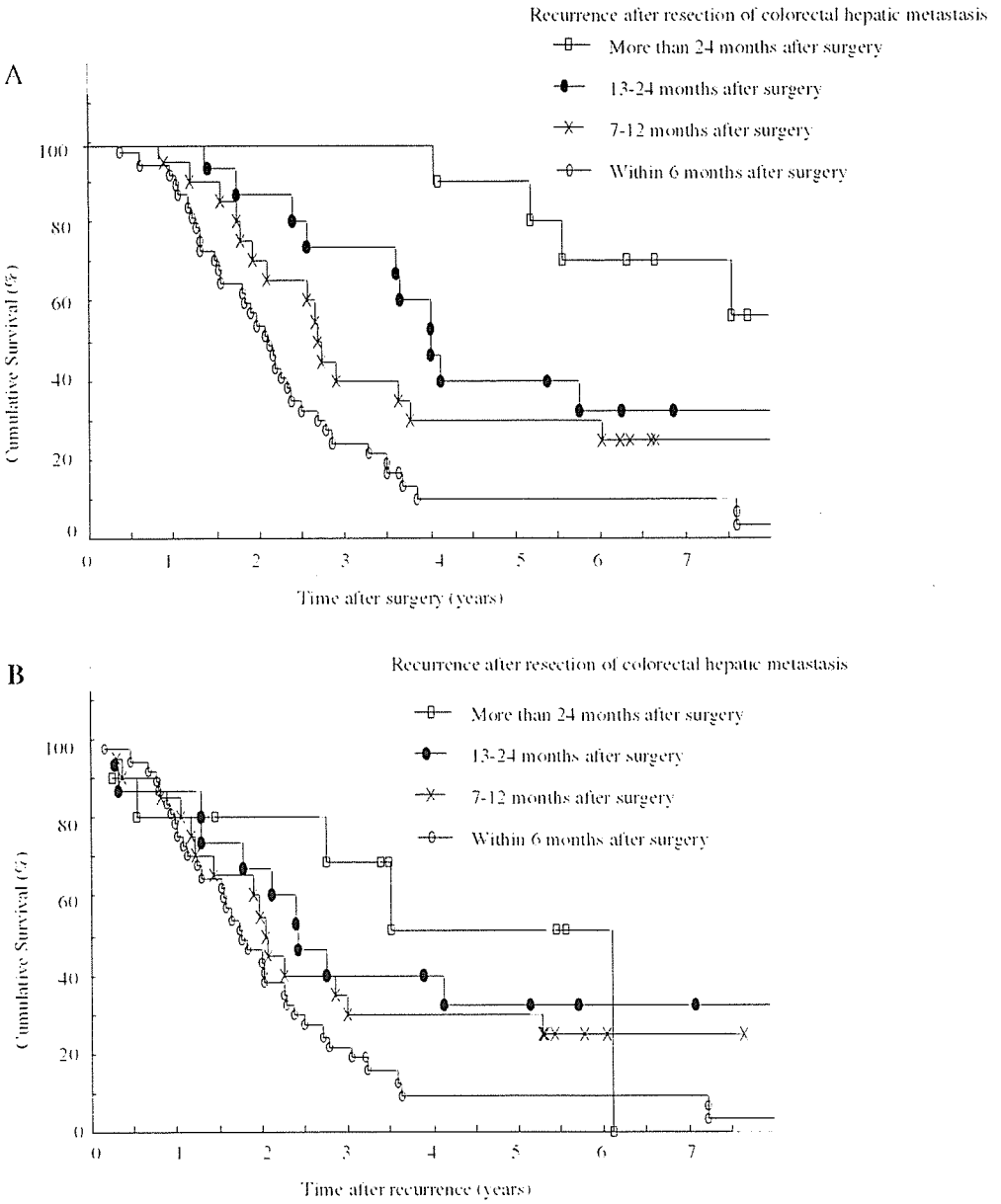

Figure 3. (A) Cumulative survival curves after resection of colorectal hepatic metastasis according to the time to recurrence. (B) Cumulative survival curves after recurrence after resection of colorectal hepatic metastasis according to the time to recurrence.

relative risk (RR) = 5.16; 95% confidence interval (CI), 2.10–12.69], bilobar metastases (P = 0.04; RR = 2.73; 95% CI, 1.03–7.27), microscopic positive surgical margin (P = 0.03; RR = 2.25; 95% CI, 1.11–4.59) and CEA level above 15 ng/ml (P = 0.02; RR = 1.96; 95% CI, 1.09–3.55) had a predictive value for decreased recurrence-free survival after CHM resection. Median disease-free survivals and 1-year recurrence rates of patients with the aforementioned factors were 4.6, 5.6, 5.0 and 8.4 months and 100, 70, 79 and 65%, respectively.

Histological type of poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor and CEA level above 15 ng/ml were also the poor prognostic factors for overall survival (data not shown).

DISCUSSION

The goal of this study was to assess the correlation between time to recurrence after CHM resection and prognosis. Results showed that prognosis of patients with recurrence within 6 months after resection was significantly worse than that of patients with recurrence after more than 6 months. Our findings indicate that short time to recurrence after CHM resection correlates with a poor prognosis.

The main reason for poor prognosis of patients with recurrence within 6 months was that only a few patients could undergo a second resection for recurrence after CHM resection. Most patients who could not undergo a second resection

Table 2. Correlation between clinicopathological factors and disease-free survival after hepatectomy for colorectal hepatic metastases

Variable	No. of patients	Median disease-free survival (months)	*P*-value
Primary colorectal lesion			
Location			
Colon	73	9.0	0.67
Rectum	28	9.5	
TNM Classification			
I, II	25	6.2	0.87
III, IV	76	9.6	
Lymph node metastasis			
Absent	35	9.0	0.79
Present	66	9.5	
Histological type of adenocarcinoma			
Well- or moderately differentiated	94	11.3	<0.01
Poorly differentiated signet ring cell or mucinous	7	5.1	
Hepatic metastases			
Number of tumors			
Solitary	58	13.6	<0.01
≥2	43	5.9	
Maximum size of the tumor (cm)			
<5	77	9.0	0.58
≥5	24	13.4	
Distribution of metastases			
Unilobar	67	13.5	<0.01
Bilobar	34	5.7	
Microscopic surgical margin			
Negative	87	10.3	0.03
Positive	14	6.4	
CEA level before treatment (ng/ml)			
<15	47	15.4	0.04
≥15	54	8.4	
Synchronous/metachronous			
Synchronous	39	9.1	0.84
Metachronous	62	9.3	
Interval between colorectal resection and hepatectomy			
<1 year	65	7.8	0.11
≥1 year	36	13.5	

CEA, carcinoembryonic antigen.

had extensive disease such as hepatic or pulmonary recurrence with much tumor burden, recurrence involving multiple organs, or distant metastases outside liver and lung that were not suitable for resection. In this series, re-resection rates of recurrence in the remnant liver and lung were relatively low (42 and 40%, respectively) when recurrences were observed within 6 months after CHM resection, whereas they were high (76 and 75%, respectively) when recurrences were observed more than 6 months after resection.

Tumor doubling time is correlated with prognosis in various cancers (17–20). In CHM, it has been reported that short tumor doubling time is a poor prognostic factor for both overall and disease-free survival (21). Short time to recurrence represents short tumor doubling time. Those results are in accord with those of the present study.

Our results suggest that recurrence-free survival can be a surrogate endpoint for adjuvant trial in resectable CHM. Moreover, recurrence within 6 months should be a major target for additional chemotherapy because of a great number and the poor prognosis of these patients. Theoretically, if we can determine which patients will have a recurrence with short recurrence-free survival, we could identify which ones would possibly benefit from neoadjuvant chemotherapy. Adam *et al.* (22) showed efficacy of neoadjuvant chemotherapy for CHM patients with four or more tumors regardless of initially resectable or not, as long as objective tumor response or stabilization was achieved by chemotherapy, and demonstrated the possibility of neoadjuvant chemotherapy for resectable CHM. However, neoadjuvant chemotherapy sometimes causes chemotherapy-associated steatohepatitis which may increase operative morbidity (23,24); then, neoadjuvant chemotherapy should be recommended for high-risk patients for recurrence.

In the present study, histological type of poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor, bilobar metastases, microscopic positive surgical margin and CEA above 15 ng/ml were the independent prognostic factors for poor recurrence-free survival. Especially, histological type of poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor exhibited the strongest power for predicting early recurrence because all patients with the factor had recurred within 10 months. Then, histological type of poorly differentiated signet ring cell or mucinous adenocarcinoma in the primary tumor, which was not considered in other large studies (2,5), should be considered as one of the preoperative predictors of early recurrence after CHM resection. Patients with the factor are recommended to receive neoadjuvant chemotherapy. Bilobar metastases and CEA above 15 ng/ml were also prognostic factors for recurrence; however, long-term recurrence-free survival was achieved in some patients with the factors. Neoadjuvant chemotherapy for patients with either of the factors is controversial. In addition, considering the correlation between positive surgical margin and early recurrence, hepatic surgeons should pay much attention to keep negative surgical margin during hepatic dissection in order to prevent early recurrence.

In a retrospective analysis of consecutive 1001 CHM patients by Fong *et al.* (5), poor prognostic factors for recurrence after CHM resection were positive surgical margin, extrahepatic disease, node-positive primary, less than

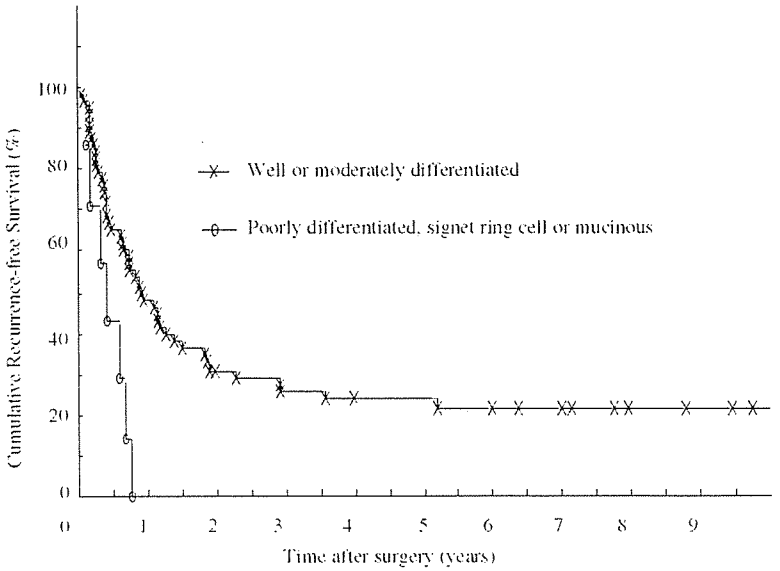

Figure 4. Recurrence-free survival curves after resection of colorectal hepatic metastasis according to the histological type of primary tumor.

Table 3. Multivariate analyses of factors affecting disease-free survival after hepatectomy for colorectal hepatic metastases

Variable	Relative risk (95% CI)	*P*-value
Primary colorectal lesion		
Histological type of adenocarcinoma		
Well- or moderately differentiated	–	<0.01
Poorly differentiated signet ring cell or mucinous	5.16 (2.10–12.69)	
Hepatic metastases		
Number of tumors		
Solitary	–	0.60
≥2	1.29 (0.50–3.38)	
Distribution of metastases		
Unilobar	–	0.04
Bilobar	2.73 (1.03–7.27)	
Microscopic surgical margin		
Negative	–	0.03
Positive	2.25 (1.11–4.59)	
CEA level before treatment (ng/ml)		
<15	–	0.02
≥15	1.96 (1.09–3.55)	

CI, confidence interval; –, reference.

12 months of disease-free interval from the primary resection, 2 or more tumors, tumor size >5 cm and CEA >200 ng/ml. The aforementioned prognostic factors for recurrence were also predictors of poor overall survival, and the fact was consistent with the concept of our results that short time to recurrence correlated with poor survival. Fong *et al.* proposed a scoring system using five poor prognostic factors and insisted that the scoring system was useful in choosing adjuvant therapy.

The difference between our results and those of Fong's might be partly due to patients' background and the number of patients examined. In the present study, patients with extrahepatic disease were excluded because CHM with extrahepatic disease was totally different from pure CHM considering pathways of metastases. Moreover, none of the patients had received adjuvant chemotherapy after primary colorectal resection or CHM resection. However, the possibility that not all of Fong's predictors could be validated well because of relatively small population of our study cannot be ruled out.

In the present study, patients were followed and examined precisely at least for 5 years in order to elucidate complete profile of recurrence, and then median follow-up of survivors was 87 months. This study has clarified frequencies of the recurrences after CHM resection in liver, lung and other organs respectively according to time to recurrence and also clarified the resection-rates for those recurrences. On the result of the present study, the organ where recurrence had occurred most frequently and the resection-rate for the recurrences differed according to time to recurrence after CHM resection. Frequency of hepatic recurrence decreased rapidly after 2 years of CHM resection; however, that of pulmonary recurrence was not low even more than 2 years after CHM resection. A periodical checkup by chest XP or chest CT adding to abdominal examination is recommended for 5 years at least.

In conclusion, short time to recurrence after CHM resection correlates with a poor prognosis. This result provides grounds for proposal that an effective neoadjuvant chemotherapy and a system using the clinicopathological factors and

pharmacogenetics which identify best candidates for the neoadjuvant chemotherapy are needed in order to reduce early recurrence. Histological type of primary tumor might be a strong predictor for early recurrence after CHM resection.

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Importance of intra-individual variation in tumour volume of hepatic colorectal metastases

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Abstract

Aims: The efficacy of surgical resection for multiple colorectal hepatic metastases (MCHM) has been controversial. We examined the survival of patients who received surgery for MCHM and examined the factors associated with survival.
Methods: A retrospective analysis was performed of 50 consecutive patients who received hepatic resections for MCHM, defined as four or more metastatic lesions of colorectal cancer.
Results: Overall survival after hepatic resection for MCHM was 48% at 3 years and 43% at 5 years (median survival, 22.3 months). Multivariate analyses revealed that a coefficient of variation (CV) in volume of hepatic metastases in each individual patient above 1.8 ($P = 0.01$, HR = 4.08, 95% CI = 1.33–12.5) was the only poor prognostic factor after resection of MCHM.
Conclusions: A CV in volume of hepatic metastases in each individual patient above 1.8 predicts poor survival after hepatectomy of MCHM. Thus, the CV in volume of hepatic metastases in each individual patient might be useful in planning the therapeutic strategy for patients with MCHM.

Keywords: Colorectal cancer; Hepatic metastases; Resection; Tumour volume; Coefficient of variation

Introduction

Hepatic resection is currently the only potentially curative treatment and the first-line therapy for colorectal hepatic metastasis.[1–5] The efficacy of hepatic resection has been reported for some cases of multiple colorectal hepatic metastases (MCHM); Bolton et al. reported that the survival of patients who underwent resection of more than four and/or bilobar hepatic metastases was equivalent to that of patients who underwent resection of fewer than four and unilobar hepatic metastases.[6] Nevertheless, hepatic resection for MCHM has been controversial because several reports demonstrated that having fewer lesions is a favorable prognostic factor after hepatic resection of colorectal hepatic metastases.[5,7–13]

Therefore, this study was conducted to evaluate the efficacy of resection for MCHM and elucidate any prognostic factors that could identify the patients who would benefit from surgical resection for MCHM. We focused on the histology of the tumour, tumour volume ratio (tumour volume/whole liver volume), and dispersion (coefficient of variation) of volume of hepatic metastases in each patient. We defined MCHM as four or more metastatic lesions of colorectal cancer of the liver, because four metastases corresponds to the limit of surgical resectability most widely used during the past decade.[6,14]

Patients and methods

Definition of MCHM

MCHM was defined as four or more metastatic lesions of colorectal cancer in the liver. Patients who showed any

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metastatic lesion outside the liver were excluded from the MCHM group. The diagnosis of MCHM was confirmed by diagnostic imaging before treatment.

Patient population

The records of 370 patients who had undergone hepatic resection for colorectal hepatic metastasis at the National Cancer Center Hospital East between September 1992 and August 2005 were examined retrospectively. Fifty of these patients met the criteria for MCHM. The patients consisted of 34 men and 16 women, ranging in age from 44 to 85 years, with a mean age of 60 years. Two of the patients had received oral uracil/tegafur and five had received 5-fluorouracil (5-FU)-leucovorin (LV) as adjuvant chemotherapy after primary colorectal resection. Few use of adjuvant chemotherapy after primary colorectal resections in our series ascribed to the fact that adjuvant chemotherapy has been rarely used after primary colorectal resections in our institution until 2002 although all patients with stage III colorectal cancer has received either 5-FU-LV or oral uracil/tegafur-LV since 2002.

The criteria for hepatectomy were as follows: metastatic lesions were confined to the liver and all lesions could be resected using oncologic principles (tumour-free margin and no residual disease) while preserving liver function. Basically, extended lobectomy plus partial resections was considered as the upper limit of hepatectomy that could be performed safely, and trisegmentectomy was applied only when the volume of the residual liver was deemed to be thoroughly abundant. Neither the number of metastatic tumours nor tumour size alone excluded patients from hepatectomy.

Irinotecan/5-FU/LV has been administered after hepatic resection of colorectal metastasis since 2003 when patients want to receive adjuvant chemotherapy; 9 patients in this study received the adjuvant therapy.

Operative procedure

After laparotomy, a careful search was performed for local recurrence, extrahepatic metastases, and peritoneal dissemination in the abdominal cavity. Any suspicious lesions were examined by biopsy. If metastasis in the regional lymph nodes (hepatoduodenal or peripancreatic lymph nodes) was suspected by preoperative imaging diagnosis or intraoperative findings, dissection of the regional lymph nodes was performed. Intraoperative bimanual liver palpation and ultrasonography were performed to confirm tumour location and size of the lesions in all patients, and all of the resections were ultrasound-guided procedures. Hepatic resection was performed with tumour-free resection margins by the forceps fracture method under inflow occlusion (Pringle's maneuver). Blood loss and operative time were recorded.

Clinical follow-up

After hepatic resection, patients were closely followed up with diagnostic imaging [chest X-ray and abdominal computed tomography (CT)] every 3 months, measurement of serum carcinoembryonic antigen (CEA) levels every month, and an annual colonoscopy to detect any tumour recurrence. The median follow-up duration of survivors was 27 months.

Measurement of tumour volume

Tumour volumes were obtained from helical CT scans of the abdomen, which were performed in all patients before initial treatment using 5-mm collimation after administration of 120 cc of non-ionic intravenous contrast injected at 2 cc per second with a 60-s delay. Images were reconstructed at 5-mm intervals using a standard soft-tissue algorithm.

Metastatic lesions and the whole liver were outlined manually on each axial slice using a computer mouse. The volume of metastatic lesions and that of whole liver were calculated automatically by multiplying the sum of the areas from each slice by the reconstruction interval. Then, tumour volume ratio was calculated (volume of tumour/volume of whole liver × 100%). All measurements were made by one radiologist.

For statistical analysis of inter-tumour variability in volume, in other words, dissimilarity in volume of metastases in each single patient, the coefficient of variation (CV; SD of the mean divided by the mean) was calculated for each case.

Histological parameters

The resected colorectal specimens and hepatic specimens were fixed in 10% phosphate-buffered formalin and cut at intervals of 5 mm and 10 mm, respectively, and then embedded in paraffin. Serial sections of 3-μm thickness were stained with hematoxylin and eosin (H&E) for morphological examination. Each case was histologically classified according to the histological type, tumour size, location, number of metastases, presence of serosal invasion, nodal status, and margin status. Histological diagnosis was performed according to the World Health Organization intestinal tumour classification.[15]

Statistical analysis

Analyses of survival were performed using the Kaplan–Meier method[16] and differences between the curves were tested using the log-rank test. The log rank test was also used to examine the significance of associations between survival curves and the following: CEA cutoff values 10 ng/ml, 20 ng/ml, 30 ng/ml, 50 ng/ml, 70 ng/ml, 100 ng/ml, and 200 ng/ml; tumour volume ratio cutoff values

1%, 3%, 5%, 8%, 10%, and 20%; and CV in tumour volume cutoff values 1.2, 1.4, 1.6, 1.8, and 2.0. Factors related to survival were analyzed with the Cox proportional hazards regression model.[17] A P value of less than 0.05 was considered to denote significance.

Results

Clinicopathological features of patients with MCHM

Fifty patients underwent resection of MCHM at the National Cancer Center Hospital East. Table 1 summarizes the primary and metastatic tumour characteristics. Four liver tumours were found in 20 patients, 5 tumours in 12, 6 tumours in 8, 7 and 8 tumours in 3 each, 9 tumours in 2, and 10 and 11 tumours in 1 each. Neither hepatoduodenal nor peripancreatic lymph node metastasis was found in any patient.

Surgical resections

Multiple partial resections were performed on 24 patients, segmentectomy on 12, lobectomy on 10, extended lobectomy on 2, and central bi-segmentectomy on 2 according to Couinaud's anatomical classification.[18] Forty-two of the 50 patients underwent multi-site resections. Microscopically positive surgical margins were observed in 11 patients. There was no perioperative mortality. Eleven complications were observed: five cases of biliary leak, two cases of intra-abdominal abscess, two cases of anastomotic leak in patients with synchronous metastases, one case of postoperative bleeding, and one case of liver failure.

Table 1
Clinicopathological findings of 50 patients with multiple colorectal hepatic metastases

	No. of patients
Primary colorectal tumour	
Stage (TNM classification)	
I	2
II	8
III	14
IV	26
Location	
Rectum	19
Colon	31
Maximum size of tumour (mean ± SD, cm)	4.9 ± 1.9
Histological type of adenocarcinoma	
Well or moderately differentiated	46
Poorly differentiated and others	4
Hepatic metastases	
Maximum size of tumour (mean ± SD, cm)	3.7 ± 2.3
Number of tumours (mean ± SD)	5.4 ± 1.8
Preoperative CEA level (mean ± SD, ng/ml)	65.4 ± 142.2
Synchronous/Metachronous	
Synchronous	24
Metachronous	26
Distribution of metastases	
Unilobar	12
Bilobar	38
Sum of the tumour volume (mean ± SD, cm^3)	61.2 ± 86.4
Tumour volume ratio* (mean ± SD, %)	4.8 ± 6.3
Coefficient of variation† in tumour volume (mean ± SD)	1.2 ± 0.6
Interval between resection of primary site and resection of hepatic metastases (median, mo)	7.9

SD, standard deviation; CEA, carcinoembryonic antigen. *Sum of tumour volume/whole liver volume × 100%. †Standard deviation of the mean divided by the mean.

Recurrences after resection of MCHM

Among the 50 patients, 37 developed recurrences. Locations of recurrence were as follows: liver in 32 patients, lung in 8, lymph node in 4, local recurrence in 3, peritoneum in 2, and bone and ovary in 1 each. Ten patients underwent resection for hepatic recurrences, 2 underwent resection for pulmonary recurrences, and one underwent resection for both hepatic and pulmonary recurrences. Of the remaining 24 patients, 19 received systemic chemotherapy, 2 received hepatic arterial infusion, and 3 received optimal supportive care.

Overall survival

Kaplan–Meier curve for overall survival after resection of MCHM is shown in Fig. 1. Actuarial overall survival after resection of MCHM was 48% at 3 years and 43% at 5 years with a median survival of 22.3 months. Meanwhile, overall survival of the entire cohort of 370 patients was 58% at 3 years and 46% at 5 years with a median survival of 27.6 months.

Association between clinicopathological factors and overall survival

To find prognostic factors for survival after resection of MCHM, clinicopathological factors and overall survival

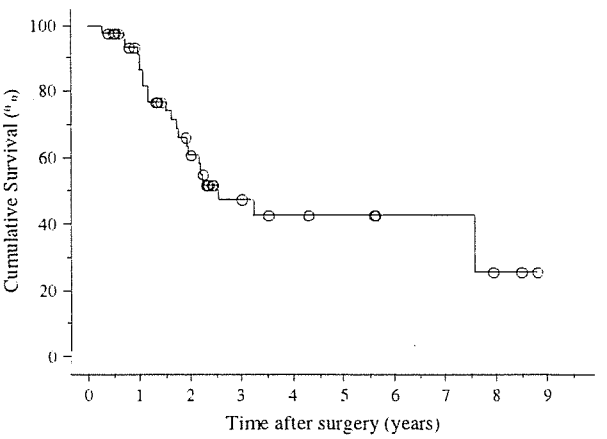

Figure 1. Cumulative survival curve for 50 patients with resected MCHM. The survival curve was generated by Kaplan–Meier analysis.

were analyzed in the 50 patients with MCHM (Table 2). Maximum tumour size above 5 cm ($P = 0.02$), CEA level before hepatectomy above 20 ng/ml ($P = 0.01$), tumour volume ratio above 8% ($P = 0.04$), and CV in tumour volume above 1.8 ($P < 0.01$) were significantly associated with poor overall survival.

We examined the independent predictive value of the aforementioned factors in overall survival. The data were analyzed using a Cox regression model (Table 3). Maximum size of the tumour was excluded from the analysis because it was strongly correlated with tumour volume. Then, only CV in tumour volume above 1.8 ($P = 0.01$; HR = 4.08; 95% CI, 1.33 to 12.5) had predictive value for decreased overall survival after resection of MCHM. Fig. 2 shows a case of MCHM with low CV (a) and another one with high CV (b) in tumour volume. The median survival of patients with CV in tumour volume below 1.8 was 25.0 months and that above 1.8 was 16.1 months (Fig. 3).

Table 2
Correlation between clinicopathological factors and overall survival after hepatectomy for multiple colorectal hepatic metastases

	No. of patients	Median survival (mo)	P
Primary colorectal lesion			
Location			
Colon	31	23.4	0.63
Rectum	19	18.5	
Stage (TNM classification)			
I, II	10	20.4	0.44
III, IV	40	22.7	
Lymph node metastasis			
Absent	18	23.4	0.82
Present	32	19.7	
Histological type of adenocarcinoma			
Well or moderately differentiated	46	23.5	0.08
Poorly differentiated and others	4	12.5	
Hepatic metastases			
Number of tumours			
<5	20	21.1	0.61
≥5	30	23.4	
Maximum size of the tumour (cm)			
<5	40	23.5	0.02
≥5	10	15.9	
Distribution of metastases			
Unilobar	12	21.1	0.60
Bilobar	38	23.4	
Microscopic surgical margin			
Negative	39	23.4	0.95
Positive	11	21.3	
CEA level before treatment (ng/ml)			
<20	27	24.6	0.01
≥20	23	17.5	
Tumour volume ratio* (%)			
<8	41	23.4	0.04
≥8	9	17.5	
Coefficient of variation† in tumour volume			
<1.8	42	25.0	<0.01
≥1.8	8	16.1	
Synchronous/Metachronous			
Synchronous	24	24.4	0.80
Metachronous	26	18.0	
Interval between colorectal resection and hepatectomy			
<1 year	39	24.6	0.91
≥1 year	11	12.1	
Adjuvant chemotherapy after hepatectomy			
Absent	41	23.5	0.61
Present	9	16.4	

CEA, carcinoembryonic antigen. *Sum of tumour volume/whole liver volume × 100%. †Standard deviation of the mean divided by the mean.

Table 3
Multivariate analyses of factors affecting overall survival after hepatectomy for multiple colorectal hepatic metastases

	Hazard Ratio (95% C.I.)	P
Hepatic metastases		
CEA level before treatment (ng/ml)		
<20	reference	0.07
≥20	2.39 (0.93–6.16)	
Tumour volume ratio* (%)		
<8	reference	0.87
≥8	1.10 (0.36–3.39)	
Coefficient of variation† in tumour volume		
<1.8	reference	0.01
≥1.8	4.08 (1.33–12.5)	

C.I., confidence interval; CEA, carcinoembryonic antigen. *Sum of tumour volume/whole liver volume × 100%. †Standard deviation of the mean divided by the mean.

Discussion

Several reports have described the efficacy of resection for MCHM. Bolton et al. analyzed clinical outcomes of 165 patients who underwent hepatic resection for colorectal metastases, and evaluated its efficacy and safety for patients with more than four and/or bilobar hepatic metastases.[6] The prognosis for such patients was almost equal to that of patients with fewer than four and unilobar hepatic metastases. Weber et al. reported that the 5-year survival rate after hepatic resection for 155 patients with four or more metastases was 23%, and twelve 5-year survivors were observed.[19] Minagawa et al. similarly reported a 32% 5-year survival of patients with four or more tumours.[13] In the present study, overall survival after hepatic resection for MCHM was 48% at 3 years and 43% at 5 years. Our results reconfirm that hepatic resection is beneficial for some patients with MCHM of colorectal cancer.

We found that a CV in tumour volume of above 1.8 was the only independent poor prognostic factor after resection of MCHM. Dispersion of tumour volume for each tumour is variable among patients. However, no previous study has attempted to quantify the dispersion of tumour volume or to evaluate its prognostic significance in colorectal hepatic metastases, and then we studied the association between the dispersion of tumour volume, quantified by CV, and survival after hepatectomy. Coefficient of variation is a statistical measure of the dispersion of data. It represents the ratio of the standard deviation to the mean, and is a useful statistic for comparing the degree of deviation from one

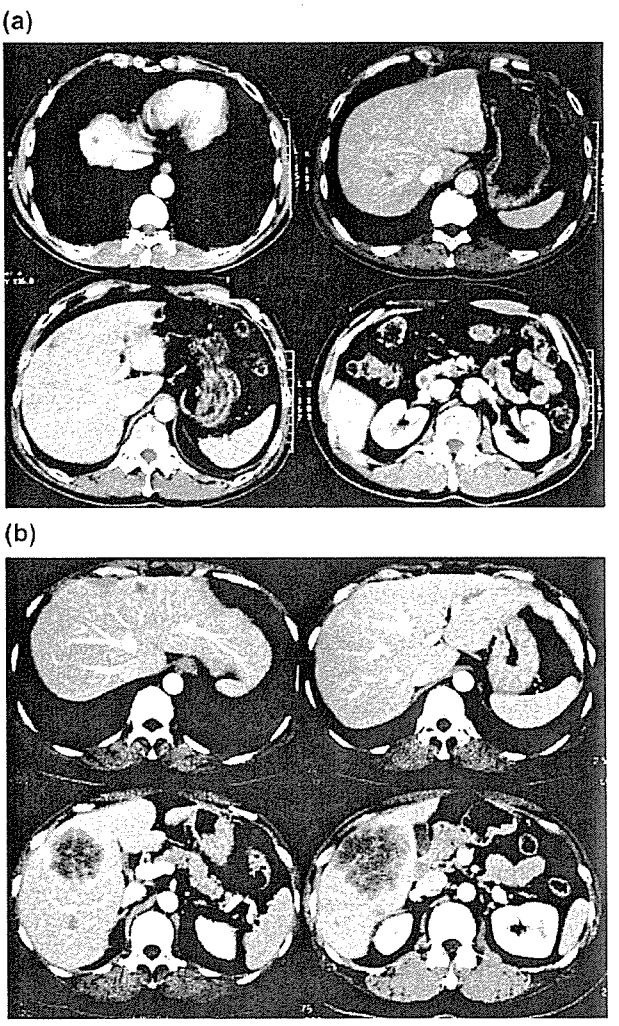

Figure 2. (a) A case of MCHM with low CV (=0.41) in tumour volume. (b) A case of MCHM with high CV (=3.20) in tumour volume.

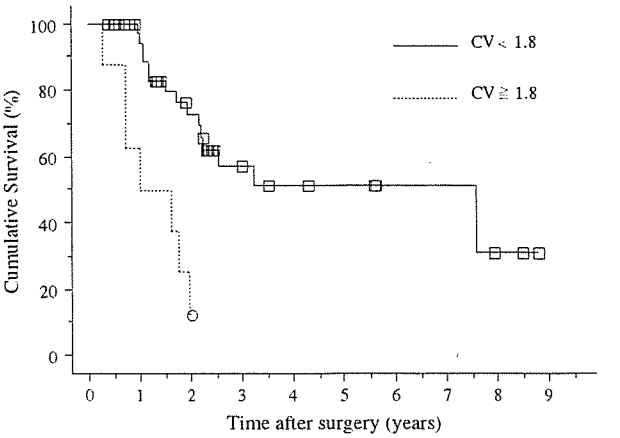

Figure 3. Cumulative survival curves after hepatic resection of MCHM according to CV in tumour volume. The median survival of patients with CV in tumour volume below 1.8 was 25.0 months and that above 1.8 was 16.1 months. Survival of patients with CV in tumour volume above 1.8 was poorer than that of patients with CV in tumour volume below 1.8 ($P < 0.01$).

data series to another, even if the means are drastically different from each other.[20,21] The mean tumour size varied widely among patients and CV was more useful than standard deviation in the present analyses.

The reason why high CV in tumour volume is strongly associated with independent poor prognosis after hepatic resection is obscure. However, a high CV may denote the coexistence of huge and tiny tumours. We propose two hypotheses to explain the association between high CV and poor prognosis. The first is that a high CV means the existence of a rapidly growing tumour; the high CV may result from the coexistence of tiny tumours growing at an ordinary rate and a huge tumour with an extremely aggressive nature and rapid growth. Another hypothesis is that high CV means a huge tumour with many intrahepatic metastases. Tiny tumours might have metastasized, not from the primary colorectal tumour, but from this huge hepatic tumour. Accordingly, a high CV might reflect progressive characteristics of MCHM.

In 8 patients with CV > 1.8, 6 suffered from severe hepatic recurrence after hepatic resection. In the remaining 2 patients, although lymph node recurrence was initially observed, hepatic recurrence with much tumour burden was recognized in the next few months. Then, severe hepatic recurrence could be a characteristic pattern of recurrence in patients with CV > 1.8. High CV might suggest extensive micro-metastases in the remnant liver.

Node-positive primary tumour,[4,22,23] serosal involvement of primary tumour,[22,23] stage of the primary tumour,[8,13] histological differentiation of primary tumour[22], a short disease-free interval from the primary tumour to metastasis,[4,11] extrahepatic disease at hepatectomy,[3,4,11,22,23] high CEA levels before hepatectomy,[4,5,8,10,22] large size of hepatic tumour,[4,8,23] the number of hepatic tumours,[4,5,8–13,22,23] bilobar distribution of hepatic tumour,[11] lymph node metastasis during hepatectomy,[3,11,13] an advanced age at hepatectomy,[8] and a positive margin of hepatectomy[4,5,8–11,22] have been reported as poor prognostic factors after resection of MCHM. However, the factors mentioned above were not found to be prognostic factors in this study. The difference between our results and those of other studies was partly due to difference of population. Patients of the present study consisted of only those with four or more metastatic lesions of colorectal cancer in the liver. Moreover, the difference might have resulted from the fact that CV in tumour volume, which had not been evaluated as a prognostic factor in other studies, affected patients' survival much more strongly than the aforementioned factors did in the present study.

In our study, the median survival of patients with CV in tumour volume above 1.8 was only 16 months and no 2-year survivors were found. Results of the present study lead us to conclude that hepatic resection is not