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NQO1 as a Marker of Chemosensitivity and Prognosis for Colorectal Liver Metastasis

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Abstract. *Background/Aim: This study aimed to evaluate how NAD(P)H: quinone oxidoreductase-1 (NQO1) affects survival after hepatectomy in patients with colorectal liver metastasis (CRLM). Patients and Methods: A retrospective analysis was conducted of 88 consecutive patients who underwent hepatectomy for CRLM. Of the 88 patients, preoperative chemotherapy was administered to 30 patients. Immunohistochemistry of the resected specimens was conducted using monoclonal anti-NQO1 antibody. Results: NQO1-positive expression in tumor cells of CRLM was associated with worse overall survival (p=0.026) and was an independent adverse prognostic factor in multivariate analysis (hazard ratio=5.296, p=0.007). Among 30 patients who received preoperative chemotherapy, patients with loss of NQO1 expression in non-neoplastic epithelial cells of the bile ducts (NQO1 polymorphism: n=19) showed significantly better response to preoperative chemotherapy for CRLM (p=0.004). Conclusion: NQO1-positive expression in tumor cells of CRLM may be an adverse prognostic factor after hepatectomy for CRLM.*

For patients with colorectal liver metastasis (CRLM), hepatectomy is the most effective treatment, with a 5-year survival rate of up to 60% (1). Favorable outcomes have been achieved through the increased use of preoperative chemotherapy for CRLM, leading to down-staging of the disease and increased resection rate (2). However, preoperative chemotherapy for CRLM is not always effective. Therefore, it is important to identify predictive markers for response to preoperative chemotherapy for CRLM to select effective drugs for each patient and avoid unnecessary treatment.

NAD(P)H: quinone oxidoreductase-1 (NQO1) is a ubiquitous flavoprotein discovered by Ernster *et al*. in 1958 (3). In normal cells, NQO1 protects cells against redox cycling and oxidative stress (3), and also against carcinogenesis by stabilizing the p53 tumor suppressor (4). Recent studies have demonstrated that NQO1 induces cell cycle progression and proliferation in melanoma and cholangiocarcinoma cell lines (5, 6) and that NQO1 expression is associated with prognosis in various types of cancer including colon, breast, pancreatic, and cholangiocarcinoma (7-9).

Recent studies of NQO1 identified an NQO1 polymorphism encoded by NQO1*2, a missense variant characterized by a C609T substitution (3). An estimated 4%-20% of the human population harbors the homozygous C609T polymorphism (10), resulting in loss of NQO1 function (11). Some studies have suggested that NQO1 polymorphism status is associated with survival (11, 12) and is also associated with response to chemotherapy in cancer patients (11, 13). However, no studies have analyzed whether NQO1 has prognostic value or is a predictive marker for response to preoperative chemotherapy in patients with CRLM. Thus, the aim of this study was to evaluate the prognostic value of NQO1 and the predictive impact of NQO1 on response to preoperative chemotherapy in patients undergoing hepatectomy for CRLM.

Patients and Methods

Patients. From January 2005 through December 2016, 95 consecutive patients with CRLM were admitted to the Niigata University Medical and Dental Hospital for surgical intervention. Of these patients, 88 underwent potentially curative hepatectomy and were included in this retrospective study. Participants comprised 59 men and 29 women with a median age of 65 years (range=33-

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83 years) at the time of initial hepatectomy. This study was approved by the ethics committee of Niigata University Graduate School of Medical and Dental Sciences (approval number: 2017-0052). The status of the primary tumor was assessed using the TNM staging system (14). Synchronous liver metastasis was defined as metastasis detected simultaneously with the primary tumor or found within 1 month after surgery for the primary colorectal tumor; metachronous liver metastasis was defined as metastasis detected later than 1 month after primary colorectal surgery.

Preoperative chemotherapy for CRLM. Of the 88 patients, 30 were administered preoperative chemotherapy for CRLM with a combination of 5-fluorouracil (5-FU) and oxaliplatin or irinotecan. Of the 30 patients who were administered preoperative chemotherapy, 13 were treated with bevacizumab, 3 with panitumumab, and 1 with cetuximab. Tumor response to preoperative chemotherapy for CRLM was evaluated using contrast-enhanced computed tomography and assessed based on the Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1 (15).

Hepatectomy procedures. All metastatic lesions were indicated for surgery when considered resectable and the patients had an acceptable operative risk. In this study, major hepatectomy was defined as the removal of ≥3 Couinaud segments and minor hepatectomy was defined as the removal of <3 Couinaud segments. The hepatectomy procedure was determined according to the hepatic tumor status (number, size, and location), hepatic functional reserve, and patient's general condition.

Patient follow-up after hepatectomy. As adjuvant chemotherapy after hepatectomy for CRLM, 38 patients received intravenous or oral administration of 5-FU or its derivatives within approximately 1 year. Patients were regularly followed-up with physical and blood biochemistry examinations and imaging investigations every 3-6 months after hepatectomy for CRLM. The median follow-up after hepatectomy was 65.4 months (range=0.2-156.9 months).

Pathologic evaluation. Resected specimens were submitted to the Department of Surgical Pathology at our hospital. Histologic grading of the hepatic tumor was done according to the areas with the highest grade. Hepatectomy margin status was assessed histologically as either R0 (no residual tumor) or R1 (microscopic residual tumor), depending on the absence or presence of histologically verified tumor cells in the hepatectomy margin. Pathologic response to preoperative chemotherapy for CRLM was evaluated based on the Japanese classification of colorectal carcinoma as follows: grade 0, no recognizable cytologic or histologic therapeutic effect was observed; grade 1, viable cells accounted for at least one-third of the tumor tissue; grade 2, viable cells accounted for less than one-third of the tumor tissue; and grade 3, no viable cells were observed.

Immunohistochemistry. Immunohistochemical staining was performed for the surgically resected specimen. A rabbit monoclonal antibody against NQO1 (Epitomics, Burlingame, CA, USA) was used at a dilution of 1:200. Three serial 3-μm sections were recut and prepared from each block: 1 each for hematoxylin-eosin staining, immunohistochemical staining, and negative control. Two independent surgical pathologists blinded to the clinical details assessed each section. Before staining, sections were microwaved for 21 min in 10 mM sodium citrate buffer (pH 6.0). After overnight incubation at 4˚C with NQO1 antibody, sections were incubated with goat anti-rabbit IgG polymerized horseradish peroxidase-labeled secondary antibody (Epitomics) at room temperature for 30 min. Diaminobenzidine was used as the chromogen and sections were counterstained with hematoxylin.

Patterns of NQO1 expression and definition of NQO1 polymorphism status. NQO1 expression was defined as the presence of cytosolic and/or nuclear staining as described previously (9, 16). Then, according to NQO1 expression in tumor specimens of CRLM, patients were classified as either those with positive expression in CRLM (Figure 1A) or those with loss of expression in CRLM (Figure 1B). Additionally, non-neoplastic interlobular biliary epithelial cells of the liver, which typically show immunopositive staining for NQO1 (Figure 1A and B) (3), occasionally showed no NQO1 immunoreactivity (Figure 1C), probably because homozygosity for the NQO1 polymorphism is associated with loss of NQO1 protein (11). Thus, in the current study, patients were classified as follows: patients with NQO1 polymorphism, characterized by no NQO1 expression in non-neoplastic intralobular biliary epithelial cells of the liver, or patients without NQO1 polymorphism, characterized by NQO1-positive expression in non-neoplastic intralobular biliary epithelial cells of the liver.

Statistical analysis. Categorical variables were compared using Fisher's exact test. The follow-up period was defined as the interval between the date of hepatectomy and last follow-up. Cumulative survival was estimated using the Kaplan–Meier method, and the log-rank test was applied to compare survival between the groups. To identify independent prognostic factors, the Cox proportional hazards regression model was used. All statistical evaluations were performed using the PASW Statistics 23 software package (SPSS, Inc., Chicago, IL, USA). All tests were two tailed and $p<0.05$ was considered statistically significant.

Results

For all 88 patients, the incidence of post-hepatectomy mortality was 0%; overall survival rates after hepatectomy were 74.5% at 5 years and 60.2% at 10 years.

Factors associated with NQO1 expression in tumor cells of CRLM and NQO1 polymorphism status. Of the 88 patients, 61 were classified as patients with NQO1-positive expression in CRLM and 27 with loss of NQO1 expression in CRLM. In addition, 69 of the 88 were classified as patients without NQO1 polymorphism and 19 with NQO1 polymorphism. All 61 patients with NOQ1-positive expression in CRLM showed NQO1-positive expression in the non-neoplastic interlobular biliary epithelial cells (without polymorphism) (Figure 1A, Table I). Of the 27 patients with loss of NQO1 expression in CRLM, 8 showed NQO1-positive expression in the non-neoplastic interlobular biliary epithelial cells (without NQO1 polymorphism) (Figure 1B, Table I), and 19 showed loss of expression in the non-neoplastic interlobular biliary epithelial cells (with NQO1 polymorphism) (Figure 1C, Table I).

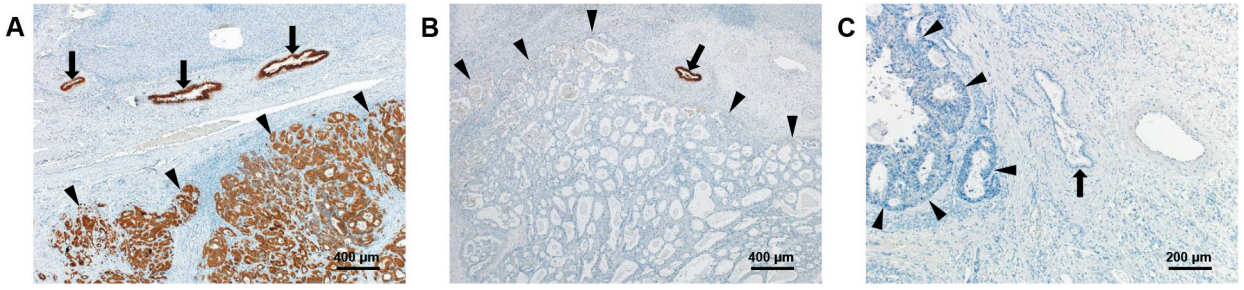

Figure 1. *NAD(P)H: quinone oxidoreductase-1 (NQO1) expression. (A) NQO1-positive expression in both non-neoplastic intralobular biliary epithelial cells of the liver (arrows) and tumor cells of colorectal liver metastasis (CRLM) (arrowheads) (B) NQO1-positive expression in non-neoplastic intralobular biliary epithelial cells of the liver (arrow), and loss of NQO1 expression in tumor cells of CRLM (arrowheads) (C) Loss of NQO1 expression in both non-neoplastic intralobular biliary epithelial cells of the liver (arrow) and tumor cells of CRLM (arrowheads).*

NQO1-positive expression in CRLM was more frequent in patients with high concentrations of preoperative serum carcinoembryonic antigen (CEA) (45/58, 93.8%) than in patients with low concentrations (16/30, 53.3%; p=0.028) (Table I). NQO1-positive expression in CRLM was more frequent in patients without NQO1 polymorphism (61/69, 88.4%) than in patients with the polymorphism (0/19, 0%; p<0.001) (Table I). NQO1 polymorphism status was significantly associated with the distribution of CRLM (Table I). Patients with NQO1 polymorphism more frequently had bilobar tumors (11/19, 57.9%) than patients without the polymorphism (21/69, 30.4%; p=0.034) (Table I).

Factors influencing overall survival after hepatectomy for CRLM. Univariate analysis revealed that NQO1 expression in CRLM (p=0.026) (Figure 2) and extrahepatic disease (p=0.023) were significant prognostic factors (Table II). Overall survival after hepatectomy for CRLM was significantly worse in patients with tumors with NQO1-positive expression (cumulative 5-year survival rate, 66.5%) than in those with tumors with loss of NQO1 expression (cumulative 5-year survival rate, 90.9%; p=0.026).Variables with p-values <0.1 in univariate analyses were entered into multivariate analyses, revealing that NQO1-positive expression in CRLM (hazard ratio, 5.296; p=0.007) and the presence of extrahepatic disease (hazard ratio, 7.384; p=0.001) were independent adverse prognostic factors (Table II).

Association between NQO1 status and response to preoperative chemotherapy for CRLM. Of the 30 patients administered preoperative chemotherapy for CRLM, 17 were classified as having partial response, 9 were classified as having stable disease, and 4 were classified as having progressive disease on RECIST. Partial response was more frequent in patients with NQO1 polymorphism (8/8, 100%) than those without the polymorphism (9/22, 40.9%; p=0.004) (Table III). There were no associations between tumor response to preoperative chemotherapy for CRLM and NQO1 expression either in CRLM (p=0.119) or in primary colorectal cancer (p=0.259) (Table III). There were also no associations between pathologic tumor response to preoperative chemotherapy for CRLM and NQO1 status (Table III).

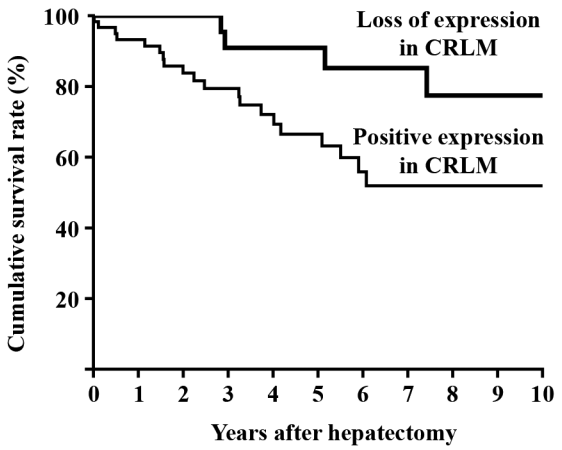

Figure 2. *Kaplan–Meier survival estimates for overall survival. The outcome after hepatectomy for colorectal liver metastasis was significantly worse in patients with tumors with NAD(P)H: quinone oxidoreductase-1 (NQO1)-positive expression (cumulative 5-year survival rate, 66.5%) than in patients with loss of NQO1 expression (cumulative 5-year survival rate, 90.9%; p=0.026).*

Discussion

NQO1 protects cells from oxidative stress, free radical damage, and toxic substrate accumulation by catalyzing the reduction of quinone compounds to their hydroquinone forms (5). Several studies have indicated that NQO1 is involved in chemosensitivity in cancer patients (11).

Table I. *Association between NQO1 status and clinicopathologic factors in 88 patients with CRLM who underwent hepatectomy.*

Variable	No. of patients		*p*-Value	No. of patients		*p*-Value
	With loss of NQO1 expression in CRLM (n=27)	With NQO1-positive expression in CRLM (n=61)		With NQO1 polymorphism (n=19)	Without NQO1 polymorphism (n=69)	
Gender			>0.999			0.784
Male	18	41		12	47	
Female	9	20		7	22	
Age (years)			0.105			0.128
≤65	18	28		13	33	
>65	9	33		6	36	
Initial stage of disease			0.452			0.252
I-IIC	6	19		3	22	
IIIA-IVC	21	42		16	47	
Site of primary tumor			0.479			0.589
Colon	16	41		11	46	
Rectum	11	20		8	23	
Preoperative serum CEA (ng/ml)			0.028			0.062
≤5	14	16		10	20	
>5	13	45		9	49	
Number of CRLM			0.158			0.296
Solitary	7	26		5	28	
Multiple	20	35		14	41	
Size of the largest CRLM (cm)			0.488			0.683
≤5	23	55		18	60	
>5	4	6		1	9	
Distribution of metastases			0.056			0.034
Unilobar	13	43		8	48	
Bilobar	14	18		11	21	
Extrahepatic disease			0.671			0.655
Absent	24	57		17	64	
Present	3	4		2	5	
Timing of the diagnosis of CRLM			>0.999			0.610
Synchronous	14	33		9	38	
Metachronous	13	28		10	31	
Hepatectomy procedure			0.798			0.383
Major hepatectomy	10	27		7	30	
Minor hepatectomy	17	34		12	39	
Preoperative chemotherapy for CRLM			0.808			0.424
Absent	17	41		11	47	
Present	10	20		8	22	
Adjuvant chemotherapy for CRLM			0.818			0.607
Absent	16	34		12	38	
Present	11	27		7	31	
Histologic grade of CRLM			0.716			0.677
G1	2	7		1	8	
G2, G3	25	54		18	61	
Hepatectomy margin status			0.550			>0.999
R0	27	58		19	66	
R1	0	3		0	3	
NQO1 expression in CRLM			-			<0.001
Loss of expression	-	-		19	8	
Positive expression	-	-		0	61	
NQO1 polymorphism status			<0.001			-
With polymorphism	19	0		-	-	
Without polymorphism	8	61		-	-	

NQO1: NAD(P)H: quinone oxidoreductase-1; CRLM: colorectal liver metastasis; CEA: carcinoembryonic antigen; G1: well-differentiated; G2: moderately differentiated; G3: poorly differentiated; R0: no residual tumor; R1: microscopic residual tumor.

Table II. *Univariate and multivariate analysis for overall survival after hepatectomy in 88 patients with CRLM.*

Variable	Categories	n	Univariate analysis		Multivariate analysis	
			5-year survival (%)	*p*-Value	Hazard ratio (95%CI)	*p*-Value
Gender	Male	59	74.8	0.836		
	Female	29	73.9			
Age (years)	≤65	46	77.2	0.648		
	>65	42	71.7			
Initial stage of disease	I-IIC	25	68.9	0.865		
	IIIA-IVC	63	76.3			
Site of primary tumor	Colon	57	71.0	0.994		
	Rectum	31	81.4			
Preoperative serum CEA (ng/ml)	≤5	30	96.2	0.058		
	>5	58	60.8			
Number of CRLM	Solitary	33	75.7	0.587		
	Multiple	55	73.3			
Size of largest CRLM (cm)	≤5	78	74.3	0.653		
	>5	10	76.2			
Distribution of metastases	Unilobar	56	76.0	0.675		
	Bilobar	32	71.7			
Extrahepatic disease	Absent	81	78.1	0.023	1.000	
	Present	7	33.3		7.384 (2.156-25.291)	0.001
Timing of the diagnosis of CRLM	Synchronous	47	67.7	0.372		
	Metachronous	41	81.8			
Hepatectomy procedure	Major	37	66.7	0.459		
	Minor	51	81.1			
Preoperative chemotherapy for CRLM	Absent	58	67.5	0.129		
	Present	30	87.4			
Adjuvant chemotherapy for CRLM	Absent	50	78.8	0.646		
	Present	38	72.1			
Histologic grade of CRLM	G1	9	64.3	0.922		
	G2, G3	79	75.4			
Hepatectomy margin status	R0	85	74.1	0.541		
	R1	3	100			
NQO1 expression in CRLM	Loss of expression	27	90.9	0.026	1.000	
	Positive expression	61	66.5		5.296 (1.579-17.759)	0.007
NQO1 polymorphism status	With polymorphism	19	93.3	0.102		
	Without polymorphism	69	69.1			

CRLM: Colorectal liver metastasis; CI: confidence interval; CEA: carcinoembryonic antigen; G1: well-differentiated; G2: moderately differentiated; G3: poorly differentiated; R0: no residual tumor; R1: microscopic residual tumor; NQO1: NAD(P)H: quinone oxidoreductase-1.

Additionally, an increasing number of studies have demonstrated that NQO1 upregulation promotes cancer progression and is associated with poor survival in cancer patients (5-9). However, the prognostic value of NQO1 status and its relationship with chemosensitivity in CRLM remains unclear. In this study, we found that NQO1 expression in the tumor cells of CRLM was an independent prognostic factor in patients with CRLM, and NQO1 polymorphism status was associated with sensitivity to preoperative chemotherapy for CRLM.

NQO1 activity depends mainly on polymorphisms in the NQO1 locus. Among NQO1 polymorphisms, NQO1*2 is the key naturally-occurring germline polymorphism, which is a missense variant with a cytosine to thymidine (C→T) substitution at nucleotide position 609 of NQO1 cDNA that codes for a proline to serine change in the amino acid structure (3). The homozygous C609T polymorphism results in no measurable NQO1 activity because of a resulting unstable protein structure (11) and occurs in 4%-20% of the human population (10). The second most frequent NQO1 polymorphism is NQO1*3, which shows a C465T change coding for an arginine to tryptophan substitution with decreased activity. However, the frequency of NQO1*3 is quite low. Recent studies demonstrated detection of homozygous NQO1*3 in 1 of 575 samples (17). Additionally, 22 other variants of NQO1 have been detected upon screening single-nucleotide polymorphism databases, but these occur less frequently and their phenotypes are

Table III. *Association between response to preoperative chemotherapy and NQO1 status in 30 patients with CRLM.*

	Primary tumor			CRLM					
	No. of patients with			No. of patients with			No. of patients		
	Loss of NQO1 expression (n=11)	NQO1-positive expression (n=19)	*p*-Value	Loss of NQO1 expression (n=10)	NQO1-positive expression (n=20)	*p*-Value	With NQO1 polymorphism (n=8)	Without NQO1 polymorphism (n=22)	*p*-Value
RECIST			0.259			0.119			0.004
PR	8	9		8	9		8	9	
SD, PD	3	10		2	11		0	13	
Pathologic Response*			>0.999			0.245			0.682
Grade 1	6	9		7	8		5	10	
Grade 2, 3	5	10		3	12		3	12	

NQO1: NAD(P)H: quinone oxidoreductase-1; CRLM: colorectal liver metastasis; RECIST: Response Evaluation Criteria In Solid Tumors version 1.1; PR: partial response; SD: stable disease; PD: progressive disease. *Pathologic response to preoperative chemotherapy for CRLM was evaluated based on the Japanese classification of colorectal carcinoma as follows: grade 0, no recognizable cytologic or histologic therapeutic effect was observed, grade 1, viable cells accounted for at least one-third of the tumor tissue, grade 2, viable cells accounted for less than one-third of the tumor tissue, grade 3, no viable cells were observed.

presently unknown (18). In this study, no NQO1 immunoreactivity in non-neoplastic intralobular biliary epithelial cells, which typically show immunoreactivity for NQO1 (3), was observed in 21.6% of patients, which is comparable to the probability of the homozygous C609T polymorphism. Thus, it is reasonable to consider that patients with no immunoreactivity for NQO1 in non-neoplastic intralobular biliary epithelial cells are homozygous for the C609T polymorphism of NQO1.

Some studies have shown that high NQO1 expression is associated with poor survival in several types of cancer such as pancreatic, gastric, and breast cancer (8, 19, 20). In contrast, we previously found that low NQO1 expression was a predictor of poor prognosis in intrahepatic cholangiocarcinoma (9). These conflicting results may be related to the different study populations or different types of cancer evaluated. Previously, we reported that NQO1-positive expression was associated with shortened survival in KRAS-wild-type colorectal cancer (7). Oh *et al*. (21) reported that by stabilizing hypoxia-inducible factor-1α, which is a master regulator of oxygen homeostasis, NQO1 promoted colon cancer growth *in vivo* and *in vitro* and influenced survival in colorectal cancer patients. In our study, NQO1-positive expression in CRLM was associated with poor prognosis after hepatectomy for CRLM. Furthermore, multivariate analysis revealed that NQO1-positive expression in CRLM was an independent dismal prognostic factor. Taken together, in addition to previously reported prognostic factors (22-25), NQO1-positive expression in CRLM can be a predictor of poor prognosis in patients with CRLM.

NQO1 has been studied as a predictor of chemosensitivity. Tian *et al*. (13) reported that the presence of NQO1 polymorphism was associated with a lower response to platinum-based therapy in non-small cell lung cancer. In contrast, Gang *et al*. (12) found no association between NQO1 polymorphism status and combination therapy of oxaliplatin, epirubicin, and 5-FU in metastatic gastric cancer. The association between NQO1 status and chemosensitivity remains controversial. In colorectal cancer, we previously reported that loss of NQO1 expression in tumor cells was an independent favorable prognostic factor among patients with advanced KRAS wild-type colorectal cancer treated using 5-FU-based chemotherapy (7). In this study, preoperative chemotherapy was a 5-FU-based regimen, and among the 30 patients administered this therapy for CRLM, the presence of NQO1 polymorphism was associated with a favorable response to preoperative chemotherapy on RECIST. Thus, the presence of NQO1 polymorphism may be a predictive factor for favorable response to 5-FU-based chemotherapy in CRLM. It may be possible to predict the efficacy of 5-FU-based chemotherapy for patients with CRLM by testing the polymorphism status using non-neoplastic tissues such as blood samples.

In this study, the response to preoperative chemotherapy for CRLM showed no significant difference between patients with NQO1-positive expression and those with loss of NQO1 expression, although those with loss of NQO1 expression were expected to show favorable response. We speculate that these unexpected results might be due to the small number of patients and sampling bias in the immunohistochemical analysis. For immunohistochemical staining, we usually select one among several blocks containing CRLM tissue for

each patient. Even if the CRLM cells in the selected block occasionally have no NQO1 immunoreactivity, CRLM cells in other blocks in the same patient may have some immunoreactivity due to the heterogeneity of the cancer. Conversely, the NQO1 polymorphism status of non-neoplastic cells in each patient is consistent throughout the body and is thus expected to show no sampling bias. These might explain why NQO1 polymorphism status, but not NQO1 expression in CRLM, was associated with response to preoperative chemotherapy for CRLM in this study.

This study had some limitations. First, this was a retrospective analysis of a small number of patients. Second, the follow-up period was short for some patients. Third, polymorphism status was not determined using genotyping, but was assessed by immunohistochemical staining. However, these biases did not appear to affect the results.

Conclusion

NQO1-positive expression may be an adverse prognostic factor after hepatectomy for CRLM. NQO1 polymorphism status is expected to be a clinically useful biomarker for predicting the response to preoperative chemotherapy for CRLM.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Study design: Yuki Hirose, Jun Sakata, Hitoshi Kameyama, Toshifumi Wakai. Data analysis: Yuki Hirose, Jun Sakata, Hiroshi Ichikawa, Masayuki Nagahashi, Yoshifumi Shimada. Data collection: Yuki Hirose, Kohei Miura, Kizuki Yuza, Mae Nakano. Drafting the manuscript: Yuki Hirose, Jun Sakata. Revising the manuscript, final approval of the version to be published: All Authors.

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