Int J Clin Exp Pathol 2017;10(5):5841-5849
www.ijcep.com /ISSN:1936-2625/IJCEP0047723

Original Article

Clinical significance of NQO1 expression in KRAS wild-type colorectal cancer

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Received December 30, 2016; Accepted January 27, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: NAD(P)H: quinone oxidoreductase-1 (NQO1) protects cells against redox cycling and oxidative stress; however, in cancer cells, NQO1 confers resistance against anticancer agents. The aim of this study was to evaluate the association between NQO1 expression and prognosis in patients with advanced (locally advanced or metastatic/recurrent) colorectal cancer (CRC). A retrospective analysis of 47 patients [28 male and 19 female; median age: 62 years (range, 17-78)] with advanced CRC was conducted. Immunohistochemical examination of tumor tissue specimens was performed using monoclonal anti-NQO1 antibody. The association of NQO1 expression with patient characteristics, chemotherapeutic response, and clinical prognosis was assessed. Therapeutic efficacy (complete response, partial response, stable disease, and progressive disease) was evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. We compared the therapeutic efficacy in KRAS wild and mutant CRC because epidermal growth factor receptor (EGFR)-signaling pathway plays a pivotal role in CRC. Of the 47 patients, 31 (66.0%) had KRAS wild CRC and 16 (34.0%) had KRAS mutant CRC. Moreover, 37 (78.7%) had NQO1-positive tumors and 10 (21.3%) had NQO1-negative tumors. Among the patients with KRAS wild CRC, NQO1-negative patients showed significantly better disease control rate (complete response + partial response + stable disease) than NQO1-positive patients (P = 0.028). Moreover, NQO1-negative patients had longer progression-free survival and overall survival than NQO1-positive patients (P = 0.041 and P = 0.043, respectively). NQO1 expression in the tumor may be a predictor of therapeutic efficacy and prognosis in patients with KRAS wild advanced CRC.

Keywords: NQO1, colorectal cancer, KRAS

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related-deaths in the United States [1]. The incidence of CRC has dramatically increased in the last decade [2, 3]. CRC accounts for the largest proportion of deaths from malignant neoplasms among women, and the third largest proportion of deaths from malignant neoplasms among men in Japan [3]. There have been significant advances in the treatment of metastatic CRC recently [4]. In the era of 5-fluorouracil, response rates of chemotherapy for CRC were 10%-15%, and the median survival time was only 10 months [5]. However, the use of newer agents, such as oxaliplatin, irinotecan, and more recently, that of biological agents (bevacizumab, cetuximab, aflibercept, panitumumab, and regorafenib), has prolonged survival of patients with metastatic CRC, reaching median survival times of more than 20 months [4]. The epidermal growth factor receptor (EGFR)-signaling pathway in particular plays a pivotal role in CRC and forms the basis for use of EGFR-targeted antibodies for treatment of patients with CRC [2]. This monoclonal antibody has been effective in patients with KRAS wild CRC [4]. However, its use needs careful consideration owing to the cost implications of its use [6].

The gene for NAD(P)H: quinone oxidoreductase-1 (NQO1), which is also referred to as DT-diaphorase, is located on chromosome 16q22 and consists of six exons and five introns [7]. Oxidative stress promotes nuclear accumula-

Table 1. Association between clinicopathological characteristics and NQO1 expression in patients with advanced colorectal cancer

Variable		No. of patients			*P*-value
		Total cases (n = 47)	NQO1-positive (n = 37)	NQO1-negative (n = 10)	
Age (yrs)	Median (range)	62 (17-78)	62 (17-78)	59.5 (55-78)	0.56
Gender	Male	28	22	6	1.00
	Female	19	15	4	
Location	Colon	19	15	4	1.00
	Rectum	28	22	6	
Serum CEA (ng/mL)	< 5	5	4	1	1.00
	≥ 5	42	33	9	
Surgical resection	Yes	23	16	7	0.17
	No	24	21	3	
Histological grade	G1/G2	36	27	9	0.41
	G3	11	10	1	
KRAS mutation status	Wild	31	25	6	0.72
	Mutant	16	12	4	
Use of anti-EGFR drugs in KRAS wild cases	Yes	13	10	3	0.68
	No	18	15	3	

NQO1, NAD(P)H: quinone oxidoreductase-1; CEA, Carcinoembryonic antigen; EGFR, Epidermal growth factor receptor; KRAS, Kirsten rat sarcoma viral oncogene homolog.

tion of nuclear factor erythroid 2-related factor 2 (Nrf2) and activates transcription of NQO1. In normal cells, NQO1 protects against oxidative stress and electrophilic attack [8]. In cancer cells, NQO1 confers resistance against anticancer agents, and, in particular, against oxidative stress inducers such as cisplatin or 5-fluororuracil, doxorubicin, and gemcitabine [9, 10]. Notably, altered expression level of NQO1 has been reported in several solid tumors such as cholangiocarcinoma, esophagus, lung, pancreas, stomach, colon, uterine, and ovarian cancers [11-18]. Some studies have investigated NQO1 expression in CRC; however, the association between NQO1 expression and prognosis in patients with advanced CRC is not well-characterized.

We hypothesized that NQO1 expression in CRC is related to chemotherapeutic resistance and clinical prognosis. The aim of this study was to evaluate the association between NQO1 expression and the therapeutic efficacy and clinical prognosis in patients with advanced CRC.

Patients and methods

Patients and clinical information

The present study included 47 consecutive Japanese patients [28 male and 19 female; median age: 62 years (range, 17-78)] with advanced CRC (locally advanced or metastatic/recurrent cancer), who underwent chemotherapy at Niigata University Medical and Dental Hospital, Niigata, Japan, from March 2007 through to January 2013. The association between NQO1 expression and clinicopatholgical characteristics of CRC was assessed retrospectively. The demographic and clinicopathological characteristics of patients are summarized in **Table 1**. Patients were divided into two groups based on the NQO1 expression status on immunohistochemical examination (NQO1-positive and NQO1-negative groups). The study was approved by the Institutional Review Board at the Niigata University Medical and Dental Hospital.

DNA extraction and KRAS mutation analysis

Five serial sections (thickness: 10 μm) were prepared from each representative histological sections of primary colorectal tumor biopsy specimens or surgically resected specimens after their deparaffinization. Cancer tissue in each slice was dissected under microscopic guidance as described previously [19]. Non-neoplastic tissue in each slice was used as the source of constitutional DNA. DNA was extracted using a DNA Isolator PS Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan),

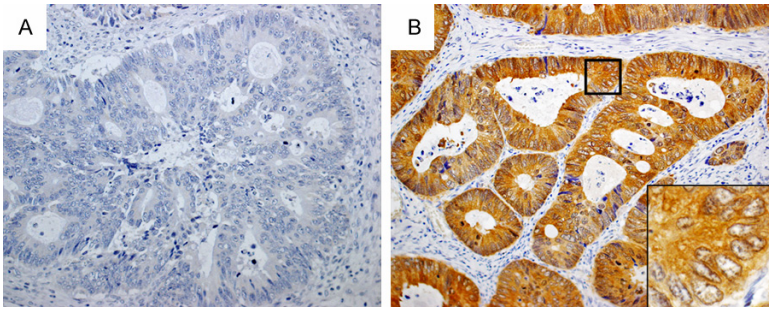

Figure 1. NQO1 immunohistochemical staining pattern in patients with advanced colorectal cancer. A. Representative section showing negative expression; B. Representative section showing positive cytoplasmic NQO1 expression in CRC cells (Original magnification ×200).

Table 2. Chemotherapy regimens as first chemotherapy for advanced colorectal cancer

Regimen	No. of patients
mFOLFOX6	7
mFOLFOX6 + anti-VEGF drug	10
mFOLFOX6 + anti-EGFR drug	3
FOLFIRI	2
FOLFIRI + anti-VEGF drug	4
FOLFIRI + anti-EGFR drug	1
XELOX	2
XELOX + anti-VEGF drug	9
SOX	1
IRIS	2
Anti-EGFR drug	2
Irinotecan	1
Irinotecan + anti-EGFR	1
UFT + leucovorin	1
UFT	1

mFOLFOX6, Modified infusional fluorouracil, leucovorin, and oxaliplatin; VEGF, Vascular endothelial growth factor; EGFR, Endothelial growth factor receptor; FOLFIRI, Infusional fluorouracil, leucovorin, and irinotecan; XELOX, Capecitabine plus oxaliplatin; SOX, S1 plus oxaliplatin; IRIS, Irinotecan plus S1.

as described previously [19]. KRAS mutation at codon 12, 13, and 61 were examined by nested polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism, and direct sequencing, as described elsewhere [20].

Pathologic evaluation

Biopsy or surgically resected specimens were submitted to the Department of Surgical Pathology in our hospital. Specimens were examined to determine the histological grade, as described in the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 7th edition [21].

Immunohistochemistry

From each resected specimen, 1 to 4 paraffin-embedded block (s) (median: 2 blocks) were used for immunohistochemistry (IHC). Three serial 3-μm thick sections, one for routine histological examination using hematoxylin-eosin staining, one for immunohistochemical staining with a rabbit monoclonal antibody against NQO1 (Epitomics, Burlingame, CA, USA), and one for a negative control, were prepared from each block. Two independent surgical pathologists who were blinded to clinical details examined each section. For IHC, sections were deparaffinized and rehydrated, then microwaved at 500 W for 21 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) to retrieve antigenic activity. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 20 minutes; subsequently, the sections were incubated overnight at 4°C with NQO1 rabbit monoclonal antibody (Epitomics, Burlingame, CA, USA; 1:200 dilution) using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Normal rabbit immunoglobulin was used as a negative control. Sections were counterstained using hematoxylin.

As described by Winski et al. [22], NQO1 expression was defined as the presence of cytoplasmic and/or nuclear staining. NQO1 expression in tumor specimens was classified as positive expression or negative expression. In this study, lack of staining or staining of only a few scattered cells (< 5%) was considered as NQO1-negative; positive staining of ≥ 5% of cells was considered as NQO1-positive.

Evaluation of prognostic factors

Eight conventional variables in addition to NQO1 expression were assessed for their potential association with outcomes in 47 patients (age, gender, location of the primary tumor [colon vs. rectum], serum CEA level before chemotherapy (< 5 ng/mL vs. ≥ 5 ng/mL), surgical resection, histological grade (G1/G2 vs. G3),

Table 3. Evaluation of therapeutic efficacy using RECIST version 1.1

	Total patients			KRAS wild			KRAS mutant		
	NQO1-positive	NQO1-negative	*P*-value	NQO1-positive	NQO1-negative	*P*-value	NQO1-positive	NQO1-negative	*P*-value
Response			0.52			0.83			0.07
CR	0	0		0	0		0	0	
PR	6	4		6	3		0	1	
SD	12	6		6	3		6	3	
PD	19	0		13	0		6	0	
DCR	48.6%	100%	0.0031	48.0%	100%	0.028	50.0%	100%	0.23

RECIST, Response Evaluation Criteria in Solid Tumor; NQO1, NAD(P)H: quinone oxidoreductase-1; CR, Complete response; PR, Partial response; SD, Stable disease; PD, Progressive disease; DCR, Disease control rate (CR + PR + SD).

KRAS mutation status, and use of anti-EGFR drugs in KRAS wild cases. Therapeutic efficacy was evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1.

Statistical analysis

Medical records and survival data were obtained for all 47 patients. Clinical features and pathological tumor-related factors were compared between the patient groups using the Fisher's exact test. The causes of deaths were determined from the medical records. The follow-up period was defined as the time from the date of initiation of chemotherapy and that of the most recent follow-up. Deaths from other causes were censored. Survival curves were constructed using the Kaplan-Meier method and differences in survival were evaluated using the log-rank test. The Cox proportional hazards model was used for multivariate survival analysis. All statistical analyses were performed using the SPSS version 23 software package (SPSS Japan Inc, Tokyo, Japan). All tests were 2-sided, and *P* value < 0.05 was considered statistically significant.

Results

NQO1 expression in CRC

IHC analysis revealed a predominantly cytoplasmic NQO1 expression. Thirty seven specimens (78.7%) showed NQO1-positive expression, while 10 (21.2%) specimens tested negative for NQO1 expression (**Figure 1**). There were no significant differences with respect to clinicopathological variables between the two groups (**Table 1**).

KRAS status in CRC patients

KRAS wild CRC was 37 (78.8%) and KRAS mutant CRC was 10 (21.3%). Of these, surgical resection was performed in 23 patients (48.9%). Thirteen patients (41.9%) with KRAS wild CRC were administered anti-EGFR antibody drugs (**Table 1**).

Therapeutic efficacy evaluation (RECIST version 1.1)

After the diagnosis of advanced CRC, various systemic chemotherapeutic regimens were administered (**Table 2**). 5-fulorouracil based chemotherapy was administered in 43 patients (91.5%) as the first regimen in our study. Biological agents were used in 32 patients (68.1%). Disease control rate (DCR; complete response + partial response + stable disease) was higher in NQO1-negative patients than in NQO1-positive patients (100% vs. 48.6%; P = 0.0031) (**Table 3**). Among KRAS wild cases, the DCR in NQO1-negative patients was higher than that in NQO1-positive patients (100% vs. 48.0%, P = 0.028). However, in patients with KRAS mutation, there was no significant difference in chemotherapeutic response between NQO1-negative and NQO1-positive patients (**Table 3**).

Survival analyses according to NQO1 expression

To further substantiate the importance of NQO1 expression in CRC, we assessed the progression-free survival (PFS) and overall survival (OS) of 47 CRC patients using Kaplan-Meier method. No significant differences were observed between NQO1-negative and NQO1-positive patients (**Figure 2A** and **2B**). However,

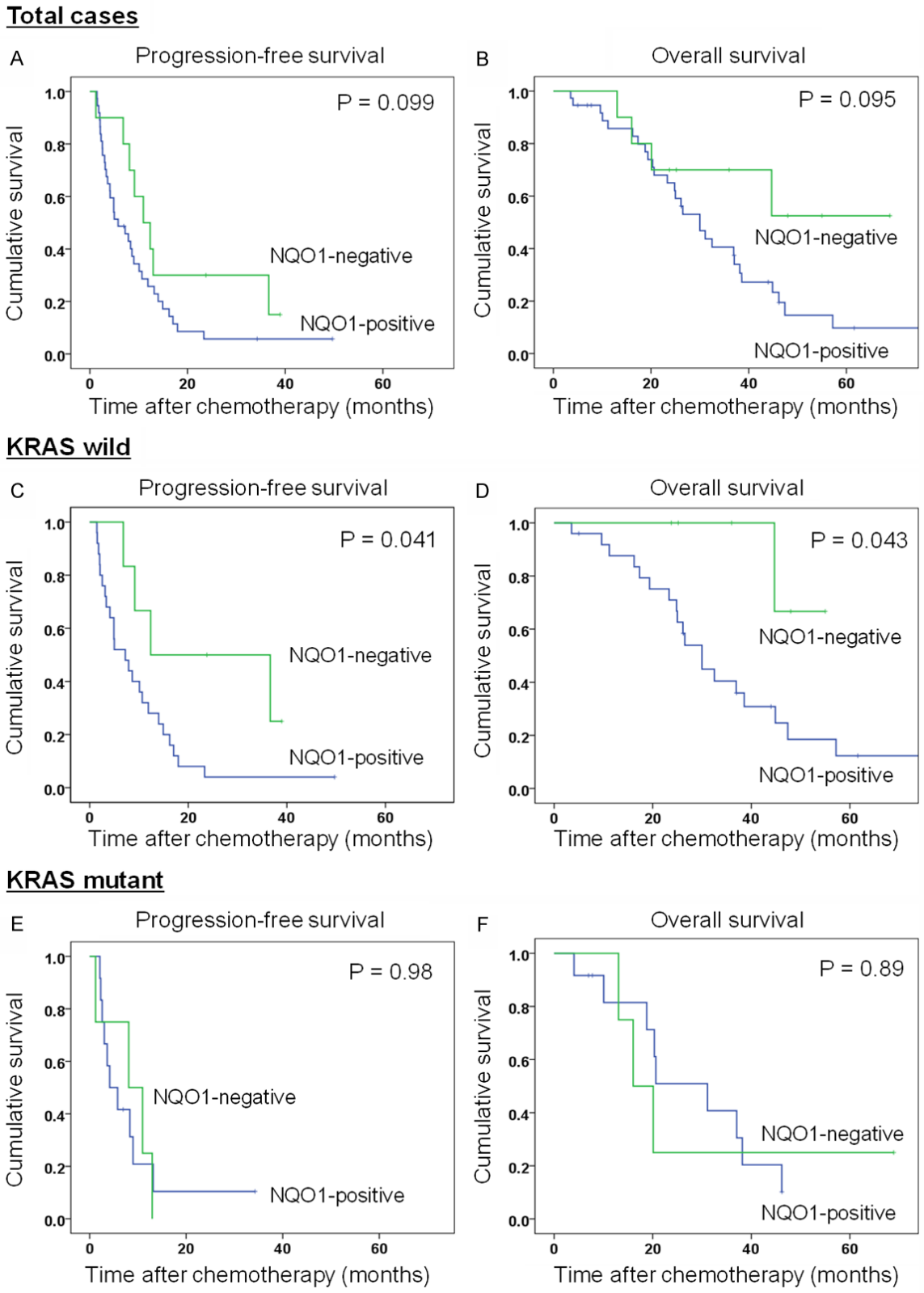

Figure 2. Kaplan-Meier survival curve for progression-free survival and overall survival after chemotherapy for advanced colorectal cancer according to NQO1 expression. Among patients with KRAS wild CRC, NQO1-negative patients had increased PFS compared with NQO1-positive patients (P = 0.041), and NQO1-negative patients had increased OS compared with NQO1-positive patients (P = 0.043).

Table 4. Prognostic factors for PFS in patients with KRAS wild colorectal cancer

	Univariate		Multivariate		
	Median PFS (months)	*P*-value	HR	95% CI	*P*-value
Age (years)					
< 65	7.9	0.19			
≥ 65	11.9				
Gender					
Male	9.1	0.74			
Female	6.8				
Location					
Colon	6.8	0.76			
Rectum	10.1				
CEA (ng/mL)					
< 5	10.6	0.99			
≥ 5	7.9				
Histological grade					
G1/G2	8.6	0.305			
G3	7.9				
Surgical resection					
Yes	9.1	0.18			
No	7.2				
Use of anti-EGFR drugs					
Yes	8.6	0.30			
No	7.9				
NQO1 expression					
Positive	7.2	0.041	1		0.049
Negative	12.3		2.9	1.01-8.3	

PFS, Progression-free survival; KRAS, Kirsten rat sarcoma viral oncogene homolog; HR, Hazard ratio; CI, Confidence interval; CEA, Carcinoembryonic antigen; EGFR, Epidermal growth factor receptor; NQO1, NAD(P)H: quinone oxidoreductase-1.

among patients with KRAS wild CRC, NQO1-negative patients had significantly longer PFS and OS than NQO1-positive patients (P = 0.041 and P = 0.043, respectively) (**Figure 2C** and **2D**). In patients with KRAS mutant CRC, PFS, and OS showed no significant difference between NQO1-negative and positive groups (**Figure 2E** and **2F**). In patients with KRAS wild CRC, NQO1 expression was the only factor associated with PFS on univariate analysis (P = 0.041) (**Table 4**). We therefore entered all variables in a Cox proportional hazards model for multivariate analysis. Negative NQO1 expression was found to be an independent prognostic factor for longer PFS in patients with KRAS wild CRC [hazard ratio (HR) = 2.9, 95% confidence interval (CI) = 1.01-8.3; P = 0.049].

Furthermore, surgical resection (P = 0.008) and NQO1 positive expression (P = 0.043) were associated with OS in the univariate analysis in KRAS wild CRC (**Table 5**). We entered all variables into a Cox proportional hazards model for multivariate analysis. Surgical resection was found to be an independent prognostic factor of longer OS in these patients (HR = 6.1, 95% CI = 1.5-24.4; P = 0.011).

Discussion

The catalytic properties of NQO1 were first reported by Ernster and Navazio in 1958 [23]. The gene for NQO1 also known as DT-diaphorase, is located on chromosome 16q22 and consists of six exons and five introns [7]. NQO1 is present in several organs and its expression is induced along with a battery of defensive genes in response to stresses including xenobiotics, oxidants, ultraviolet light, ionizing radiation and toxic substances [24]. NQO1 function is primarily to protect normal cells against these stressors. On the other hand, high NQO1 expression is associated with many solid tumors [11-18]. Awadallah et al. reported upregulation of NQO1 expression in pancreatic ductal adenocarcinoma [25]. An association between NQO1 expression and chemotherapy resistance has been widely reported [15, 26]. NQO1 knockdown or inhibition of its enzymatic activity was shown to enhance cisplatin-induced cytotoxicity in urogenital cancer cell lines [26]. Lin et al. demonstrated an association between NQO1 overexpression and increased tumor size, serosal invasion, and late-stage tumors, which suggests a role of NQO1 expression in tumorigenesis and malignant progression of gastric cancer [15]. Therefore, NQO1 might be useful as a poor prognostic biomarker in patients with gastric cancer [15].

In CRC, Mikami et al. attributed that NQO1 expression was associated with malignant attributes of colorectal tumors. CRC with nodal me-

Table 5. Prognostic factors for OS in patients with KRAS wild colorectal cancer

	Univariate		Multivariate		
	Median OS (months)	*P*-value	HR	95% CI	*P*-value
Age (years)					
< 65	17.7	0.36			
≥ 65	5.4				
Gender					
Male	36.9	0.863			
Female	30.0				
Location					
Colon	38.6	0.373			
Rectum	32.5				
CEA (ng/mL)					
< 5	47.4	0.473	1		0.152
≥ 5	32.5		0.19	0.02-1.85	
Histological grade					
G1/G2	26.1	0.687			
G3	36.9				
Surgical resection					
Yes	57.2	0.008	6.1	1.5-24.4	0.011
No	26.1		1		
Use of anti-EGFR drugs					
Yes	36.9	0.771			
No	30.0				
NQO1 expression					
Positive	30.0	0.043	1		0.068
Negative	-		7.4	0.86-62.5	

OS, Overall survival; KRAS, Kirsten rat sarcoma viral oncogene homolog; HR, Hazard ratio; CI, Confidence interval; CEA, Carcinoembryonic antigen; NQO1, NAD(P)H: quinone oxidoreductase-1.

tastases showed significantly higher NQO1 (DT-diaphorase) expression than CRC without metastases [16]. However, the association between NQO1 expression and clinical prognosis in patients with CRC is not clear.

Targeted therapies for CRC have rapidly evolved in recent years. However, less than 20% of patients with CRC respond to clinically available targeted drugs when used as monotherapy [27]. In this study, we found a direct association between NQO1 expression and resistance to chemotherapy. To the best of our knowledge, this is the first study to evaluate the association between NQO1 expression and the KRAS status in patients with advanced CRC.

Some studies have shown association between high NQO1 expression and poor prognosis in the context of solid tumors [15, 16]. Patients with high expression levels of NQO1 in cervical squamous cell carcinoma had lower disease-free survival (DFS) and 5-year OS [16]. In patients with gastric adenocarcinoma, high NQO1 expression was associated with lower DFS and OS than their counterparts with low NQO1 expression [15]. These findings suggested a potential diagnostic and prognostic value of NQO1 expression in solid tumors. However, the function of NQO1 in malignancies remains controversial. Marco et al. demonstrated that NQO1 expression induced cell cycle progression via upregulation of cyclin A2, B1 and D1 [24]. Asher et al. showed that NQO1 played an important role in the regulation of p53 functions by inhibiting its degradation [28]. These findings suggested a relation between high level of NQO1 expression and tumorigenesis as well as malignant progression of cancer [24].

In our study, there was no association between the NQO1 expression and therapeutic efficacy/clinical prognosis in KRAS mutated CRC. It is well known that various molecular pathways are involved in CRC development. In particular, the mitogen-activated protein kinases (MAPK) and phosphoinositide-3 kinase (PIK3) pathways, which are modulated by RAS protein, are two of the main pathways involved in the development of CRC [29]. MAPK and PIK3 cascades associated with “RAS” are very important to carcinogenesis and progression of CRC [17].

Recently, NQO1 has been used as a therapeutic target in tumor cells, which exemplifies the enzyme directed approach to anticancer drug development [30]. β-lapachone which targets NQO1, was shown to induce programmed necrosis in solid tumors. Huang et al. reported that deoxynyboquinone kills a wide spectrum of cancer cells in an NQO1-dependent manner with greater potency than β-lapachone [31, 32]. Therefore, deoxynyboquinone

is a very promising anticancer therapeutic agent for treatment of solid tumors. Further studies are necessary to verify whether NQO1 targeted drugs may be of clinical benefit to patients with solid tumors.

This study had several limitations. First, this study was a retrospective analysis of a small number of patients in a single center. Second, the validity of IHC for NQO1 expression in CRC has not been demonstrated. However, IHC has been used to evaluate NQO1 expression in the context of cervical and gastric carcinoma [15, 16]. Third, minor mutations such as NRAS, BRAF were not investigated in our study. Nevertheless, we believe that the results of the present study are informative for prediction of therapeutic efficacy and prognosis in patients with KRAS wild CRC. Further prospective, large-scale, multicenter studies are needed to clarify the role of NQO1 expression in CRC.

In conclusion, our obtained data suggested that NQO1 expression confers chemotherapeutic resistance in patients with KRAS wild CRC. Only a few studies have investigated the association between KRAS and NQO1, and the underlying mechanism by which NQO1 status determined the prognosis of KRAS wild type is unknown. Oncogenic activation of KRAS in murine embryonic fibroblasts was recently shown to induce Nrf2 activity by binding to Jun and Myc oncogenes [33]. Together with our data, it is plausible that KRAS-independent NQO1 expression may determine the prognosis in KRAS wild CRC. NQO1-negative expression using IHC may be a favorable predictive prognostic factor in KRAS wild CRC patients.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (No. 26462006) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure of conflict of interest

None.

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