

Forkhead Box Q1 Expression Is Associated With Tumor Location of Right-Sided Colon, But Not With Acquisition of Oxaliplatin Resistance in Colorectal Cancer

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Research Article

Keywords: FOXQ1, drug resistance, oxaliplatin, colorectal cancer, tumor location

Posted Date: February 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-154413/v1>

Abstract

Oxaliplatin (OHP) is a reagent for the standard treatment of advanced and recurrent colorectal cancer (CRC), although OHP resistance mechanisms are not fully elucidated. We found that OHP-resistant clones derived from HCT116, but not DLD1 were also resistant against the other drugs used for CRC treatment (5-fluorouracil, OHP, and trifluorothymidine) and their xenograft tumors were resistant against OHP treatment. Among the candidate genes derived from microarray analysis using the samples of OHP-resistant cells and their xenografts derived from HCT116, Forkhead box Q1 (FOXQ1) was further assessed for validation of OHP resistance and its association with clinicopathological features. Modification of FOXQ1 via siRNA knockdown and expression vector could not confirm the involvement of FOXQ1 in OHP resistance. In 173 CRC patients, FOXQ1 was upregulated in most CRC tumors compared to normal colonic mucosa. FOXQ1 expression was significantly different by tumor location of the right-sided colon cancer compared with left-sided and rectal cancer. Moreover, expression level was significantly associated with prognosis in advanced and recurrent patients. TCGA data also showed significant association of FOXQ1 expression with tumor location. Our results indicated that FOXQ1 expression is associated with tumor location of right-sided colon, but not with acquisition of OHP resistance in colorectal cancer.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide¹.

Combination of anticancer drugs (oxaliplatin [OHP], irinotecan [CPT], and 5-fluorouracil [5-FU]) and antibodies targeting vascular endothelial growth factor and epidermal growth factor receptor (EGFR) has become the standard therapy, improving the prognosis of advanced CRC^{2,3}.

However, advanced CRC is still a fatal disease because resistance against these anticancer drugs is common, despite the initial effectiveness of these drugs. Therefore, elucidating the mechanisms involved in drug resistance is indispensable for further improvements in the prognosis of CRC patients.

The mechanisms of resistance against OHP for advanced CRC treatment have been assessed to improve the prognosis of CRC⁴. However, no molecular marker is available for clinical use. Differing from molecular targeted therapy, cytotoxic reagents do not have target genes or proteins. Moreover, multidrug resistance after standard therapy for CRC is also a critical problem, although no molecular marker for multidrug resistance has yet to be identified for clinical use⁵.

Tumor location in the right-sided colon was reported as a prognostic marker before the era of molecular targeted therapy, although the background was unclear⁶. Recently, EGFR-targeted antibodies have been shown to be ineffective for right-sided colon cancer (RCC) patients regardless of the existence of *RAS* mutations⁷⁻⁹. However, the genetic background of patients with advanced RCC suffering from poor prognosis has not yet been elucidated^{10,11}.

In this study, we established OHP-resistant clones from CRC cell lines (DLD1 and HCT116) to elucidate the mechanism of OHP resistance. Then, we assessed molecular markers associated with OHP resistance in HCT116-derived clones by microarray using both *in vitro* (cells) and *in vivo* (xenografts) samples, because the clones derived from HCT116 but not DLD1 also became resistant to the other drugs (5-FU, CPT and trifluorothymidine (TFT)). Among the identified genes, we selected Forkhead box Q1 (FOXQ1) as a candidate gene, because it was reported to be upregulated in CRC tissues and is associated with CRC progression and drug resistance¹²⁻¹⁹. Then, we performed validation of FOXQ1 involvement in OHP resistance and assessed the association of FOXQ1 with the clinical features of CRC patients treated at our department and TCGA samples.

Results

Drug sensitivity and proliferation rate

The drug sensitivity of DLD1, HCT116, and OHP-resistant clones is shown in Table 1. IC₅₀ against the other drugs in OHP-resistant clones derived from DLD1 were less than two times compared to those in DLD1, although there were statistically significant differences between DLD1 and OHP resistant clones in limited cases. On the other side, IC₅₀ against the other drugs in OHP-resistant clones derived from HCT116 were more than two times compared to those in HCT116 except for HCT/OHP3 against 5-FU. There was statistically significant difference between HCT116 and OHP resistant clones except for HCT/OHP5 against TFT. Thus, OHP-resistant clones derived from HCT116 but not DLD1 had become multidrug resistant. This different pattern of drug sensitivity other than OHP suggested that DLD1 and HCT116 might use different mechanisms for acquisition of OHP resistance. This was also supported by principal component analysis of microarray data of these OHP resistant cells (Figure S1). Therefore, the mechanism used for OHP resistance in HCT116 seems more critical than in DLD1, because this mechanism may induce multidrug resistance during treatments for the patients with advanced CRC.

Table 1
Drug sensitivity of oxaliplatin resistant clones

Drug	Cell	IC ₅₀ (mean ± SD)	* <i>P</i>	Cell	IC ₅₀ (mean ± SD)	** <i>P</i>
Oxaliplatin (μM)	DLD1	4.38 ± 1.46	–	HCT116	0.49 ± 0.22	–
	DLD/OHP1	12.14 ± 3.06	0.005	HCT/OHP1	3.13 ± 1.4	0.002
	DLD/OHP4	10.0 ± 3.31	0.049	HCT/OHP3	7.73 ± 2.83	< 0.0001
	DLD/OHP5	18.70 ± 11.04	< 0.0001	HCT/OHP5	5.82 ± 0.87	0.0005
5-fluorouracil (μM)	DLD1	0.76 ± 0.19	–	HCT116	1.26 ± 0.41	–
	DLD/OHP1	1.23 ± 0.29	0.001	HCT/OHP1	3.11 ± 1.03	0.0005
	DLD/OHP4	0.99 ± 0.41	0.10	HCT/OHP3	2.4 ± 0.45	0.01
	DLD/OHP5	0.84 ± 0.14	0.59	HCT/OHP5	2.71 ± 0.13	0.008
Irinotecan (μg/ml)	DLD1	2.25 ± 0.37	–	HCT116	0.74 ± 0.15	–
	DLD/OHP1	3.35 ± 0.84	0.002	HCT/OHP1	2.73 ± 0.44	0.0003
	DLD/OHP4	2.26 ± 0.6	0.94	HCT/OHP3	2.69 ± 0.99	0.0003
	DLD/OHP5	3.16 ± 0.79	0.01	HCT/OHP5	1.79 ± 0.74	0.04
TFT (μM)	DLD1	3.41 ± 0.21	–	HCT116	1.14 ± 0.58	–
	DLD/OHP1	4.08 ± 0.38	0.11	HCT/OHP1	20.22 ± 6.74	0.0001
	DLD/OHP4	3.72 ± 0.28	0.43	HCT/OHP3	17.67 ± 9.83	0.0005
	DLD/OHP5	4.23 ± 0.75	0.06	HCT/OHP5	5.56 ± 3.34	0.30
* <i>P</i> : statistically evaluated compared to DLD1 by t-test, ** <i>P</i> : statistically evaluated compared to HCT116 by t-test, TFT: Trifluorothymidine						

There was no significant difference between the doubling time of HCT116 and OHP-resistant clones. Doubling time was 19.0 ± 4.0 h in HCT116, 19.0 ± 1.0 h in HCT/OHP1, 22.5 ± 2.8 h in HCT/OHP3, and 18.6 ± 2.5 h in HCT/OHP5, respectively (mean \pm standard deviation).

Xenograft model of OHP-resistant clones

Unlike cell cultures, tumor growth was significantly different in tumors derived from HCT/OHP1 ($P < 0.0001$) and HCT/OHP3 ($P < 0.0001$) compared with those derived from HCT116, although tumor growth was similar between tumors derived from HCT116 and HCT/OHP5 ($P = 0.63$; Fig. 1A). These experiments have been performed twice and showed the same results. Thus, drug resistance induced slower tumor progression, although drug resistance was believed to enhance tumor progression. OHP treatment induced significant growth inhibition ($P = 0.003$) in HCT116-derived tumors with a relatively low tumor growth inhibition (TGI) value of 0.37 (Fig. 1B). OHP treatment was ineffective in both HCT/OHP1-derived and HCT/OHP5-derived tumors with TGI values of 0.12 and 0.07, respectively. CPT treatment induced significant growth inhibition in HCT116-derived tumors ($P < 0.0001$) with a TGI value of 0.8 (Fig. 1B). CPT treatment was statistically effective in HCT/OHP5-derived tumors ($P < 0.0001$) with a TGI value of 0.76. However, CPT treatment was not effective in HCT/OHP1-derived tumors ($P = 0.17$) with a TGI value of 0.53 (Fig. 1C and 1D). These different sensitivities *in vivo* should be associated with the differences in CPT sensitivity *in vitro* (more resistance in HCT/OHP1 than in HCT/OHP5; Table 1).

Candidate genes responsible for OHP resistance

Eighty-eight genes showed more than two times higher expression in both three OHP resistant clones compared to HCT116 and two tumors derived from OHP resistant clones compared to tumor derived from HCT116 (Table 2). Twenty-nine genes showed less than half of expression in both three OHP resistant clones compared to HCT116 and two tumors derived from OHP resistant clones compared to tumor derived from HCT116 (Table S1). Among these genes, we considered FOXQ1 was the most suitable candidate gene because of its association with drug resistance and tumor progression^{12,19}. Upregulation of FOXQ1 in OHP-resistant clones derived from HCT116 and tumors derived from these clones was confirmed by qRT-PCR (Table 3). However, upregulated FOXQ1 expression in tumors derived from OHP resistant clones was not associated with enhanced tumor growth (Fig. 1C and 1D). In DLD1 and its OHP-resistant clones, FOXQ1 expression has not changed by OHP resistance. (Table 3).

Table 2
Upregulated genes in Oxaliplatin resistant clones derived from HCT116

ADAMTS14	C6orf15	CTSD	FOXQ1	IGFL1	LGALS9	PCDH1	SNAR-G1
AKR1C1	CALB2	CXCL1	GATSL3	IKZF2	LGALS9C	PMEPA1	SOAT2
AKR1C3	CCL28	CXXC4	HERC6	IL15	LMO7	PPAP2B	SRPX2
ALCAM	CD274	CYP2J2	HOXA3	IL7	LOC100507165	PTHLH	STRA6
APOBEC3C	CFH	DMBT1	HOXB3	INHBB	LOC643072	RAB27B	TMEM164
APOBEC3D	CLIP4	DNER	HOXB8	ITGB2	MALT1	RPS6KA2	TMX4
APOBEC3F	CMPK2	DUSP10	HRCT1	KIAA1244	MAST4	SAMD9L	WNT7A
APOBEC3G	COL13A1	EDN1	HSPA1A	KLF12	MGP	SERPINA1	WWC3
ARHGEF37	COL9A2	EPSTI1	IFI44	KRT86	MIA	SGK1	XDH
BCAM	CPAMD8	FILIP1L	IFIT3	KRTAP3-1	MILR1	SH3BP4	XLOC_I2_012847
C10orf11	CSF2RA	FOXA1	IGFBP6	LAMP3	MX1	SH3TC2	ZNF365

Table 3
Relative FOXQ1 gene expression in vitro and in vivo

Sample		Oxaliplatin	FOXQ1	Oxaliplatin	FOXQ1
Cell/Tumor		treatment	expression	treatment	expression
Cell	HCT116	(-)	1	(+)	2.6
	HCT/OHP1	(-)	48.7	(+)	92.3
	HCT/OHP3	(-)	106.4	(+)	193.9
	HCT/OHP5	(-)	15.6	(+)	27.1
	DLD1	(-)	1	(+)	0.80
	DLD/OHP1	(-)	1.30	(+)	1.32
	DLD/OHP4	(-)	0.75	(+)	0.55
	DLD/OHP5	(-)	0.75	(+)	0.49
Tumor	HCT116	(-)	1	Not performed	
	HCT/OHP1	(-)	8.5	Not performed	
	HCT/OHP3	(-)	47.4	Not performed	
	HCT/OHP5	(-)	15.7	Not performed	

Influence of modified FOXQ1 expression on OHP sensitivity

The IC₅₀ of OHP in HCT116 cells and HCT/OHP5 cells were 1.35 μ M and 7.25 μ M following FOXQ1 siRNA knockdown and 1.82 μ M and 9.3 μ M following the control siRNA treatment (Table 4). The IC₅₀ of OHP in HCT116 cells and HCT/OHP cells was 3.43 μ M and 18.61 μ M following FOXQ1 expression vector treatment and 2.05 μ M and 17.94 μ M following the control vector (Table 4). Thus, modification of FOXQ1 gene expression could not change IC₅₀ of OHP in

both HCT116 cells and HCT/OHP5 cells. In case of DLD1 and DLD/OHP5, IC₅₀ of OHP has increased by FOXQ1 siRNA knockdown compared to the control siRNA treatment.

Table 4
Effect of FOXQ1 modification on IC50 of Oxaliplatin

Cell	Treatment	IC50 of Oxaliplatin (μM)
HCT116	siRNA(Control)	1.82
HCT116	siRNA(FOXQ1)	1.35
HCT/OHP5	siRNA(Control)	9.3
HCT/OHP5	siRNA(FOXQ1)	7.25
DLD1	siRNA(Control)	2.05
DLD1	siRNA(FOXQ1)	5.04
DLD/OHP5	siRNA(Control)	17.86
DLD/OHP5	siRNA(FOXQ1)	35.93
HCT116	Plasmid(Control)	5.53
HCT116	Plasmid(FOXQ1)	3.43
HCT/OHP5	Plasmid(Control)	17.94
HCT/OHP5	Plasmid(FOXQ1)	18.61

Relative FOXQ1 expression in colorectal cancer compared with normal mucosa

Relative FOXQ1 expression was not influenced by clinical features including age, sex, existence of preoperative chemotherapy, clinical stage, and recurrence/stage IV (Table 5). However, tumor location was significantly associated with FOXQ1 expression (Table 5). FOXQ1 expression in RCC was significantly lower than left-sided colon ($P=0.006$) and rectal cancer ($P=0.01$). Microsatellite instability was not associated with FOXQ1 expression. KRAS mutation or BRAF mutation alone was not associated with FOXQ1 expression, although the combined status of KRAS mutation and BRAF mutation was associated with FOXQ1 expression ($P=0.02$).

Table 5. Association of Clinical features and relative FOXQ1 expression in CRC patients

Clinical features	Category	Number	Relative FOXQ1 expression	<i>P</i> value
Age class	<65	60	38.8	0.84
	65–74	53	40.7	
	75≤	60	45.3	
Gender	Male	100	47.6	0.13
	Female	73	33.5	
Tumor location	Right-sided colon	62	23.5	0.0088
	Left-sided colon	36	58.1	
	Rectum	75	48.8	
Preoperative chemotherapy	No	155	43.1	0.34
	Yes	18	28.7	
Clinical stage	I	16	29.3	0.34
	II	34	53.9	
	III	36	49	
	IV	87	36.1	
Stage IV/recurrence	Yes	101	40.7	0.81
	No	72	43.0	
Microsatellite status	MSS	159	41.7	0.92
	MSI	13	43.4	
KRAS mutation	No	115	47.3	0.09
	Yes	57	30.8	
BRAF mutation	No	158	42.8	0.17
	Yes	12	18.0	
KRAS/BRAF mutation	No	101	49.7	0.024
	Yes	69	28.6	

In recurrent or stage IV CRC patients, overall survival (OS) was significantly better in those with a relative FOXQ1 expression of $20 \leq$ than in those with < 20 ($P = 0.0007$; Fig. 2A). This significance was also shown when cases were limited to the right-sided colon ($P = 0.014$, Fig. 2B), but not left-sided colon (Fig. 2C) nor rectum (Fig. 2D). Then, the extent of FOXQ1 upregulation was associated with tumor location and poor prognosis in advanced cancer.

FOXQ1 expression in TCGA samples

We assessed FOXQ1 expression in colorectal samples to confirm our findings in TCGA samples. Advanced clinical stage was not associated with FOXQ1 expression, although there was significant difference in FOXQ1 expression between stage II and stage IV. Tumor location was significantly associated with FOXQ1 expression (Table 6). FOXQ1 expression in RCC was significantly lower than left-sided colon ($P < 0.0001$) and rectal cancer ($P = 0.0001$). Microsatellite instability was significantly associated with FOXQ1 expression ($P < 0.0001$). KRAS mutation, BRAF

mutation, and the combined status of KRAS mutation and BRAF mutation were significantly associated with FOXQ1 expression. There was no significant difference in OS by extent of FOXQ1 expression. Then, significant association of FOXQ1 expression was shown with tumor location and combined status of KRAS mutation and BRAF mutation both in our department samples and TCGA samples.

Table 6. Association of Clinical features and relative FOXQ1 expression in TCGA data

Clinical features	Category	Number	Δ z-scores relative to normal samples between FOXQ1 and GAPDH	<i>P</i> value
Age class	<65	241	5.29	0.13
	65–74	179	5.09	
	75≤	167	4.60	
Gender	Male	309	5.13	0.47
	Female	278	4.93	
Tumor location	Right-sided colon	247	4.15	<0.0001
	Left-sided colon	144	6.08	
	Rectum	160	5.55	
Clinical stage	I	102	5.11	0.006
	II	218	4.40	
	III	171	5.25	
	IV	84	6.14	
Microsatellite status	MSS/MSI-L	504	5.43	<0.0001
	MSI-H	83	2.47	
KRAS mutation	No	397	5.25	0.024
	Yes	193	4.58	
BRAF mutation	No	534	5.20	0.0002
	Yes	56	3.42	
KRAS/BRAF mutation	No	342	5.53	<0.0001
	Yes	248	4.36	

Discussion

Here we assessed the mechanisms associated with OHP resistance in CRC cells. We found that OHP resistant clones derived from DLD1 and HCT116 showed different patterns of drug sensitivity against 5-FU, CPT, and TFT, which were used for CRC treatment after OHP resistance. We found that OHP resistant clones derived from HCT116 were also resistant against 5-FU, CPT, and TFT, although OHP resistant clones derived from DLD1 showed the same sensitivity

as DLD1. Then, the mechanisms used in HCT116 seemed more critical than DLD1, because further treatments after OHP resistance maybe ineffective. These data indicated that there might be several mechanisms for OHP resistance in CRC.

Then, we further assessed the genes which have changed significantly in three OHP resistant clones compared to parental HCT116 cells both *in vitro* and *in vivo* using microarray analysis. Among the genes significantly changed in three OHP resistant clones compared to HCT116, we selected FOXQ1 as the candidate gene associated with acquisition of OHP resistance. This is because upregulation of FOXQ1 in CRC tissue and its association with tumor progression and drug sensitivity has been reported^{12–18}. However, expression and influence of FOXQ1 on CRC cell lines were not consistent in these reports.

In this study, inhibition of FOXQ1 expression did not change IC₅₀ of OHP in HCT116 and its OHP resistant clone, which was not consistent with previous report using SW480 cells¹⁵. Enhancement of FOXQ1 by expression vector also could not change IC₅₀ of OHP. Thus, we considered that upregulation of FOXQ1 in OHP resistant clones derived from HCT116 might result from OHP treatment but was not the cause of OHP resistance. These results also showed the limitations of exploring drug resistance mechanisms using drug-resistant cells.

Regarding influence of FOXQ1 expression on tumor progression, tumor growth derived from OHP resistant clones was similar or downregulated compared to those derived from HCT116. These data were consistent^{14,16} and inconsistent^{12,18} with the previous reports, although upregulation of FOXQ1 was acquired by drug resistance in this study and by genetic modification in the previous reports. In clinical samples of our department and TCGA, FOXQ1 expression was not associated with clinical stage. These data are not inconsistent with the previous report¹⁵.

On the other hand, upregulation of FOXQ1 in CRC tissue compared with normal tissue was consistent with previous studies^{12–15, 17,18}. Moreover, we found that FOXQ1 expression differed by tumor location both in our cohort and in TCGA samples. This is the first report indicating the association of FOXQ1 with tumor location of RCC, which was considered prognostic marker in advanced and recurrent CRC patients^{6–9}. In advanced and recurrent CRC patients of our cohort, FOXQ1 expression was associated with overall survival of RCC, which was not shown in stage IV patients of TCGA samples. This difference in TCGA samples, but not in our cohort, FOXQ1 expression was significantly lower in MSI-H patients compared to MSS patients. In patients with KRAS or BRAF mutations, FOXQ1 expression was significantly lower than those without mutations in both our cohort and TCGA samples. The differences between our cohort and TCGA samples include more frequency of stage IV and recurrent patients in our cohort.

Our study has several limitations. First, we have not elucidated the mechanisms of OHP resistance, although this study started this purpose at first. FOXQ1 was not associated with acquisition of OHP resistance in DLD1 and HCT116 cells, and genes other than FOXQ1 have not yet been assessed. Second, we have not assessed the mechanisms associated with the differential expression of FOXQ1 by tumor location, although these may elucidate the reason of tumor location of RCC for the prognostic marker of CRC.

In conclusion, our study shows that several mechanisms are associated with acquisition of OHP resistance and FOXQ1 expression is associated with tumor location of RCC. Further study is necessary to elucidate these mechanisms.

Methods

Drugs and chemicals

OHP, CPT, and 5-FU were purchased from NIPRO (Osaka, Japan), TOWA Pharmaceutical Co., (Kadoma, Japan), and KYOWA KIRIN (Tokyo, Japan), respectively. TFT was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and dissolved in DMSO at a concentration of 20 mM. All drugs were diluted in culture medium immediately before use.

Cell lines and cloning of drug-resistant cells

Human CRC cell line DLD1 was purchased from Japan Health Sciences Foundation (Osaka, Japan). Human CRC cell line HCT116 was a kind gift from Dr. Yamamoto (Department of Surgery and Clinical Oncology, Osaka University Graduate School of Medicine, Osaka, Japan). These cells were authenticated by American Type Culture Collection using DNA profiling (Manassas, Virginia, USA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 10,000 units penicillin, 10 mg/ml streptomycin, and 25 µg/ml amphotericin B. Culture media and fetal bovine serum were obtained from Life Technologies Japan (Tokyo, Japan). All cells were grown at 37°C in a humidified incubator with 5% CO₂. DLD1 and HCT116 cells were co-cultured with 20 µM OHP or 10 µM OHP, respectively. Then, OHP-resistant clones were isolated by limited dilutions and named as DLD/OHP1, DLD/OHP4, and DLD/OHP5, when derived from DLD1, and HCT/OHP1, HCT/OHP3, and HCT/OHP5, when derived from HCT116.

Drug sensitivity and proliferation rate

Cells were seeded in 200 µl medium in 96-well flat-bottom plates at a density of 2×10^3 cells per well. The next day, the medium was removed and serial dilutions of OHP (0.1 – 500 µM), 5-FU (0.1 – 500 µM), CPT (0.1 – 100 µg/ml), and TFT (0.1 – 100 µM) were added to each well. After 96 h, cells were counted using a cell counting kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Half maximal inhibitory concentration (IC₅₀) values were calculated as the concentrations that corresponded to a 50% reduction in cellular proliferation compared with untreated cells. The proliferation rate was calculated as the doubling time of the cell number as measured by the cell counting kit. Experiments were performed independently at least three times, and data are shown as means ± standard deviations.

Animal experiments

Four-week-old female BALB/cAJcl-nu/nu mice were purchased from Japan Clea Inc. (Tokyo, Japan). Mouse care and experiments were performed under specific pathogen-free conditions at the Institute of Experimental Animal Science, Hyogo College of Medicine. All animal protocols were approved by the Institutional Animal Care and Use Committee of Hyogo College of Medicine (12–067). All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Hyogo College of Medicine. Then, the study was carried out in compliance with the ARRIVE guidelines (<http://www.nc3rs.org.uk/page.asp?id=1357>).

To assess the tumor growth of xenografts derived from HCT116 and OHP-resistant clones, a total of $5 \cdot 10^6$ HCT116 cells and OHP-resistant clones (HCT/OHP1, HCT/OHP3, and HCT/OHP5 cells) were subcutaneously inoculated in the right and left flanks of seven mice, respectively. Tumor size was measured twice a week. Tumor volume was calculated as $a \times b^2$ (where a represents the tumor length and b represents its width).

To assess OHP resistance in xenografts, a total of $5 \cdot 10^6$ HCT116 cells and OHP-resistant clone HCT/OHP5 cells were subcutaneously inoculated in both flanks of 12 mice (24 sites). Xenograft tumors derived from HCT/OHP1 cells were minced and inoculated into both flanks of the 12 mice because the growth of HCT/OHP1-derived xenograft tumors was so slow compared with that of HCT116 and HCT/OHP5. Tumor size was measured twice a week and tumor volume was calculated as described above. When the tumor diameter was > 5 mm, the mice were randomized into no treatment (control) or drug treatment groups (5 mg/kg OHP or 10 mg/kg CPT injected intraperitoneally twice a week for a total of five weeks, four mice [eight tumors] per each group) after adjusting the mean tumor volume among the

groups. The rate of TGI was calculated as follows: $1 - (\text{increase in tumor volume in the drug treatment group}) / (\text{increase in tumor volume in the control group})$. TGI was assessed after the completion of drug treatment.

Microarray data analysis

A total of 1×10^6 HCT116, HCT/OHP1, HCT/OHP3, and HCT/OHP5 cells were seeded onto P10 tissue culture plates. Twenty-four hours later, total RNA was extracted using an RNeasy Mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. Total RNA was also extracted from human tumor xenografts derived from HCT116, HCT/OHP1, and HCT/OHP5 without drug treatment.

Gene expression profiles were analyzed by Agilent SurePrint G3 Human GE 8x60K v2 Microarray kit (Agilent). The data set is available at Gene Expression Omnibus under accession number GSE77932 for cell experiments and GSE124808 for tumor experiments.

Signal data were imported into GeneSpring (Agilent) for analysis. Signal evaluation was performed depending on signal uniformity and the significant difference between signal and background. Signal data were normalized by the 75th percentile among arrays. Genes for which signal data were ≥ 2 in OHP resistance clones compared to HCT116 both *in vitro* and *in vivo* were selected as upregulated genes. Genes for which signal data were $0.5 \geq$ in OHP resistance clones compared to HCT116 both *in vitro* and *in vivo* were selected as downregulated genes.

Validation of FOXQ1 involvement in OHP resistance

Among the upregulated and downregulated genes, *FOXQ1* was selected as a candidate gene because of its association with drug resistance and tumor progression^{12–19}. *FOXQ1* expression in cells and tumors was evaluated by quantitative real-time reverse transcription PCR (qRT-PCR), as previously described^{17,20}. The primer sequences were *FOXQ1*-forward: CTTCCCTCCCCCTAAGTACAT and *FOXQ1*-reverse: ATGCCACATACGTACACGGATG. *GAPDH* was used as an internal control. ΔCT was calculated using the CT (Threshold cycle) value of *FOXQ1* and that of *GAPDH* in each sample. Data was calculated from triplicate wells.

The influence of *FOXQ1* on OHP resistance was assessed by inhibition of *FOXQ1* expression by siRNA knockdown (siRNA (h): sc-60660; Santa Cruz, Dallas, USA) and control siRNA-A (sc-37007; Santa Cruz). Hily MAX was used for transfection (Dojindo). A total of 8×10^3 HCT116, HCT/OHP5, DLD1, and DLD/OHP5 cells in a volume of 200 μ l medium were seeded into 96-well flat-bottom plates. The next day, the medium was removed and serial dilutions of OHP (0.1 – 100 μ M), 1.2 μ L Hily Max, and 20 pmol siRNA were added to each well in a total volume of 200 μ l medium. The influence of *FOXQ1* expression on OHP resistance was also assessed by enhancement of *FOXQ1* using *FOXQ1* expression plasmid and control plasmid, which were constructed previously¹⁸. Then 8×10^3 HCT116 and HCT/OHP5 cells in 200 μ l medium were seeded into 96-well flat-bottom plates. The next day, the medium was removed and serial dilutions of OHP (0.1 – 100 μ M), 1.2 μ L Hily Max, and 0.2 μ g *FOXQ1* expression plasmid or control plasmid were added to each well in a total volume of 200 μ l medium. After 48 h of *FOXQ1* expression modification, cells were counted using a cell counting kit according to the manufacturer's instructions and IC_{50} was measured as described above.

Association of FOXQ1 expression with clinical features in CRC patients

Specimens were collected from 173 CRC patients who underwent surgery at our department. All protocols were approved by the ethics committee of Hyogo College of Medicine and all patients provided written informed consent (No. 0120 by the Institutional Review Board of Hyogo College of Medicine). All experiments were performed depending on the Declaration of Helsinki, the guidelines and the associated laws in Japan. The CRC specimens consisted of 155

tumors without chemotherapy, 13 tumors after chemotherapy including OHP, and five after chemoradiotherapy. The CRC specimens were obtained with adjacent normal mucosal tissues for comparison and stored at -80°C in RNA later before use (Qiagen K.K.). Relative FOXQ1 expression was assessed by $\Delta\Delta\text{CT}$ generated from difference of ΔCT values in CRC tumors and normal mucosal tissue.

Association of FOXQ1 expression with clinical features in CRC patients of TCGA data

We further assessed FOXQ1 expression in CRC samples of TCGA. RNA-seq data of colorectal adenocarcinoma were collected from TCGA PanCancer Atlas in cBioPortal for Cancer Genomics (https://www.cbioportal.org/study/summary?id=coadread_tcg_pan_can_atlas_2018). Differences between mRNA expression z-scores of FOXQ1 relative to normal samples and those of GAPDH relative to normal scores were used to evaluate association of FOXQ1 expression with clinical features as in CRC patients of our department.

Data analysis

Differences in IC_{50} values and doubling times between OHP-resistant clones and parental cells were assessed by t-test. Tumor volumes of xenografts at seven weeks after inoculation were assessed between each clone and HCT116 cells by t-test. The tumor volumes by OHP treatment were also compared between the control and treatment groups using a t-test. Association of FOXQ1 expression with clinical features was assessed by t-test in case of two categories and by analysis of variance in case of more than three categories. Influence of FOXQ1 expression on overall survival was assessed by Kaplan-Meier curve and evaluated by Log-rank test. A P value of < 0.05 was considered significant for all analyses.

Declarations

Acknowledgements

We thank Ms. Miki Fukumoto, MS. Takako Ishikawa, Ms. Shino Tanaka, the members of Center for Comparative Medicine and Joint-Use Research facilities at Hyogo College of Medicine for collecting data. This work was supported by JSPS KAKENHI grant number 26462031, JP17K10658 and Grant-in-Aid for Researchers, Hyogo College of Medicine, 2012. We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Author Contributions

TY conceived the study; TY, TK, AY performed experiments; TY, YT, JS, KK, MY, AB, KK, NB, MI collected samples and data, TY, SK, TK, KN, NT analyzed and interpreted data; TY wrote the manuscript; all authors reviewed the manuscript.

Competing interests

TY is a temporary employee of Shionogi & CO., LTD.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

Figure 1.

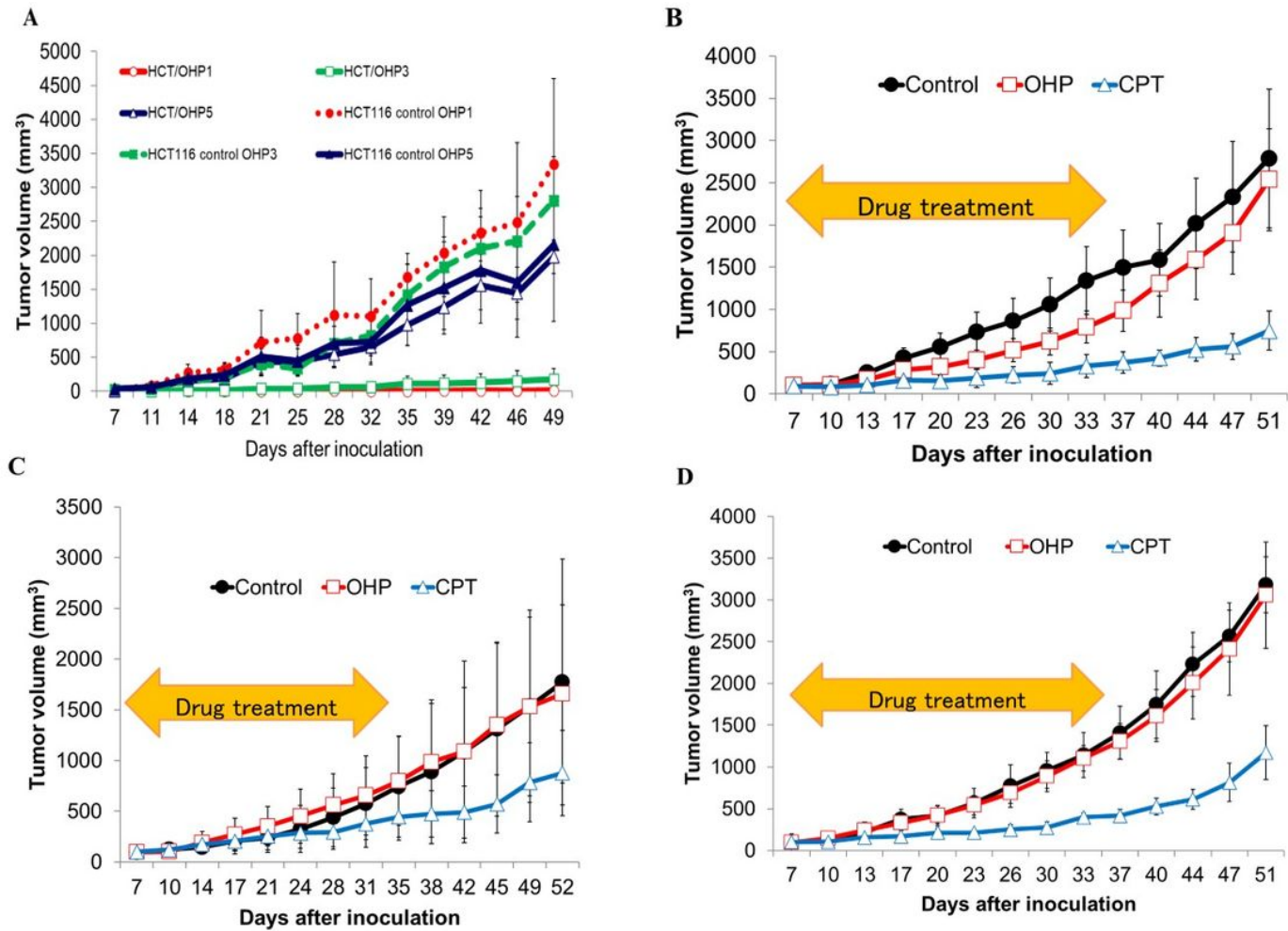


Figure 1

A. The growth of tumors derived from HCT/OHP1 and HCT/OHP3 was significantly inhibited compared with those derived from HCT116. The growth of xenograft tumors derived from HCT/OHP5 was similar to those derived from HCT116. B. The growth of tumors derived HCT116 was significantly inhibited by oxaliplatin (OHP) or irinotecan (CPT) treatment. C. The growth of tumors derived from HCT/OHP1 was significantly inhibited by CPT but not OHP. D. The growth of tumors derived from HCT/OHP5 was significantly inhibited by CPT but not OHP.

Figure 2.

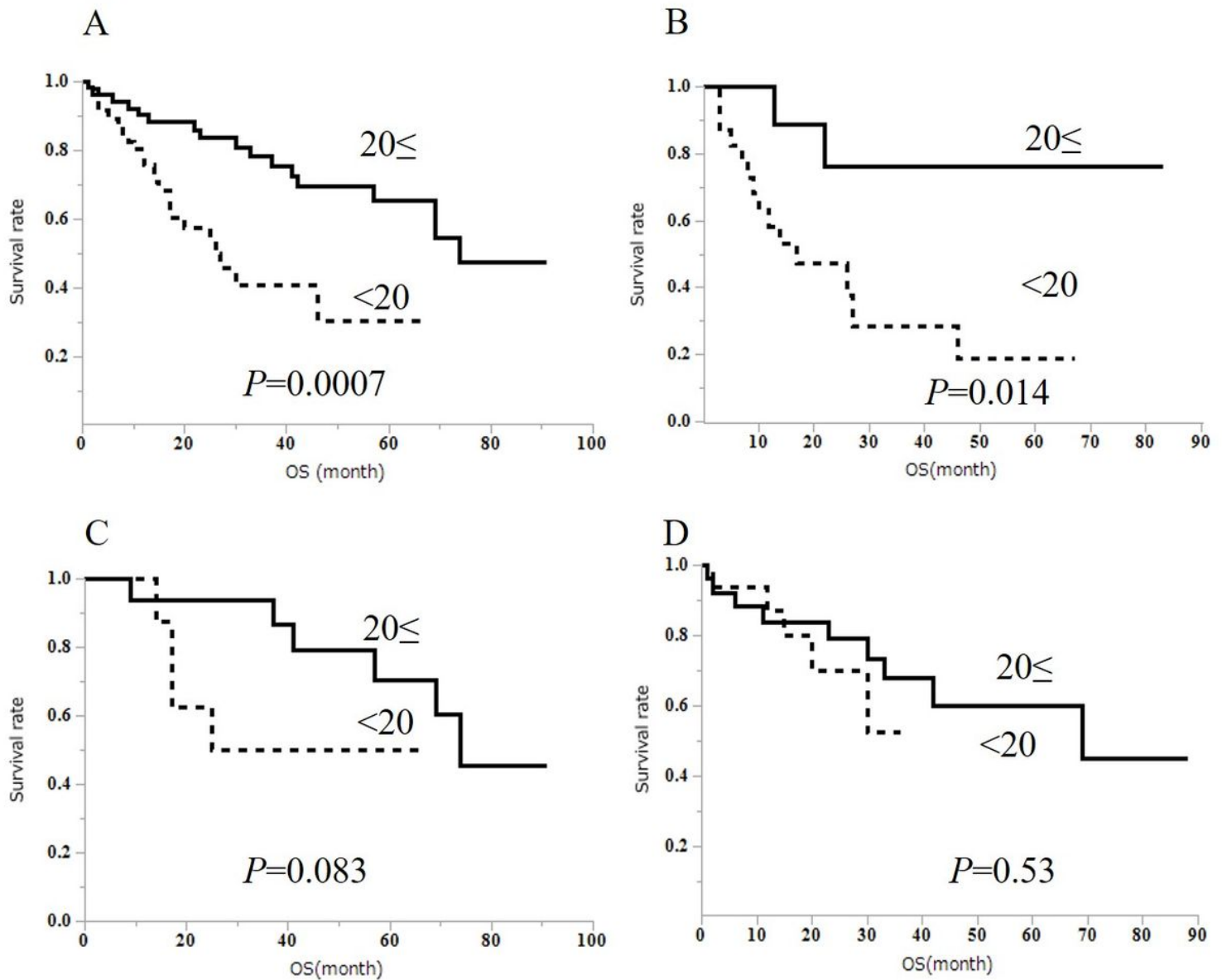


Figure 2

In advanced colorectal cancer patients, overall survival significantly differed according to the extent of FOXQ1 upregulation (which was upregulated by 20 times in CRC tissue compared with normal mucosa, A). This difference was also significant in right-sided colon (B), but not left-sided colon (C), or rectal cancer (D).

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