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ORIGINAL ARTICLE

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Gene expression of ferredoxin reductase predicts outcome in patients with metastatic colorectal cancer treated by 5-fluorouracil plus leucovorin

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Abstract *Purpose*: Ferredoxin reductase (FDXR) is a putative contributor to p53-mediated apoptosis from 5-fluorouracil (5-FU) through the generation of oxidative stress in the mitochondria. However, the influence of FDXR gene expression levels on the outcome of 5-FU chemotherapy has been relatively little studied. The aim of this study is to investigate the association between FDXR gene expressions and the clinical outcome when treated by 5-FU chemotherapy, as well as the correlation of FDXR gene expressions and p53 mutation. *Methods*: Pre-chemotherapeutic fresh frozen samples of 33 patients with metastatic colorectal cancer, who received bolus 5-FU and leucovorin (LV) as first line chemotherapy, were studied. FDXR gene expression and p53 mutation were evaluated by real-time RT-PCR and direct sequencing, respectively. *Results*: FDXR gene expression was significantly higher in responding tumors compared with non-responding ones ($P=0.0379$). Patients with FDXR values above the cutoff value of 13.52 had a statistically longer survival than those with FDXR gene expressions below the cutoff value ($P=0.0148$). The 9 tumors with wild-type p53 had statistically higher FDXR gene expressions than the 14 tumors with mutant-type p53 which had sequence alterations within the "hot spot" codons, the L2–L3 loops, or frameshift ($P=0.0463$). Conclusions: FDXR gene expression did not affect clinical outcome in patients with wild-type p53 tumors, whereas, among patients with p53 mutant-type tumors, patients with tumors with low FDXR gene expression had a worse outcome than those with a high FDXR gene expression ($P=0.0200$). FDXR gene expression, which is regulated at least in part by p53, is associated with both response and survival when metastatic colorectal cancer is treated with 5-FU plus LV. In addition, analysis of p53 mutation combined with FDXR gene expression might be useful in estimating the outcome in 5-FU-treated patients.

Keywords Ferredoxin reductase · p53 mutation · 5-FU · Colorectal cancer

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Introduction

5-Fluorouracil (5-FU) has continued to play an important role in the treatment of colorectal cancer for several years [19]. Its main mode of action is inhibition of thymidylate synthase, a de novo DNA synthetic enzyme, followed by DNA damage [17]. After DNA damage by activated 5-FU, it is commonly assumed that cell death is primary the result of apoptosis [18]. p53 can trigger elimination of the DNA damaged cells by promoting apoptosis [17]. In vitro studies have reported that loss of p53 function reduced cellular sensitivity of 5-FU [3, 16].

In human cancer cell lines, the gene encoding Ferredoxin reductase (FDXR) was indicated to be a putative contributor to p53 mediated apoptosis from 5-FU through the generation of oxidative stress in the mitochondria [11, 15]. FDXR is a 50,000 kDa mitochondrial flavoprotein attached to the matrix side of the inner

mitochondrial membrane. FDXR transports electrons from NADPH via the soluble single electron shuttle ferredoxin to a membrane-integrated cytochrome P450 enzyme. Under substrate-limiting conditions, excess electrons can leak from this shuttling system, and overexpressed FDXR can deplete the reduced NADPH, and both processes together increase the amount of superoxide in cells [10, 11, 15, 21]. These previously described biochemical properties imply a role for FDXR in p53-dependent apoptosis through oxidative stress.

Yu and McLeod et al. [28]demonstrated that RNA expression levels of FDXR in normal tissues were lower than those in colorectal cancer tissues, which correlated with p53 expression at the RNA level. The p53 response element has been found within the promoter of FDXR in human cancer cell lines [11, 15]. Taking these data together, increased FDXR RNA expression in human colorectal tumors might be seen as induced, at least in part, by p53. The expression of the p53 gene was also increased in the majority of human colorectal cancers, but some p53 proteins lose the natural function of p53 because of gene mutation [12]. To date, little information is available on associations between FDXR gene expression and p53 mutation in colorectal cancer tissues, or the clinical outcome of 5-FU chemotherapy.

To investigate these associations, the FDXR gene expression and p53 mutation were examined in 33 colorectal cancer tissues which were obtained from surgically resected specimens. Additionally, we investigated the relation between FDXR gene expression and clinical outcome of patients treated with 5-FU and leucovorin (LV).

Patients and methods

Patients and samples

The study population consisted of 33 patients with metastatic colorectal cancer who were treated with 5-FU and LV as first-line chemotherapy after resection of primary tumors at the Department of Digestive and General Surgery, Saitama Medical School, between February 2002 and January 2003. Eligible patients had (a) a histologically proven colorectal cancer with at least one measurable metastatic lesion; (b) Eastern Cooperative Oncology Group performance status of 0–2 with adequate hematologic, hepatic, and renal function; and (c) no prior treatment during the preceding 4 weeks. All patients were treated with the Roswell Park regimen (600 mg/m^2/day weekly 5-FU bolus for 6 weeks combined with 250 mg/m^2/day *l*-LV, followed by 2 weeks of no drug administration). Table 1 outlines patient characteristics.

Before the treatment and after every two cycles of treatment, measurable disease was reassessed. Response criteria were based on the standard definitions for bidimensionally measurable disease [8]. To be classified as a responder, the tumor had to have a 50% reduction in the sum of the products of the orthogonal diameters of the indicator lesion without growth of other diseases or the appearance of new lesions [8]. There were 10 responders and 23 nonresponders, with a response rate of 30.3% [95% confidence interval (CI), 14.6–46.0%].

Table 1 Patients' characteristics

No. of patients	33
Age (years)	
Median	65
Range	36–78
Primary tumors	
Colon	18
Rectum	15
Metastasis	
Synchronous	6
Metachronous	27
Adjuvant chemotherapy	
Yes	19
No	14
No. of metastatic sites	
1	15
2	16
3	2
No. of treatment course of 5-FU and leucovorin	
Median	3
Range	1–8
Second line chemotherapy of irinotecan	
Yes	13
No	20

Archival fresh frozen samples had been obtained from the primary colorectal tumors at the time of surgery. No patients had received any chemotherapy preoperatively. Immediately after resection, the tumor sample was divided into two equal portions of at least 500 mg each, after removal of necrotic tissues. One portion was fresh frozen in liquid nitrogen until the time of RNA extraction, and the other portion was embedded in paraffin to confirm histologically that it contained less than 5% contamination of normal tissues, necrotic tissues, and lymphocytes by one pathologist (M. Shimizu).

This study was approved by the Institutional Review Board of Saitama Medical School and Taiho Pharmaceutical Co. Ltd., and all patients gave written informed consent.

Quantitative real-time reverse transcription-PCR and measurement of relative RNA expression level

Total RNA isolation and reverse-transcription into cDNA using a High Capacity cDNA Archives Kit (Applied Biosystems, Foster City, CA, USA) were done as described previously [13, 14]. The mRNAs of FDXR and β-actin (ACTB), as an internal reference gene, were determined by a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems], as described previously [14]. TaqMan Gene Expression Assays (Applied Biosystems), pre-validated assay including the specific

primers and probe for each gene, were used for cDNA quantitation of these genes (Assay ID, FDXR; Hs00244590_m1, ACTB; Hs99999903_m1). For real-time PCR, Taqman Universal PCR Master Mix was used (Applied Biosystems). The PCR profile consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 30 s at 97°C and 1 min at 60°C in the ABI Prism 7900 (Applied Biosystems).

Taqman analysis yielded values that were expressed as ratios between two relative measurements (FDXR/ACTB).

Detection of p53 mutation

cDNA was analyzed to detect mutation within the entire p53 gene, exons 2–11 (except for exon 1, which is noncoding), according to the report of Forslund et al. [6]. In brief, 1 µl of cDNA (50 ng) was added to the PCR reaction mixture containing 2 µl of 10× Ex Taq Buffer (Takara, Shiga, Japan), 5 pmol each primer, 1.6 µl of 0.2 mM dNTP, and 0.8 µl of Ex Taq (4U), 14.6 µl of distilled H_2O. The cycling conditions consisted of 38 cycles with 15 s at 94°C, 30 s at 58°C, and 45 s at 72°C in a GeneAmp 9700 (Applied Biosystems). A 5-min-incubation at 72°C was added to ensure complete elongation. Four sets of primers were used to amplify the complete coding region within exon 2–11 of the p53 cDNA [23].

All PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and sequenced using an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with the same primers used for PCR.

Statistical analysis

Statistical analysis was performed using JMP software, version 5.01 (SAS Institute, Inc., Cary, NC, USA). The Mann–Whitney *U* test was used to compare FDXR gene expressions in terms of response and p53 mutation status. To evaluate the association with response, two-sided Fisher's exact test was used. Survival was calculated from the onset of chemotherapy until death. The overall survival curve was calculated using the Kaplan–Meier method. Statistical differences were assessed by the log-rank test. A *P* value of less than 0.05 was taken to evaluate significant difference.

The accuracy of gene expression to predict the response was evaluated by receiver operating characteristic (ROC) analysis [29]. The optimal cutoff value for differentiation of responding and nonresponding tumors was defined by the point of the ROC curve with minimum distance from the 0% false-positive rate and 100% true-positive rate, as well as the previous report [27]. After ROC analysis, sensitivity, specificity, and positive and negative predictive values of gene expression were calculated using standard formulas. In addition, 95% CI for these parameters were calculated by the F distribution.

Results

FDXR gene expression and association with clinical outcome

The median values of FDXR gene expression was 9.63, ranging from 1.48 to 42.15. Responding tumors had statistically higher FDXR gene expressions than nonresponding ones [median values of FDXR gene expression; 15.34 (range 4.22–42.15) and 8.34 (range 1.48–23.11) for responding tumor and nonresponding tumors, respectively, $P = 0.0379$; Mann–Whitney *U* test; Fig. 1]. Segregation of tumors according to the median value of 9.63 for the FDXR gene expression revealed no association with response ($P = 0.259$; Fisher's exact test). In the ROC analysis, FDXR gene expression of more than 13.52 was found to provide the highest accuracy

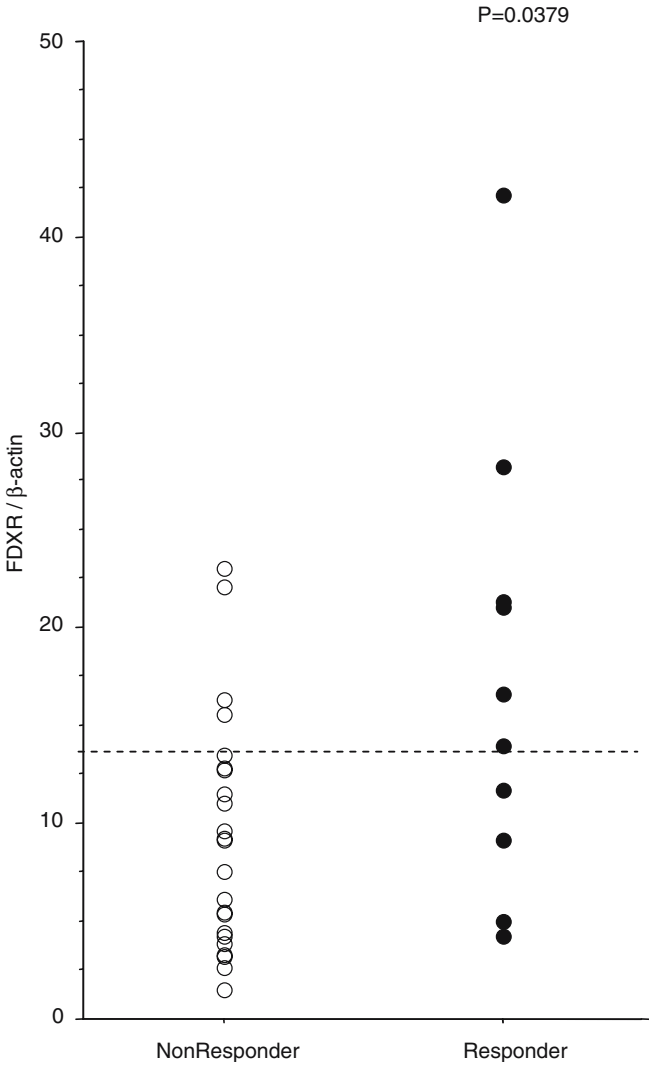

Fig. 1 FDXR gene expression grouped to response (*closed circle*) and nonresponse (*open circle*). Respective cutoff values for gene expression determined by the ROC analysis (*dotted lines*). *P* values calculated by the Mann–Whitney *U* test are indicated

for differentiation of clinically responding and nonresponding tumors. By applying this cutoff value, 6 of the 10 responding tumors and 19 of the 23 nonresponding tumors were correctly identified, providing a sensitivity and specificity of 54.5% (95%CI, 23.5–83.1%) and 82.6% (95%CI, 61.2–94.9%). An optimized cutoff value of ≥13.52 showed a clear association with response; response rates of 60% (6/10) and 17% (4/23) for tumor with a high FDXR expression level (≥13.52) and a low FDXR expression level (<13.52), respectively ($P=0.0349$; Fisher's exact test). Patients whose tumors had high FDXR expression levels showed clearly a better survival ($P=0.0148$; log-rank test; Fig. 2).

p53 mutations and association with FDXR gene expression

Among the 33 colorectal cancers, we found a total of 28 sequence alterations in the p53 exons 2–11 by direct sequencing. Because 3 tumors (No. 4, 19, 25) had 2 sequence alterations, 25 (75.8%) of all 33 colorectal cancers were found to harbor sequence alterations (Table 2). There were 22 missense mutations, 4 frameshift mutations (deletions), and 2 had silent mutations which did not result in an amino acid alteration. Eight tumors had no sequence alteration. The majority of mutations (12/28; 43%) was found within the "hotspot" codons 175, 245, 248, and 273, between exon 4 and 9, which are highly conserved throughout evolution and contain the DNA binding domain of the protein essential to p53 functional activity [5]. Eleven mutations occurred within the L2–L3 loops (codons 163–195 and 236–251, respectively). Among 22 silent mutations, there were 5 missense mutations outside the "hot spot" codons or the L2–L3 loops. Because the function of these 5 missense mutations has not been well recognized, tumors possessing these missense mutations (No.3, 9, 11, 13, 14) were excluded (Table 2). Thus, there were 19 (58%) mutant-type p53 tumors which had sequence alterations within the "hot spot" codons, the L2–L3 loops, or frameshifts, whereas 9 tumors with no sequence alterations were classified as wild-type p53 tumors.

Tumors with wild-type p53 had statistically higher FDXR gene expressions than those with mutated-type p53 [median values of FDXR gene expression; 7.55 (range 1.48–21.09) and 11.74 (range 3.84–28.22) for mutant-type p53 tumors and wild-type p53 tumors, respectively, $P=0.0463$; Mann–Whitney U test; Fig. 3].

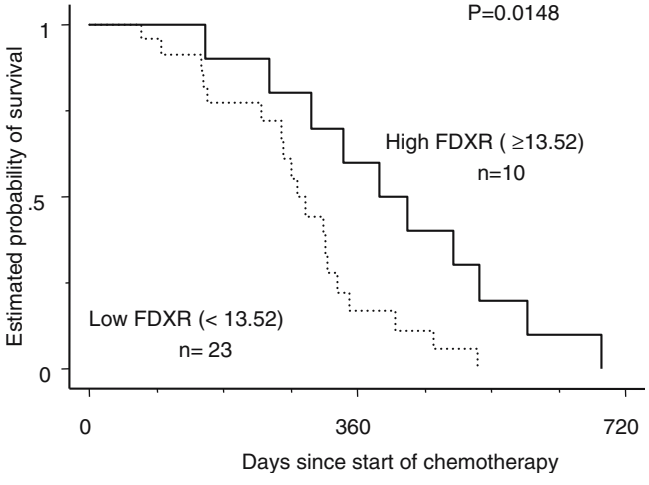

Fig. 2 Kaplan–Meier curves for patients with tumors with high FDXR expression levels (*solid line*) and those with low expression levels (*dotted line*). *P* values calculated by the log-rank test are indicated

Combination of FDXR gene expression and p53 mutation and association with clinical outcome

There was no relationship between p53 mutation alone and clinical outcome, such as response or survival (data not shown).

When analysis was performed with the combination of FDXR gene expression and p53 mutation, no difference in FDXR gene expressions was observed in terms of response among 9 wild-type p53 tumors ($P=0.624$; Mann–Whitney U test; Fig. 4). The optimal cutoff value for response among wild-type p53 tumors was not defined by the ROC analysis. In 19 mutant-type p53 tumors, 5 responding tumors had higher FDXR gene expression than the 14 nonresponding tumors, but not with statistical significance [median values of FDXR gene expression; 14.04 (range 4.22–21.09) and 5.80 (range 1.48–15.55) for responding tumor and nonresponding tumors, respectively, $P=0.0641$; Mann–Whitney U test; Fig. 4]. In the ROC analysis for mutant-type p53 tumors, FDXR gene expression of more than 9.19 was found to provide the highest accuracy for differentiation of clinically responding and nonresponding tumors. By applying this cutoff value, 4 of the 5 responding tumors and 11 of the 14 nonresponding tumors were correctly identified, providing a sensitivity and specificity of 57.1% (95%CI, 20.5–93.8%) and 91.6% (95%CI, 76.0–107.3%). An optimized cutoff value of ≥9.19 showed a clear association with response; response rates of 80.0% (4/5) and 21.5% (3/14) for tumor with a high FDXR expression level (≥9.19) and a low FDXR expression level (<9.19), respectively ($P=0.0427$; Fisher's exact test). Patients whose tumors had high FDXR expression levels showed a clearly better survival ($P=0.0200$; log-rank test; Fig. 2).

Discussion

In this study, we demonstrated a clear association between FDXR gene expression and clinical outcome, i.e. response and survival, when treated with 5-FU and LV. The increased FDXR gene expression in tumor tissues might enhance the initiation of tumor cells apoptosis and might increase the sensitivity to 5-FU through the resulting oxidative stress, as evaluated by in vitro

Table 2 Sequence alterations in the p53 exon 2–11 in 33 colorectal tumors

No	Exon	Base Change	Nucleotide	Effect	Amino acid Change	Codon	p53 domain[a]	Genotype	Response
1								Wt	Yes
2	7	742 C > T	742	Missense	R248W	248	L3	Mt	Yes
7	8	817 C > T	817	Missense	R273C	273		Mt	Yes
8								Wt	Yes
9	5	376 T > A	376	Missense	Y126N	126		–	Yes
12	5	555 C > T	555	Silent	–	185	L2	Wt	Yes
16								Wt	Yes
23	8	817 C > T	817	Missense	R273C	273		Mt	Yes
27	7	725 G > T	725	Missense	C242F	242	L3	Mt	Yes
29								Wt	Yes
3	5	469 G > T	469	Missense	V157F	157		–	No
4	2	54 A > T	54	Silent	–	18		Mt	No
	3	Deletion 22bp	75	Frameshift	–	26–32			
5	7	743 G > A	743	Missense	R248Q	248	L3	Mt	No
6	7	743 G > A	743	Missense	R248Q	248	L3	Mt	No
10	8	818 G > A	818	Missense	R273H	273		Mt	No
11	6	659 A > G	659	Missense	Y220C	220		–	No
13	5	434 T > C	434	Missense	L145P	145		–	No
14	8	797 G > T	797	Missense	G266V	266		–	No
15	5	524 G > A	524	Missense	R175H	175	L2	Mt	No
17	5	524 G > A	524	Missense	R175H	175	L2	Mt	No
18								Wt	No
19	8	797 G > T	797	Missense	G266V	266		Mt	No
	5	524 G > A	524	Missense	R175H	175	L2		
20								Wt	No
21	7	742 C > T	742	Missense	R248W	248	L3	Mt	No
22	6	Deletion 1bp	596	Frameshift	–	199		Mt	No
24	5	524 G > A	524	Missense	R175H	175	L2	Mt	No
25	8	Deletion 1bp	880	Frameshift	–	294		Mt	No
	8	789 T > G	789	Missense	N263K	263			
26	8	Deletion 1bp	880	Frameshift	–	294		Mt	No
28	8	818 G > A	818	Missense	R273H	273		Mt	No
30	8	818 G > A	818	Missense	R273H	273		Mt	No
31								Wt	No
32								Wt	No
33	6	581 T > C	581	Missense	L194P	194	L2	Mt	No

Wt wild type, *Mt* mutant type
[a] P53 zinc-binding domain: L2 (codon 163–195) and L3 (codon 236–251)

studies [11, 15]. Inclusion of more patients would enhance statistical analysis, which is now impossible because the standard regimen for metastatic colorectal cancer has been till now changing into the combination therapy of 5-FU and LV with irinotecan or oxaliplatin, from simple 5-FU and LV only, throughout the world [19]. To date, there is no information on any association between FDXR gene expression and antitumor effect by irinotecan or oxaliplatin, even in in vitro studies. The next step for us will include studying possible correlation of FDXR gene expression with clinical outcome when treated by 5-FU and LV combined with irinotecan or oxaliplatin.

In this study, cDNA, not genomic DNA, was analyzed to detect p53 mutation. This method has been validated, because there was no statistical significance in p53 mutation in cDNA and genomic DNA extracted from 123 human colorectal tumors [6]. The usage of cDNA provided the subsequent evaluation of FDXR gene expressions and the detection of p53 mutation in the same samples, allowing avoidance of contamination by heterogeneous tissues. We showed that levels of FDXR gene expression in colorectal cancer tissues correlated closely with p53 mutation (Fig. 3). Namely, tumors with wild-type p53 had higher FDXR gene expression than those with mutant-p53. This result was in accordance with the p53 response element found within the promoter of FDXR in human cancer cell lines [11, 15], and the positive relationship between FDXR and p53 expression in RNA levels in human colorectal cancers [28]. However, the range of FDXR gene expressions of wild-type tumors was relatively wide, compared with that of mutant-type tumors, suggesting that increased FDXR RNA expression in colorectal cancer tissues may be regulated at least in part by p53.

The p53 tumor suppressor protein plays a key role in coordinating cell cycle arrest, DNA repair, and programmed cell death after DNA damage, and mutations in p53 are seen in 40–50% of colorectal cancers [24]. However, the definition of mutant p53 is still controversial [22, 24, 25]. Indeed, various p53 mutations were grouped according to the site of mutation or the possible

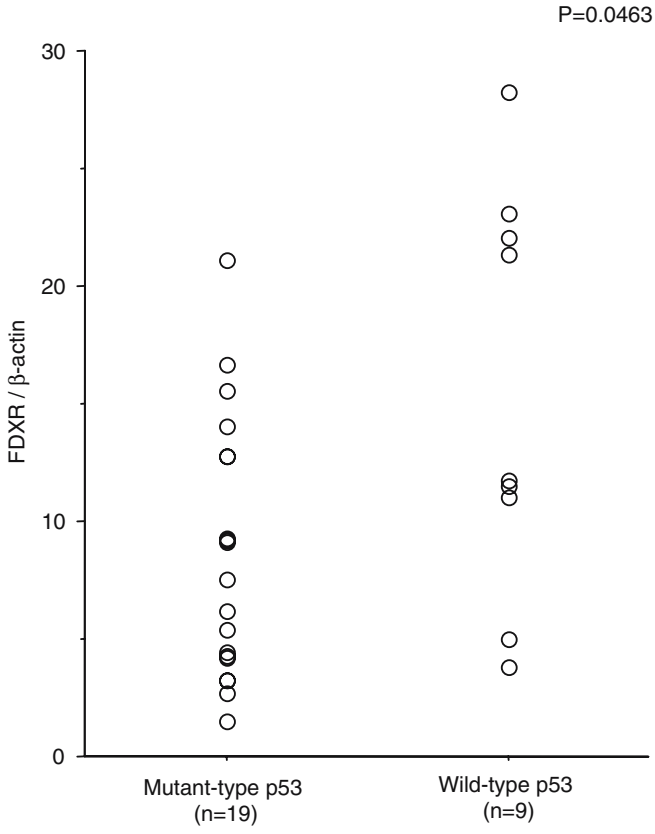

Fig. 3 Ferredoxin reductase gene expression in terms of p53 mutation status. *P* values calculated by the Mann–Whitney *U* test are indicated

function effect of the mutation [2, 20]. Mutations in five "hotspot" codons between exon 4 and 9 account for approximately 43% of all p53 mutations in colorectal cancer [25, 26]. This region is highly conserved throughout evolution and contains the DNA binding domain of the protein essential to p53 functional activity [5]. The L2–L3 loop domains (codons 163–195 and 236–251, respectively) together chelate a zinc atom and are critical to DNA binding [5]. Frameshift mutation (deletion and insertion) located upstream of the p53 tetramerization or L2–L3 loop domain could not produce a functional protein [9]. We therefore categorized tumors with sequence alterations within the "hot spot" codons, or the L2–L3 loops, and frameshift as mutant-type p53 tumors, whereas five tumors possessing missense mutation outside the "hot spot" codons and the L2–L3 domain were excluded from the analyses, as well as the report by Geisler et al. [7].

The existence of p53 mutations was not related to response when considered globally (mutated versus wild type). No association between clinical outcome, such as response and survival, and FDXR gene expression was observed in wild-type p53 tumors (Figs. 4, 5). Of note—albeit based on a small number of patients and without statistical significance—among p53 mutant-type tumors, responding tumors had higher FDXR gene expression than nonresponding ones (Fig. 4). In p53 mutant-type tumors, those with low FDXR gene expression had statistically significantly worse survival than those with high FDXR gene expression (Fig. 5). These results might indicate the functional difference of "intrinsic" and "induced" FDXR gene expressions in colorectal tumors in terms of anti-tumor effect in 5-FU treatment.

Liu et al. reported that "intrinsic" FDXR gene expression, which was over-expressed using a tetracycline-regulated promoter, had no effect on cell proliferation and increased the sensibility to 5-FU-mediated apoptosis in human lung H1229 cell line with mutant-type p53 and colorectal HCT116 cell line with wild-type p53 [15]. FDXR influences the redox state of the mito-

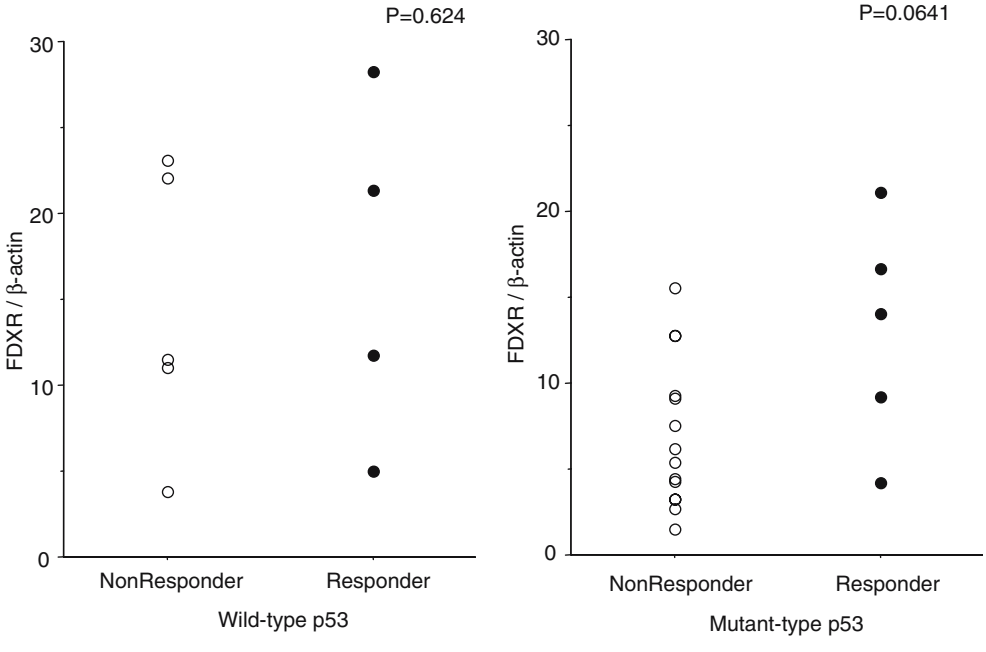

Fig. 4 Ferredoxin reductase gene expression in responding (*closed circle*) and nonresponding (*circle*) in terms of p53 mutantation status. *P* values calculated by the Mann–Whitney *U* test are indicated

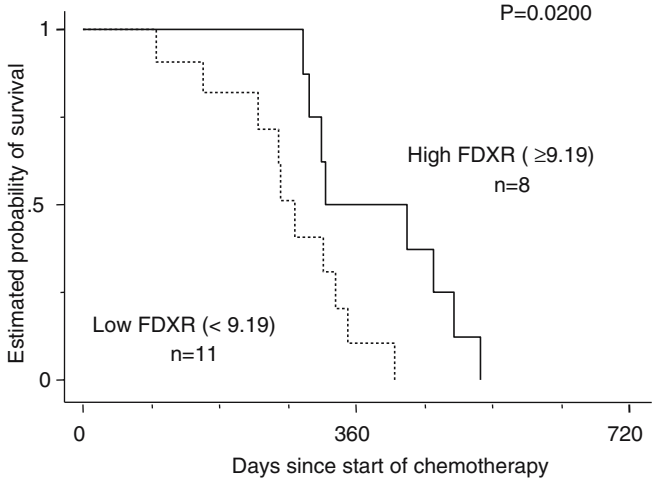

Fig. 5 Kaplan–Meier curves for patients with p53 mutant-type tumors with high FDXR expression levels (*solid line*) and those with low expression levels (*dotted line*). *P* values calculated by the log-rank test are indicated

chondria and mediates reactive oxygen species (ROS)-induced cell death [1, 4]. Thus, "intrinsic" FDXR alone sensitizes cancer cells to ROS-induced apoptosis, regardless of p53 mutation status. This is a possible explanation for the better outcome in patients with tumors expressing high levels of FDXR gene, compared with those with low FDXR gene expression, among mutant-p53 tumors. On the other hand, there were no differences in "intrinsic" FDXR gene expression in terms of response among wild-type p53 tumors. Some wild-type p53 tumors with low "intrinsic" FDXR gene expression responded to 5-FU therapy. When exposed to 5-FU, FDXR gene was induced only in HCT116 cells with wild-type p53, not in cells with mutant-p53 [11]. In cell lines with apparently intact p53 function, the pretreatment of an antioxidant specifically targeted to mitochondria completely blocked 5-FU-induced apoptosis [11]. Both "intrinsic" and "induced" FDXR might generate oxidative stress in the mitochondria of cancer cells, followed by p53-dependent apoptosis, in wild-type p53 tumors. Responding tumors with low "intrinsic" FDXR gene expression having wild-type p53 expression might "induce" FDXR more than nonresponding ones. This hypothesis agrees with the model of the feed forward loop proposed by Hwang et al. [11]. Namely, p53 is activated and induces apoptosis in response to various cellular stresses including exposure of 5-FU, and simultaneously p53 also induces FDXR, which in turn enhances the p53 function by increasing ROS-induced apoptosis [11, 15].

In conclusion, the present study clearly confirms the value of FDXR gene expression for predicting outcome in patients with metastatic colorectal cancer when treated with 5-FU and LV alone. In addition, this study suggests that FDXR RNA expression is regulated at least in part by p53 in human colorectal cancer. Our results also indicate that 5-FU cytotoxicity involves more than the status of p53 mutation, or any of its downstream mediators, such as FDXR which potentiates ROS-induced cell death. Analysis of p53 mutation, combined with FDXR expression, might be useful in predicting the outcome in patients treated with 5-FU-based chemotherapy. However these conclusions have been drawn from a limited retrospective study of a relatively small number of patients. Prospectively, randomized, translational treatment trials are needed to corroborate our results.

Conflict of interest statement

All authors disclose no financial and personal relationships with people or organizations that could inappropriately influence work.

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