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Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistance to 5-fluorouracil (5-FU) in human cancer xenografts *in vivo*

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Abstract

A human tumour sub-line resistant to 5-fluorouracil (5-FU) was established by once a day and every 5, with at least 50 administrations of 5-FU to KM12C human colorectal xenografts in nude mice. KM12C tumours treated with 5-FU showed less sensitivity to 5-FU with an inhibition rate (IR) of 7.9%, while non-treated tumours were highly sensitive to 5-FU with an IR of 81.8%. To clarify the mechanism of 5-FU-resistance, the activities of various enzymes and gene expressions involved in the metabolism of 5-FU in both parental and 5-FU-treated KM12C tumours were measured. A 2- to 3-fold increase in thymidylate synthase (TS) activity and 4- to 5-fold decrease in ribonucleotide reductase (RNR) activity were observed in 5-FU-resistant KM12C tumours, while the activities of orotate phosphoribosyltransferase (OPRT) thymidine and uridine phosphorylases (TP,UP) and thymidine kinase (TK) were not markedly changed as a consequence of repeated treatment of KM12C tumours with 5-FU. The expression of *TS* mRNA was also amplified in accordance with the increased TS activity in a 5-FU-treated tumour sub-line (KM12C/5-FU) compared with that in parental tumours, but changed expressions of both *RNR-R1* and *RNA-R2* mRNA could not be detected in the 5-FU-resistant tumour sub-line compared with the parental tumours, suggesting possible post-transcriptional regulation of RNR. Moreover, RNR, in addition to TS and OPRT, seemed to be related to the inherent insensitivity to 5-FU in human cancer xenografts. From these results, it may be concluded that RNR activity is one of the acquired or inherent resistant factors, including TS, to 5-FU in human cancer xenografts *in vivo*.

Keywords: Thymidylate synthase; Ribonucleotide reductase; Dihydropyrimidine dehydrogenase; Orotate phosphoribosyltransferase; mRNA; 5-Fluorouracil; Acquired resistance; 5-Fluorouracil metabolism; Colorectal carcinoma; Human xenograft; S-1

1. Introduction

5-Fluorouracil (5-FU) is used to treat patients with gastrointestinal, breast and head and neck cancers. Further attempts to potentiate 5-FU cytotoxicity, by improving the dosing schedule and biochemical modulation of 5-FU, are ongoing. However, the most negative factor in clinical use of 5-FU, whether alone or combined with other anticancer drugs is the development of 5-FU resistance by tumours and the existence of tumours innately resistant to 5-FU. To date, a number of *in vitro* studies have demonstrated the major mechanism of acquired-resistance to 5-FU to be an increase in thymidylate synthase (TS) activity and/or its gene expression [1–5].

TS has been recognised as the rate-limiting enzyme in *de novo* pyrimidine biosynthesis, and as being inhibited by 5-fluoro-2′-deoxyuridylate (FdUMP) formed from 5-FU, which thereby leads to inhibition of DNA synthesis [6–9]. Accordingly, high expression of TS induced by continuous treatment of cancer patients with 5-FU results in a decrease in the cytotoxic effect of 5-FU on the tumour cells. Spears and colleagues [10] reported that innate resistance to 5-FU in cancer patients receiving intravenous (i.v.). 5-FU may be attributable to the low formation of FdUMP and high TS expression. A number of clinical evaluations subsequently referred to

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the relationships of TS expression in tumours to clinical response and survival of cancer patients receiving 5-FU-based chemotherapy [11–15]. On the other hand, in several *in vitro* studies, the major mechanism of 5-FU-resistance was suggested to be a marked decrease in 5-FU-metabolising enzymes such as orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10) and uridine kinase (UK; EC 2.7.1.48) in human tumour cells treated with relatively high doses of 5-FU [16,17].

There are, however, no reports concerning *in vivo* establishment of 5-FU-resistant human tumours and the characterisation of the resistance mechanism, despite there being several descriptions of *in vitro* 5-FU-resistance mechanisms.

We have endeavoured herein to establish 5-FU-resistant human tumour xenografts by consecutive and repeated administrations of 5-FU to human cancer-bearing nude mice. Herein, we report the enzymatic and genetic changes involved in 5-FU metabolism in human tumour xenografts showing acquired resistance to 5-FU and possible mechanisms of 5-FU-resistance *in vivo*.

2. Materials and methods

2.1. Chemicals

[6-^{3}H]-5-FU (525 GBq/mmol), [6-^{3}H]-thymidine (dThd; 2.41 TBq/mmol), [6-^{3}H]-uridine (Urd; 0.88 TBq/mmol), [6-^{3}H]-FdUMP (625 GBq/mmol), and [(U)-^{14}C]-cytidine-5′-diphosphate (CDP; 2.04 GBq/mmol) were obtained from Moravek Biochemicals, Inc. (CA, USA). 5-FU was obtained from Sigma Co. (MO, USA). S-1, 5-FU preparations composed by tegafur (as 5-FU analogue), 2,4-dihydroxypyridine (as a DPD inhibitor) and potassium oxonate (as an OPRT inhibitor) was prepared in our laboratory. All other chemicals and biochemicals used were commercial products of the highest quality available.

2.2. Tumour xenografts

Human colorectal tumours KM12C and KM20C, were provided by Dr K. Morikawa (Iwamizawa Worker's Compensation Hospital, Hokkaido, Japan) and maintained by implantation into the right axilla of nude mice at 3 week-intervals. Human head and neck cancers, HEp-2 and PHA-1, were obtained from Dai-Nippon Pharmaceutical Co., Ltd. (Osaka, Japan) and the Central Institute for Experimental Animals (Kawasaki, Japan), respectively.

2.3. Treatment of tumour xenografts with 5-FU

In general, 5-FU is clinically used once a day or every 5 days in 3-week, or long-term continuous administrations. Considering that 5-FU is a time-dependent drug, we selected a 5-day administration of 5-FU to establish *in vivo* 5-FU-resistant tumours. Approximately 3-mm cubed KM12C tumour fragments were implanted into nude mice, and 1 week later, 20 mg/kg of 5-FU was administered intravenously (i.v.) once a day and every 5. At 3 weeks after implantation, the tumours were removed, then implanted into other mice by the method described above. This treatment was repeated 57 times to ensure resistance to 5-FU as 20 mg/kg 5-FU still showed an antitumour activity against KM12C tumours when the tumour was treated 20 times with 5-FU. Control tumours were maintained, on the same schedules, without 5-FU administration.

2.4. Antitumour experiments

Groups of eight nude mice were used. Parental and 5-FU-treated KM12C tumours (58 filical generations; F-58) were prepared by subcutaneous (s.c.) implantation of approximately 3-mm cubed fragments into the right axilla of mice (day 0). After 7 days, 5-FU, 20 mg/kg, was administered i.v. for 5 consecutive days, and then this schedule was repeated once more. The tumour volume [$1/2\times$(the major axis)$\times$(the minor axis)^2] was monitored twice a week throughout the experiments, and relative tumour volume (RTV) was calculated as follows: RTV = (mean tumour volume during therapy)/(mean tumour volume at the start of therapy). On day 21 the antitumour effects of 5-FU were estimated by the following equation: inhibition rate of tumour growth (IR,%) = [1 − (mean RTV of drug-treated group/mean RTV of control group) $\times$ 100].

2.5. Enzyme assay

Both parental and 5-FU-resistant KM12C (F-60) tumours were homogenised with 3 volumes of 50 mM Tris–HCl (pH 7.6) containing 10 mM 2-mercaptoethanol, 25 mM KCl and 5 mM $MgCl_2$, centrifuged at 105 000 *g* for 60 min, and the resulting supernatant was used to measure enzyme activity. Enzymes measured in this study were TS (EC 2.1.1.45), ribonucleotide reductase (RNR; EC 1.17.4.1), OPRT (EC 2.4.2.10), thymidine kinase (TK; EC 2.7.1.21), thymidine phosphorylase (TP; EC 2.4.2.4) and uridine phosphorylase (UP; EC 2.4.2.3).

TS was measured by [6-^{3}H]-FdUMP binding assay based on the method of Spears and colleagues [18]. OPRT activity was determined according to the method of Shirasaka and colleagues [19] using [6-^{3}H]-5-FU as the substrate. TK activity was measured by the method of Ikenaka and coworkers [20] except that the reaction product, [6-^{3}H]-thymidine-5′-monophosphate, was separated from [6-^{3}H]-dThd by Silica gel $60F_{254}$ (2×10 cm) thin layer chromatography (TLC) with a mixture of

chloroform, methanol and acetic acid (17:3:1, v/v/v) as the mobile phase. TP was measured according to the modified method described by Maehara and colleagues [21]. Briefly, the reaction mixture, containing 50 mM potassium phosphate buffer (pH 7.0), 0.6 mM [6-^{3}H]-dThd (74 KBq) and 50 μl of enzyme extract in a final volume of 0.125 ml, was incubated at 37°C for 30 min and immediately heated at 100°C for 2 min, followed by centrifugation. Then, 10 μl aliquots of the supernatant were subjected to TLC as described above. UP activity was measured in the same way as TP except for the use of [6-^{3}H]-Urd instead of dThd.

RNR activity was determined using [(U)-^{14}C]-CDP as the substrate. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 4 mM adenosine triphosphate (ATP), 10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 5 mM NaF, 1 mM iron(III) chloride hexahydrate, 40 μM [(U)-^{14}C]-CDP (74 KBq) and 50 μl of enzyme extract in a final volume of 150 μl, and the mixture was incubated at 37°C for 60 min, followed by boiling at 100°C for 2 min. Then, the mixture was cooled in an ice bath and further incubated for at least 30 min by adding 50 μl each of 4 mM 2′-deoxycytidine (dCyd), potato apyrase (20 mg/ml) and alkaline phosphatase (20 mg/ml). After the second incubation, the mixture was heated again at 100°C for 2 min, followed by centrifugation. A 20 μl aliquot of the supernatant fluid was subjected to Silica gel $60F_{254}$ (3×10 cm) TLC with a mixture of chloroform, methanol and triethylamine (6:4:1, v/v/v) as a mobile phase. Spots of [(U)-^{14}C]-dCyd, the final products of this assay, were scraped and their radioactivity was measured in 10 ml of the scintillation fluid. DPD activity was determined using [6-^{3}H]-5-FU as the substrate according to the method described previously [21].

2.6. Western blot analysis

TS proteins in the parental and 5-FU-resistant KM12C (F-60) tumours were also analysed by the western-blot method using 0.5 μg/ml of purified anti-rhTS polyclonal antibody [22].

2.7. Northern blot analysis

Total RNA (5 μg) extracted from each parental and 5-FU-resistant KM12C (F-60) tumours were separated on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary action under 20×SSC (3 M NaCl, 0.3 M sodium citrate). Gene-specific cDNA fragments containing the entire coding regions (approximately 300–900 bp) for use as hybridisation probes were obtained by polymerase chain reaction (PCR) amplification. cDNA probes labelled with [α-^{32}P]dCTP (3000 Ci/mmol, American Life Science) were synthesised using a rediprime II DNA labelling system (Amersham Pharmacia Biotech) and applied to column chromatography to remove unincorporated nucleotides. Membranes were prehybridised at 65°C in hybridisation buffer (Rapid-Hyb buffer, Amersham Pharmacia Biotech) for at least 30 min prior to probe addition. Hybridisation was performed with the probe at 65°C for approximately 2 h in a rolling bottle.

2.8. Statistical analysis

The significance of differences between groups with/without treatment was assessed using Welch's test.

3. Results

3.1. Effects of 5-FU on parental and 5-FU-treated KM12C tumours in mice

When 5-FU, 20 mg/kg, was administered i.v. to parental and 5-FU-treated KM12C (F-58) tumour-bearing mice, parental KM12C tumours showed a high sensitivity to 5-FU with an IR value of 82.1%, while KM12C/5-FU (F-58) tumours were almost resistant to 5-FU, as shown in Table 1. Thus, we were able to establish a human colorectal tumour sub-line highly resistant to 5-FU. Furthermore, the rate of tumour

Table 1
Antitumour effects of 5-FU on parental and 5-FU-treated KM12C tumours in nude mice

Tumour	Drug	Dose (mg/kg/day)	*n*	RTV[a] (mean±S.D.	IR[b] (%)	Body weight gain or loss (mean±S.D.)
KM12C	Saline	–	8	11.28±2.27	–	−1.8±1.1[c]
	5-FU	20	8	2.02±0.32	82.1	+1.0±2.6
KM12C/5-FU	Saline	–	8	9.10±0.80	–	0.9±1.1
(F-58)	5-FU	20	8	8.38±2.13	7.9	−4.7±1.0

S.D., standard deviation; 5-FU- 5-fluorouracil; F = no. of treatments with 5-FU; RTV, relative tumour volume; IR, inhibition rate.

Tumours were implanted into nude mice. 5-FU was administered i.v. starting 7 days after tumour implantation. On day 21, the antitumour effects of 5-FU were evaluated.

[a] Relative tumour volume.

[b] Inhibition rate of tumour growth.

[c] Body weight on day 15−body weight on day 0.

growth was nearly the same in the KM12C and KM12C/5-FU (F-58) tumour xenografts.

3.2. Activities of pyrimidine-metabolising enzymes in KM12C and KM12C/5-FU tumours

To elucidate the molecular mechanism of resistance to 5-FU in KM12C/5-FU (F-60) tumour xenografts, activities of TS, TK, OPRT, TP, UP and RNR in parental and 5-FU-resistant KM12C tumours were measured. The results are shown in Table 2. TS activity in KM12C/5-FU (F-60) tumours was approximately 2-fold higher than that in parental tumours. In contrast, the activity of RNR, catalysing the conversion of ribonucleoside-5′-diphosphate to the corresponding deoxyribonucleoside-5′-diphosphate, in KM12C/5-FU (F-60) tumours, was 4- to 5-fold lower than that in parental KM12C tumours. TP activity also increased approximately 2-fold in the 5-FU-resistant KM12C (F-60) tumours compared with that in the parental tumours, while TP activity was apparently far lower in both of these cells than in primary tumours from cancer patients [21]. The activity of OPRT converting 5-FU to 5-fluorouridine 5′-monophosphate (FUMP), UP converting 5-FU to 5-fluorouridine (FUrd), and TK, a key enzyme in *salvage* DNA synthesis, was not markedly changed throughout the consecutive and repeated treatment of KM12C tumours with 5-FU. We could not detect the activity of dihydropyrimidine dehydrogenase (DPD) in both parental and 5-FU-resistant KM12C (F-60) tumours (data not shown).

3.3. Western blot analysis of TS proteins

As the TS activity in the KM12C/5-FU (F-60) tumours was found to be higher than that in the parental tumours, the TS protein content in both tumour xenografts were also detected by western blotting using an anti-rhTS polyclonal antibody. As shown in Fig. 1, TS proteins in 5-FU-treated KM12C (F-60) tumours was apparently increased in comparison with that in the parental tumours. As KM12C/5-FU tumours have been continually treated with 5-FU for 5 days in a 3-week period, FdUMP-bound TS was also detected by the anti-TS antibody.

Table 2
Activities of 5-FU metabolising enzymes in parental and 5-FU-resistant KM12C tumours

Enzymes	KM12C	KM12C/5-FU(F-60)
TS[a]	0.182±0.01	0.413±0.111**
RNR[b]	11.90±1.34	2.43±.291**
OPRT[b]	9.35±1.20	13.60±1.49*
TP[c]	0.024±0.024	0.047±0.04
UP[c]	0.938±0.125	1.296±0.1
TK[b]	2.75±11.02	106.59±27.47

5-FU- 5-fluorouracil; TS, thymidylate synthase; RNR, ribonucleotide reductase; OPRT, orotate phosphoribosyltransferase; TP, thymidine phosphorylase; UP, uridine phosphorylase; TK, thymidine kinase.

After 60 treatments with 20 mg/kg 5-FU, enzyme activities involved in 5-FU metabolism were measured.

Values are means±standard deviation for five tumours.

*$P<0.05$, **; $P<0.01$ (Welch test).

[a] pmol/mg protein.

[b] pmol/mg protein/min.

[c] nmol/mg protein/min.

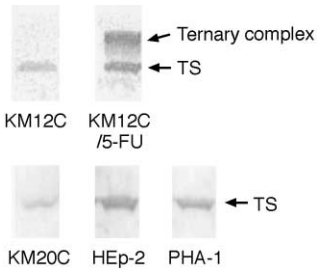

Fig. 1. Expressions of thymidylate synthase (TS) proteins in human tumour xenografts. The TS aliquot (50 μg protein) was also immunochemically analysed by the western-blot method. Upper lane shows the TS proteins from parental KM12C, 5-FU-treated KM12C, and the lower lane those from KM20C, HEp-2 and PHA-1 tumours, respectively.

3.4. Expressions of TS mRNA, TK mRNA and RNR mRNA in the parental and 5-FU-resistant KM12C tumours

Based on the amplification of TS activity and decreased RNR activity in the 5-FU-resistant KM12C

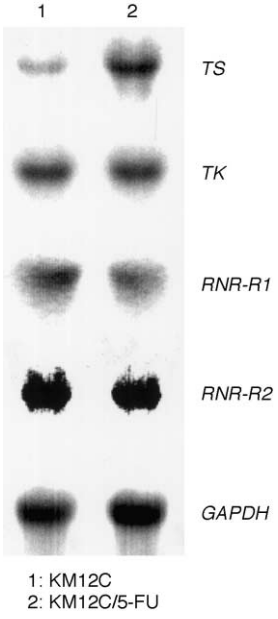

Fig. 2. Northern-blot analysis of thymidylate synthase (*TS*), thymidine kinase (*TK*) and ribonucleotide reductase (*RNR*) mRNAs from parental and 5-FU-resistant KM12C tumours. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was measured as a housekeeping gene.

tumours, the expressions of *TS* mRNA and *RNR* mRNA in the tumours were semi-quantified and compared with those in the parental KM12C tumours. In the case of RNR, as RNR consists of four components containing two R1 proteins and two R2 proteins, both *RNR-R1* mRNA and *RNR-R2* mRNA were measured. The results are shown in Fig. 2. The *TS* mRNA level in the KM12C/5-FU (F-60) tumours was 2-fold higher than that in the parental KM12C tumours, suggesting that *TS* mRNA in 5-FU-resistant KM12C tumours is distinctly amplified in accordance with the increased TS activity. In contrast, the relative expressions of *RNR-R1* and *RNR-R2* mRNA in the 5-FU resistant, compared with the parental KM12C (F-60) tumours were 0.9 and 0.8, respectively, suggesting that the expression of *RNR* mRNA does not change markedly during the treatment of KM12C tumours with 5-FU, which is not consistent with the decreased RNR activity in the KM12C/5-FU (F-60) tumours. *TK* mRNA also did not change markedly.

3.5. TS and RNR activities in human tumour xenografts are inherently insensitive to 5-FU

To further investigate whether TS and RNR activities are related to innate resistance or low sensitivity to 5-FU in human tumour xenografts, we evaluated the antitumour activity of oral 5-FU preparation, S-1 [23–25], on three species of human tumour xenografts (KM20C colorectal carcinoma, and HEp-2 and PHA-1 head and neck carcinomas) and measured the activities of TS, RNR and other enzymes in the corresponding tumour xenografts. S-1 has been demonstrated to have a higher antitumour potency than i.v. 5-FU on parental KM12C tumours and to be effective with approximately 50% of the IR value on 5-FU-treated KM12C tumours. As shown in Table 3, the antitumour effect of S-1 was high in the KM20C tumours with an IR of 91.9%, but low against both the HEp-2 and PHA-1 tumours with IRs of 43.7% and 36.9%, respectively. TS activities in HEp-2 and PHA-1 were approximately 5- and 2-fold higher than those in the KM20C tumours. Furthermore, this increased TS activity in the HEp-2 and PHA-1 tumours was confirmed by western-blot analysis of TS proteins, as shown in Fig. 1. On the other hand, RNR activity in the PHA-1 tumours was approximately 2-fold lower, while that in the HEp-2 tumours was approximately 4-fold higher than that in the KM20C tumours. In HEp-2 tumours, the activity of OPRT, one of the 5-FU-metabolising enzymes, was approximately 4-fold lower than that in the KM20C tumours. Other enzymes involved in 5-FU metabolism were nearly the same in these three tumours (data not shown).

These results suggest the expressions of RNR, in addition to TS and OPRT, could possibly be closely related to 5-FU insensitivity in human tumour xenografts *in vivo*.

4. Discussion

The appearance of acquired resistance or intrinsic resistance to anticancer drugs is a challenging problem in the long-term treatment of cancer patients. In the case of 5-FU, overexpression of TS has been suggested to be the main factor in acquired-resistance to 5-FU in human cancer cells [1–3]. Since Spears and colleagues [10] reported the mechanism of innate resistance to 5-FU in cancer patients receiving this drug, a number of reports also have discussed the relationship of intratumoral TS expression to the drug response or survival of cancer patients after 5-FU-based chemotherapy [11–15]. Although the molecular target of 5-FU cytotoxicity is thought to be TS, which forms a ternary complex with 5,10-methylenetetrahydrofolate (CH_2FH_4) and FdUMP derived from 5-FU, the expression of several enzymes involved in 5-FU metabolism may also be important in deciding whether or not 5-FU would be effective against human cancer cells because 5-FU is anabolised to its nucleotide form by these enzymes. In fact, Inaba and colleagues [17] reported reduced activities of 5-FU-metabolising enzymes, such as OPRT and UK, to be a major factor in acquired 5-FU resistance of human gastric cancer cells. In addition, Findlay and colleagues [26] reported that primary tumour TS expression did

Table 3
Antitumour activity of oral 5-FU drug, and activities of TS, RNR and OPRT in human tumour xenografts in mice

Tumour line	Type	TGI (%)	OPRT (pmol/mg/min)	RNR (pmol/mg)	TS (pmol/mg)
KM20C	Colon	91.9	9.40±2.00	4.04±0.97	0.21±0.02
HEp-2	H&N	43.7	2.31±0.60*	16.59±4.13*	0.98±0.06**
PHA-1	H&N	36.9	17.47±1.00**	1.84±0.36*	0.45±0.02**

TGI, tumour growth inhibition; H&N, head and neck cancer; OPRT, orotate phosphoribosyltransferase; RNR, ribonucleotide reductase; TS, thymidylate synthase.

Tumours were implanted into nude mice. S-1(15 mg/kg), a novel 5-FU formulation, was orally administered once daily for 14 days starting on day 15 after tumour implantation. On day 22, tumour volume was measured and various enzyme activities in each of the control tumours were determined.

*$P<0.05$, **; $P<0.01$ (Welch's test).

not correlate with clinical endpoints, such as time from diagnosis to relapse, and response to 5-FU-based chemotherapy in disseminated disease, and that primary tumoral TS expression may not give a good indication of TS expression in metastatic sites. This suggests additional factors relating to resistance or inefficacy of 5-FU in human cancers.

Recently, it was suggested that the expression of intratumoral DPD, catalysing 5-FU degradation, in addition to TS, is also related to the 5-FU sensitivity of human gastrointestinal cancers [27,28]. Therefore, it is necessary to further investigate whether only TS or other enzymes, in addition to TS, are involved in resistance and/or low-sensitivity to 5-FU in cancer patients. To date, there have been no reports concerning *in vivo* establishment of 5-FU-resistant human cancer xenografts and the determination of its mechanism. Thus, we attempted to establish *in vivo* 5-FU-resistant human colorectal cancer cells (KM12C) to allow elucidation of factors involved in acquired-resistance to 5-FU. The resulting 5-FU-resistant KM12C tumour xenograft, KM12C/5-FU, was found to be less sensitive to 5-FU treatment than the parental tumour (Table 1). When the activities of enzymes involved in 5-FU metabolism were measured, we found increased TS activity and decreased RNR activity in 5-FU-resistant KM12C tumours (Table 2). Overexpression of TS was also confirmed by measurement of its mRNA level and protein content in KM12C/5-FU tumours (Figs. 1 and 2). In our parental and 5-FU-treated KM12C tumours, we regretfully could not detect the activity of DPD. Consequently, no DPD activity in these tumours may be likely (or similar) to the inhibition of DPD in the tumour receiving DPD inhibitory fluoropyrimidines [29].

Previously, we reported that 5-FU is initially converted to FUMP and subsequently to FdUMP via FUDP and FdUDP in human gastrointestinal cancer cells *in vitro* and *in vivo* [30], which suggests that RNR may be involved in 5-FU sensitivity. In the present study, we found decreased RNR activity to be linked to decreased FdUMP formation in the KM12C/5-FU tumours. RNR is a conjugated protein with two subunits each of R1 and R2 proteins, and R1 is expressed throughout the cell cycle, while R2 increases only in the S-phase of the cell cycle [31]. The expression of both *R1* and *R2* mRNA in the KM12C/5-FU tumours tended to decrease, but did not change markedly in comparison to those in the parental KM12C tumours (Fig. 2), indicating that *RNR-R1* and *RNR-R2* mRNA expressions do not correlate with RNR activity. Although the reason for this difference is as yet unclear, a possible explanation may be the post-transcriptional regulation of either *RNR-R1* or *-R2* mRNA in the KM12C/5-FU tumours. In this experiment, we did not check how long resistance to 5-FU in the KM12C/5-FU tumours was maintained during the consecutive passages of the tumour without treatment of 5-FU. Therefore, in future experiments we will have to investigate the length of time that 5-FU resistance is maintained in established KM12C/5-FU tumours in the absence of 5-FU.

To confirm that the expression of RNR, in addition to TS, is involved in determining the inefficacy of 5-FU in other human tumour xenografts, RNR activity was measured in human head and neck cancer xenografts, PHA-1 and HEp-2, showing low sensitivity to one of 5-FU preparations, S-1 [23–25] and compared with that in a human colorectal cancer xenograft, KM20C, which is highly responsive to S-1 (Table 3). In PHA-1 tumours, decreased RNR and increased TS activities were suggested to account for the low sensitivity to 5-FU, essentially the same as the result in the KM12C/5-FU tumours. On the other hand, increased RNR and TS activities were observed in the HEp-2 tumours compared with the KM20C tumours. In this case, a marked decrease in OPRT and high expression of TS were apparently related to the low sensitivity to 5-FU. At any rate, based on our results, we can propose that increased TS and decreased RNR activities are one of possible mechanisms of acquired- or intrinsic-resistance to 5-FU in human cancer xenografts *in vivo*. Namely, we speculate that KM12C/5-FU tumour cells reduce RNR expression to prevent cell death in response to 5-FU and induce TS expression to supply thymidine-5′-triphosphate (TTP) for DNA synthesis via salvage routes, but not *de novo* pathways.

Paradiso and colleagues [32] recently reported TS and p53 primary tumour expression to be prognostic factors in advanced colorectal cancer patients. Furthermore, Tanaka and colleagues [33] found a RNR gene, *R2* to be involved in a p53-dependent cell-cycle checkpoint for DNA damage. They described the induction of p53R2, 80% homologous to RNR-R2, in p53-deficient cells as causing a G2/M arrest and preventing cell death in response to doxorubicin, and noted that inhibition of endogenous p53R2 expression in cells that have an intact p53-dependent DNA damage checkpoint reduces RNR activity, DNA repair and cell survival after exposure to various genotoxins. Therefore, it would be worthwhile to investigate the relationships between of *TP53*, *TP53R2*, *RNR-R1* and *-R2* gene expressions, as well as RNR activity to the 5-FU sensitivity of parental and 5-FU-resistant human cancers.

Although Salonga and colleagues [34] and Huang and coworkers [35] have reported relationships between the responsiveness to 5-FU and the expressions of enzymes including TS and DPD, we guess that RNR may be one of the *in vivo* sensitivity factors to 5-FU, in addition to TS, DPD and OPRT, according to our results in human tumour xenografts. Therefore, we plan to further investigate the role of RNR, as well as TS and DPD, in the responsiveness of cancer patients to 5-FU.

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