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Mechanism of acquired 5FU resistance and strategy for overcoming 5FU resistance focusing on 5FU metabolism in colon cancer cell lines

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Abstract. Fluorouracil (5FU) is converted to its active metabolite fluoro-deoxyuridine monophosphate (FdUMP) through the orotate phosphoribosyl transferase (OPRT)-ribonucleotide reductase (RR) pathway and thymidine phosphatase (TP)-thymidine kinase (TK) pathway and inhibits thymidylate synthase (TS), leading to inhibition of thymidine monophosphate (dTMP) synthesis through a *de novo* pathway. We investigated the mechanism of 5FU resistance and strategies to overcome it by focusing on 5FU metabolism. Colon cancer cell lines SW48 and LS174T and 5FU-resistant cell lines SW48/5FUR and LS174T/5FUR were used. FdUMP amount was measured by western blotting. The FdUMP synthetic pathway was investigated by combining TP inhibitor (tipiracil hydrochloride; TPI) or RR inhibitor (hydroxyurea; HU) with 5FU. Drug cytotoxicity was observed by crystal violet staining assay. FdUMP was synthesized through the OPRT-RR pathway in SW48 cells but was scarcely synthesized through either the OPRT-RR or TP-TK pathway in SW48/5FUR cells. FdUMP amount in SW48/5FUR cells was reduced by 87% vs. SW48 cells. Expression levels of OPRT and TP were lower in SW48/5FUR when compared with these levels in the SW48 cells, indicating decreased synthesis of FdUMP-led 5FU resistance. These results indicated that fluoro-deoxyuridine (FdU) rather than 5FU promotes FdUMP synthesis and overcomes 5FU resistance. Contrastingly, FdUMP was synthesized through the OPRT-RR and TP-TK pathways in LS174T cells but mainly through the TP-TK pathway in LS174T/5FUR cells. FdUMP amount was similar in LS174T/5FUR vs. the LS174T cells. OPRT and RR expression was lower and TK expression was higher in LS174T/5FUR vs. the LS174T cells, indicating that dTMP synthesis increased through the salvage pathway, thus leading to 5FU resistance. LS174T/5FUR cells also showed cross-resistance to FdU and TS inhibitor, suggesting that nucleoside analogs such as trifluoro-thymidine should be used to overcome 5FU resistance in these cells. 5FU metabolism and mechanisms of 5FU resistance are different in each cell line. Both synthesized FdUMP amount and FdUMP sensitivity should be considered in 5FU-resistant cells.

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Abbreviations: 5FU, fluorouracil; FdUMP, fluoro-deoxyuridine monophosphate; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TP, thymidine phosphatase; TK, thymidine kinase; TS, thymidylate synthase; dTMP, thymidine monophosphate; FdU, fluoro-deoxyuridine; FTD, trifluridine; TPI, tipiracil hydrochloride; HU, hydroxyurea

Key words: fluorouracil, colon neoplasms, drug resistance

Introduction

Colorectal cancer (CRC) is the world's fourth most deadly cancer with almost 900,000 deaths annually (1). Despite recent advances in the development of diagnostic tools and adjuvant therapy, many patients with CRC are still diagnosed as having an advanced stage, and recurrent tumors are often detected even after initial treatment. Thus, the continued development of drug therapy for CRC is important.

Fluorouracil (5FU) is currently a key drug for both adjuvant therapy and metastatic CRC according to guidelines such as those of the European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer Network (NCCN) (2-5). Although new chemotherapeutic agents including anti-vascular endothelial growth factor (VEGF) monoclonal antibody, anti-epidermal growth factor receptor (EGFR) therapies and programed cell death-1 (PD-1) blockade with immunotherapies have shown improvement in metastatic CRC (6,7), 5FU or its derivatives are used in almost all regimens. Thus, overcoming 5FU resistance is especially important.

Three mechanisms of 5FU action have been proposed: DNA uptake (8), RNA uptake (9) and the inhibition of thymidine synthase (TS) leading to inhibition of DNA *de novo* synthesis (10). However, many aspects of the mechanism of RNA uptake remain unclear, and 5FU is not easily taken up by DNA (because it is a uracil derivative). Therefore, inhibition

of DNA synthesis is considered the main pharmacological mechanism.

5FU is converted to its active metabolite fluoro-deoxyuridine monophosphate (FdUMP) through nucleotide metabolic pathways for thymidine monophosphate (dTMP) and forms a ternary complex with TS and 5,10-methylene-tetrahydro-folate (5,10-CH_2THF), leading to the inhibition of TS (11). Because dTMP can be synthesized through two pathways such as a *de novo* pathway and a salvage pathway, FdUMP can also be synthesized through two pathways: i) 5FU is converted to 5-fluorouridine monophosphate (FUMP) by orotate phosphoribosyl transferase (OPRT) and then converted to FdUMP by several enzymes, including ribonucleotide reductase (RR), which are derived from a *de novo* pathway for dTMP and known as the OPRT-RR pathway; or ii) 5FU is converted to fluoro-deoxyuridine (FdU) by thymidine phosphorylase (TP) and then converted to FdUMP by thymidine kinase (TK), which are derived from a salvage pathway and known as the TP-TK pathway. These mechanisms are illustrated in Fig. 1.

We previously elucidated the mechanism of acquired 5FU resistance by focusing on the changes in the expression levels of enzymes for 5FU metabolism in gastric cancer cell lines (12,13). In this study, we investigated the mechanisms of acquired 5FU resistance in colon cancer cell lines by investigating the changes in the related enzymes and the amount of synthesized FdUMP. Furthermore, we suggest a strategy to overcome 5FU resistance.

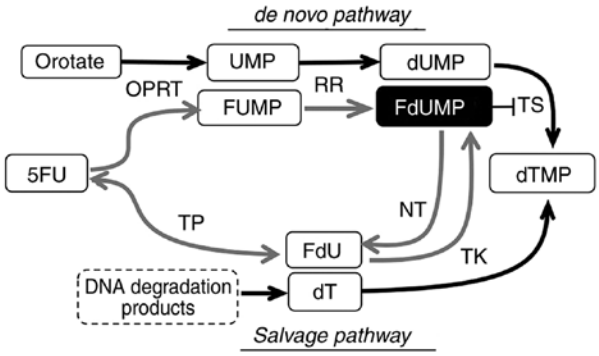

Figure 1. Diagram of fluorouracil (5FU) metabolism. FdU, fluoro-deoxyuridine; FdUMP, fluoro-deoxyuridine monophosphate; FUMP, fluorouridine monophosphate; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase.

Materials and methods

Drugs. 5FU was kindly provided by Kyowa Hakko (Tokyo, Japan). Trifluridine (FTD), FdU, tipiracil hydrochloride (TPI), hydroxyurea (HU), raltitrexed (TS inhibitor), and 3AP were purchased from Sigma-Aldrich; Merck KGaA.

Cell lines and cell culture. SW48 cells (human CRC cell line obtained from ATCC) were cultured in RPMI-1640 medium with 5% fetal bovine serum (FBS) [both obtained from Wako Pure Chemical Industries, Ltd. (Wako)] and sodium pyruvate (Sigma-Aldrich; Merck KGaA). LS174T cells (human CRC cell line obtained from ATCC) were cultured in EMEM with 10% FBS (both from Wako) and sodium pyruvate (Sigma-Aldrich; Merck KGaA). SW48/5FUR and LS174T/5FUR cells are 5FU-resistant cell lines that were established by continuously exposing these cells to increasing concentrations (0.1-2 μM) of 5FU over one year. These cells were routinely maintained in 2 μM 5FU, and prior to the study, the resistant cells were cultured in drug-free EMEM with 10% FBS for at least 2 weeks to eliminate the effects of 5FU in the experiments. All four cell lines were incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Western blot analyses and antibodies. The cells were lysed in RIPA buffer (Sigma-Aldrich; Merck KGaA) for 15 min on ice. The protein concentration of the lysates was measured using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.). The cell lysates were boiled in sample buffer solution (Wako). Total cell protein extracts (10 μg/lane) were separated by 10% SDS-PAGE using SuperSep™ ACE (Wako) and electrophoretically transferred onto polyvinyl difluoride (PVDF) membranes (EMD Millipore). The membranes were blocked with PVDF blocking reagent (Toyobo Co., Ltd.) for 1 h. The membranes were then incubated with primary antibodies, such as β-actin (13E5) rabbit mAb #4970 (1:5,000; Cell Signaling Technology, Inc.), RRM1 (D12F12) XP rabbit mAb #8637 (1:5,000; Cell Signaling Technology, Inc.), anti-thymidine kinase 1 [EPR3193] antibody (ab76495) (1:50,000; Abcam), rabbit polyclonal to thymidine phosphorylase (ab69120) (0.4 μg/ml; Abcam), dNT-1 (C-10): sc-390041 (1:100; Santa Cruz Biotechnology, Inc.), anti-thymidylate synthase, clone TS106 (MAB4130) (1:5,000; EMD Millipore), or anti-UMPS antibody (ab155763) (1:5,000; Abcam) for 2 h at room temperature. The primary antibodies were diluted with Can Get Signal Solution 1 (Toyobo Co., Ltd.). The membranes were then washed with Dako Washing Buffer (Agilent Technologies, Inc.) and incubated with goat anti-mouse IgG, peroxidase conjugated, heavy chain + light chain (AP124P) (EMD Millipore) or goat anti-rabbit IgG, peroxidase conjugate (AP132P) (EMD Millipore) diluted to 1:25,000 with Can Get Signal Solution 2 (Toyobo Co., Ltd.) for 1 h at room temperature. Immunoreactive proteins were visualized with the ImmunoStar LD reagent (Wako), and images were captured using a GeneGnome HR system (Syngene Europe, UK). Each result was confirmed with three independent experiments. Western blotting result was scaled for each band with ImageJ 1.52v software (NIH) and calculated with Microsoft Excel 2016 software program (Microsoft Corp.).

Crystal violet-staining (CVS) assay for the effects of 5FU, FdU or FTD. For SW48 and SW48/5FUR cells, 5.0×10^3 cells were seeded into each well of 96-well plates and cultured for 24 h at 37˚C. For LS174T and LS174T/5FUR cells, 2.5×10^3 cells were seeded into each well of 96-well plates and cultured for 24 h at 37˚C. These cells were then treated with 5FU, FdU or FTD for 72 h, after which 10 μl of glutaraldehyde solution (Sigma-Aldrich; Merck KGaA) was added to the culture medium. The media of the plates were then removed after 20 min and washed with water 3 times. The solution was replaced with 100 μl of 0.05% of crystal violet (Wako)/20% methanol per well for 20 min, after which the solution was removed and the wells were washed with water 3 times. After

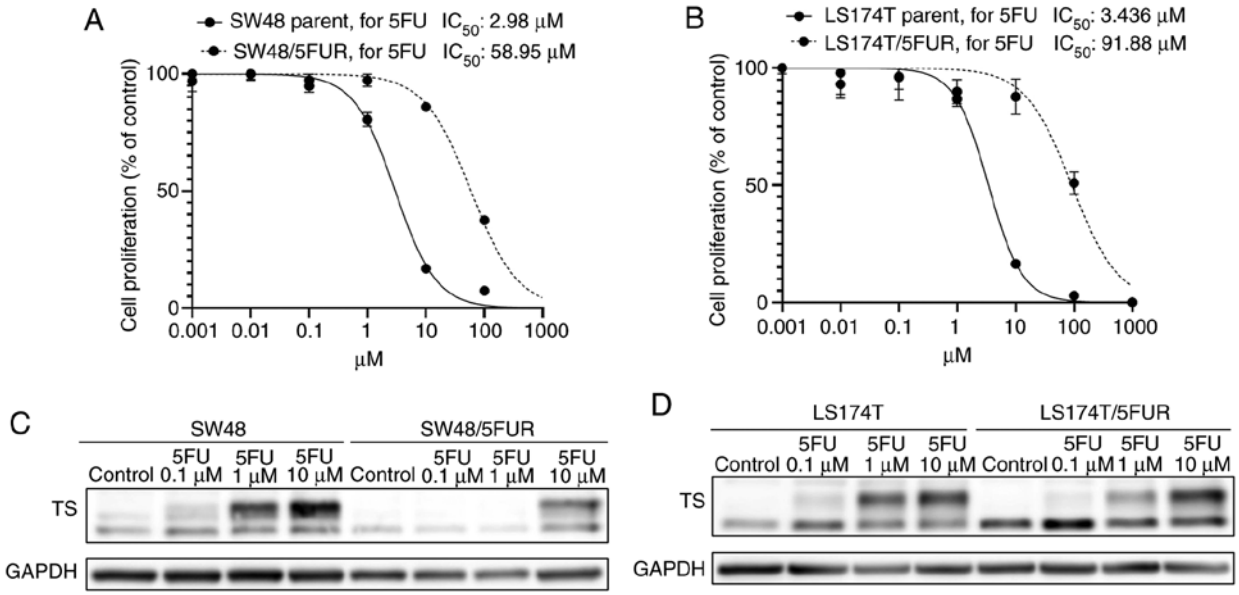

Figure 2. (A) CVS assay for fluorouracil (5FU) in SW48 and SW48/5FUR cells. (B) CVS assay for 5FU in LS174T and LS174T/5FUR cells. (C) Western blot analysis of TS after treatment with 5FU in SW48 and SW48/5FUR cells. (D) Western blot analysis of TS after treatment with 5FU in LS174T and LS174T/5FUR cells. CVS, crystal violet-staining; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IC_{50}, 50% inhibition concentration; TS, thymidylate synthase.

drying, 100 μl of 0.05 μM of sodium dihydrogen phosphate dihydrate (Wako)/50% ethanol was added per well, and the absorbance at 540 nm was measured using a Sunrise Rainbow RC-R (Tecan Group Ltd.). Each assay was repeated eight times.

Statistical analyses. The mean half maximal inhibitory concentration (IC_{50}) values were calculated based on each result of the CVS assays using the Graphpad Prism 9 software program (GraphPad Software, Inc.) and are presented as the mean ± standard error (SE).

Results

Sensitivities to 5FU and changes in amounts of synthesized FdUMP in SW48, SW48/5FUR, LS174T and LS174T/5FUR cells. SW48/5FUR cells showed an IC_{50} of 58.95 μM, which represented a 20-fold increased resistance compared with parental SW48 (IC_{50}, 2.98 μM) (Fig. 2A). LS174T/5FUR cells showed an IC_{50} of 91.88 μM, which represented a 27-fold increased resistance compared with parental LS174T cells (IC_{50}, 3.44 μM) (Fig. 2B).

After 5FU treatment, each cell line showed upper bands of TS on a western blot analysis, which represents TS in ternary complexes composed of TS, 5,10-CH_2THF and FdUMP; the density of the upper band is correlated with the intracellular concentration of FdUMP (11,14,15). The amount of FdUMP after treatment with 1 μM of 5FU in SW48/5FUR cells was decreased by 87% compared with the parental SW48 cells (Fig. 2C). However, in LS174T/5FUR cells, the amount of FdUMP was decreased only by 27% after treatment with the same concentration of 5FU compared with parental LS174T cells on western blot analysis (Fig. 2D). These results demonstrated that although SW48/5FUR and LS174T/5FUR cells showed similar extents of 5FU resistance, the mechanisms of acquiring this resistance were different.

Changes in enzymes and pathways of 5FU metabolism after acquiring 5FU resistance in each cell line. HU is the inhibitor for RR, and TPI is the inhibitor for TP. We investigated the changes in the amount of FdUMP after treatment with 5FU combined with HU or TPI to clarify which pathway is important for the synthesis of FdUMP. As shown in Fig. 3A, parental SW48 cells showed a decreased upper band of TS after treatment with 1 μM of 5FU only when combined with HU. In contrast, SW48/5FUR cells showed a decreased upper band of TS when combined with either HU or TPI, and we observed an upper band of TS only when the concentration of 5FU was increased to 10 μM. SW48/5FUR cells showed decreases in OPRT, TP and nucleotidase (NT), an increase in TK and equal level of RR when compared with these levels in the parental SW48 cells (Fig. 3B and C). These results indicated that in parental SW48 cells, FdUMP was synthesized through the OPRT-RR pathway and after acquisition of 5FU resistance, synthesis of FdUMP decreased due to decreased OPRT and TP levels in SW48/5FUR cells.

Meanwhile, parental LS174T cells showed a decreased upper band of TS after treatment with 1 μM of 5FU only when combined with both HU and TPI at about half the level (Fig. 3D). In the LS174T/5FUR cells, the upper band of TS was decreased only when the cells were treated with 1 μM of 5FU combined with TPI. LS174T/5FUR cells showed decreased OPRT and RR, increased TK, TP and NT compared with those in the parental LS17T cells (Fig. 3E and F). These results demonstrated that FdUMP in LS174T cells was synthesized through both the OPRT-RR and TP-TK pathways, and after the acquisition of 5FU resistance, FdUMP in LS174T/5FUR cells was synthesized mainly through the TP-TK pathway.

Sensitivity of synthesized FdUMP is preserved in SW48/5FUR cells and decreased in LS174T/5FUR cells. SW48/5FUR cells did not show cross-resistance to the specific TS inhibitor (Fig. 4A), whereas LS174T/5FUR cells did show

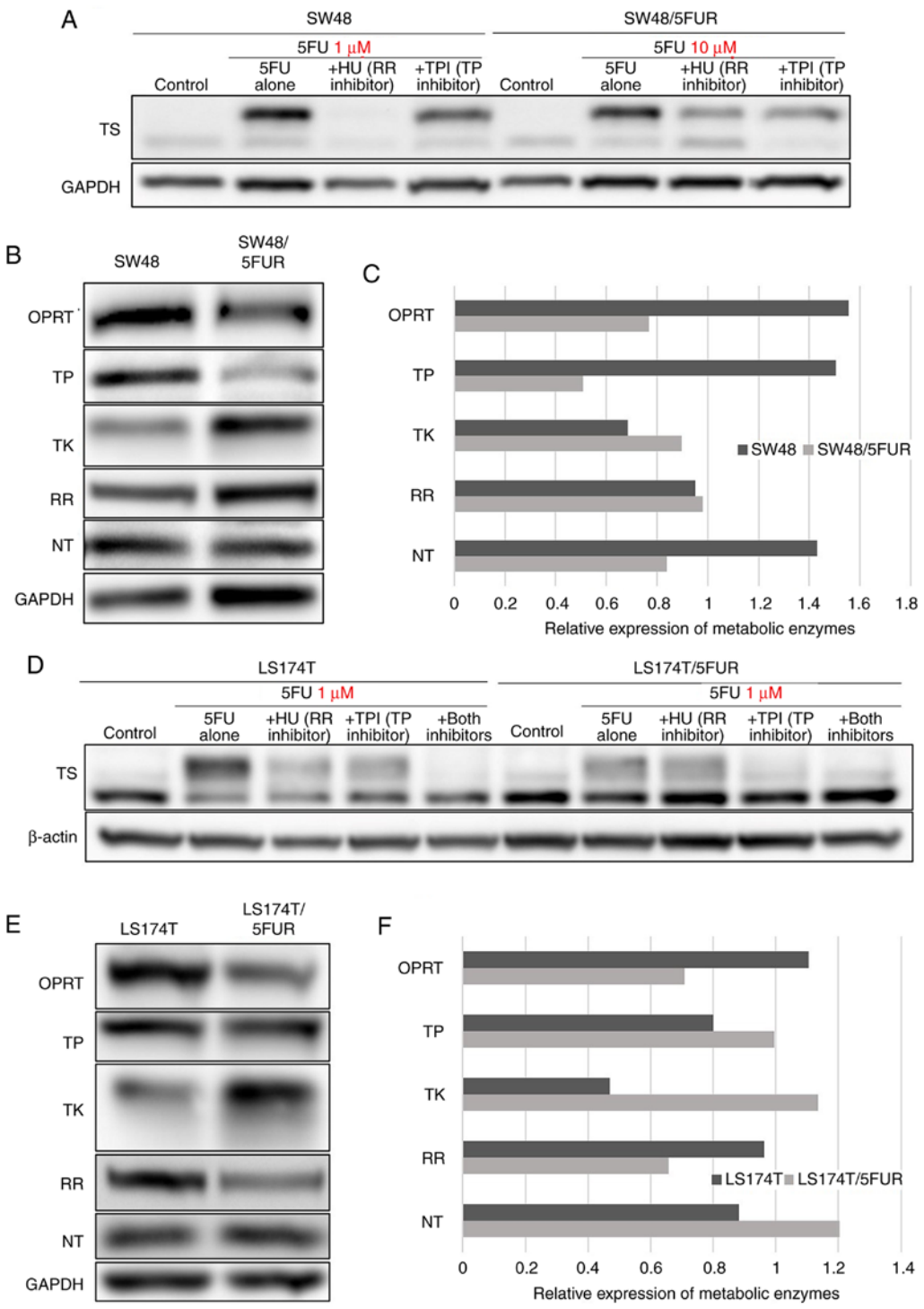

Figure 3. Changes in the amount of FdUMP after treatment with fluorouracil (5FU) when RR or TP was inhibited. (A) Western blot analysis of TS after treatment with 5FU with/without HU or TPI in SW48 and SW48/5FUR cells. (B) Western blot analysis of the enzymes for 5FU metabolism in SW48 and SW48/5FUR cells. (C) Relative expression of metabolic enzymes based on western blot analysis in SW48 and SW48/5FUR cells. (D) Western blot analysis of TS after treatment with 5FU with/without HU or TPI in LS174T and LS174T/5FUR cells. (E) Western blot analysis of the enzymes for 5FU metabolism in LS174T and LS174T/5FUR cells. (F) Relative expression of metabolic enzymes based on sestern blot analysis in LS174T and LS174T/5FUR cells. FdUMP, fluoro-deoxyuridine monophosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HU, hydroxyurea; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; TPI, thymidine phosphorylase inhibitor; TS, thymidylate synthase.

this cross-resistance (Fig. 4B). Because FdUMP inhibits TS by forming a ternary complex as described above, the cross-resistance to TS inhibitor represents decreased sensitivity to FdUMP. Therefore, these results suggested that the sensitivity to synthesized FdUMP was preserved in SW48/5FUR cells and was decreased in LS174T cells.

FdU is the derivative of 5FU, which is converted to FdUMP by TK and leads to the inhibition of TS. SW48/5FUR cells did not show cross-resistance to FdU (Fig. 4C), whereas LS174T/5FUR cells did show this cross-resistance (Fig. 4D). As shown in Fig. 3D, almost all FdUMP was synthesized through the TP-TK pathway in LS174T cells, indicating that

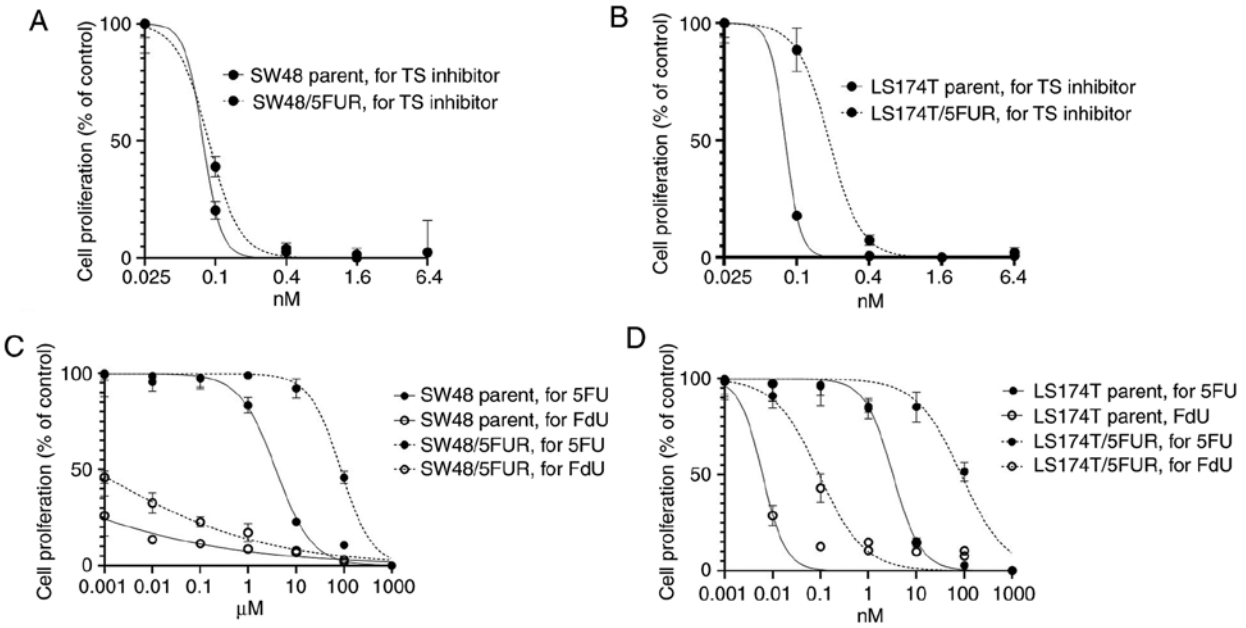

Figure 4. Investigation of the sensitivity of FdUMP by using TS inhibitor or FdU. (A) CVS assay for TS inhibitor in SW48 and SW48/5FUR cells. (B) CVS assay for TS inhibitor in LS174T and LS174T/5FUR cells. (C) CVS assay for 5FU or FdU in SW48 and SW48/5FUR cells. (D) CVS assay for 5FU or FdU in LS174T and LS174T/5FUR cells. CVS, crystal violet-staining; FdU, fluoro-deoxyuridine; FdUMP, fluoro-deoxyuridine monophosphate; TS, thymidylate synthase.

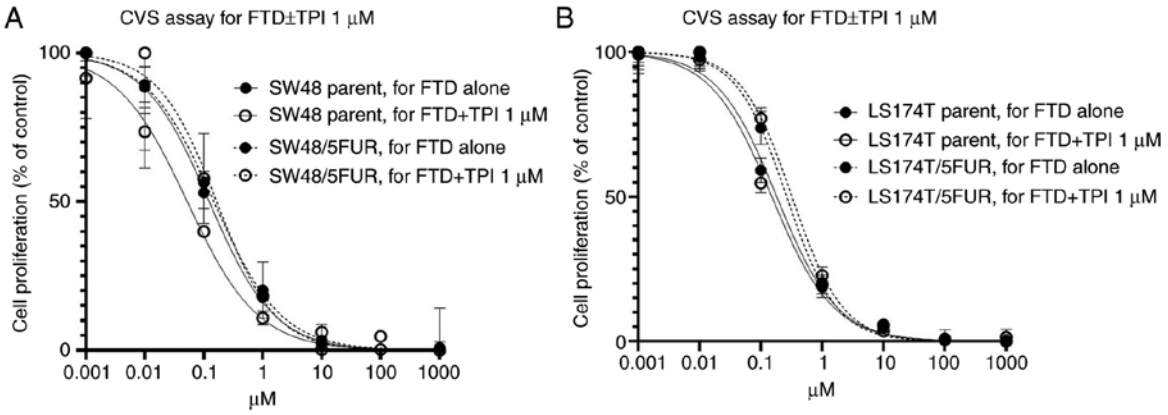

Figure 5. Overcoming fluorouracil (5FU) resistance by using FTD. (A) CVS assay for FTD and TPI in SW48 and SW48/5FUR cells. (B) CVS assay for FTD and TPI in LS174T and LS174T/5FUR cells. CVS, crystal violet-staining; FTD, fluorouridine; TPI, thymidine phosphorylase inhibitor.

the synthesis of dTMP occurred only through the salvage pathway, and the *de novo* pathway seemed to be stopped. As dTMP can be synthesized through the salvage pathway without TS, these results suggested that LS174T/5FUR cells could not be killed by the FdUMP or TS inhibitor, thus leading to 5FU resistance.

Strategies to overcome 5FU resistance. In the CVS assay for FTD with/without TPI, which is activated by TK, neither of the 5FU-resistant cells showed cross-resistance to FTD (Fig. 5A and B). As described above, the sensitivity to FdUMP was preserved in SW48/5FUR cells, and 5FU derivatives such as FdU can be used to overcome 5FU resistance. Meanwhile, whereas LS174T cells showed that decreased sensitivity to FdUMP cannot be overcome by 5FU derivatives, nucleoside analogs such as FTD can be used to overcome 5FU resistance because the expression of TK was increased.

Discussion

In fluorouracil (5FU)-resistant colorectal cancer (CRC) SW48/5FUR cells, intracellular fluoro-deoxyuridine monophosphate (FdUMP) was reduced due to decreases of orotate phosphoribosyl transferase (OPRT) and thymidine phosphatase (TP), which led to 5FU resistance. In addition, fluoro-deoxyuridine (FdU) was effective in SW48/5FUR cells because of an increased amount of thymidine kinase (TK). However, in 5FU-resistant CRC LS174T/5FUR cells, the sensitivity to thymidylate synthase (TS) inhibitor and FdUMP appeared to be decreased, and the effect of FdU was poor. Nucleoside analogs such as trifluridine (FTD) should be used to overcome 5FU resistance in LS174T/5FUR cells. Decreased sensitivity to TS inhibitor and FdUMP appears to be associated with an inactivated *de novo* pathway and an activated salvage pathway for dTMP. These hypotheses are illustrated in Fig. 6.

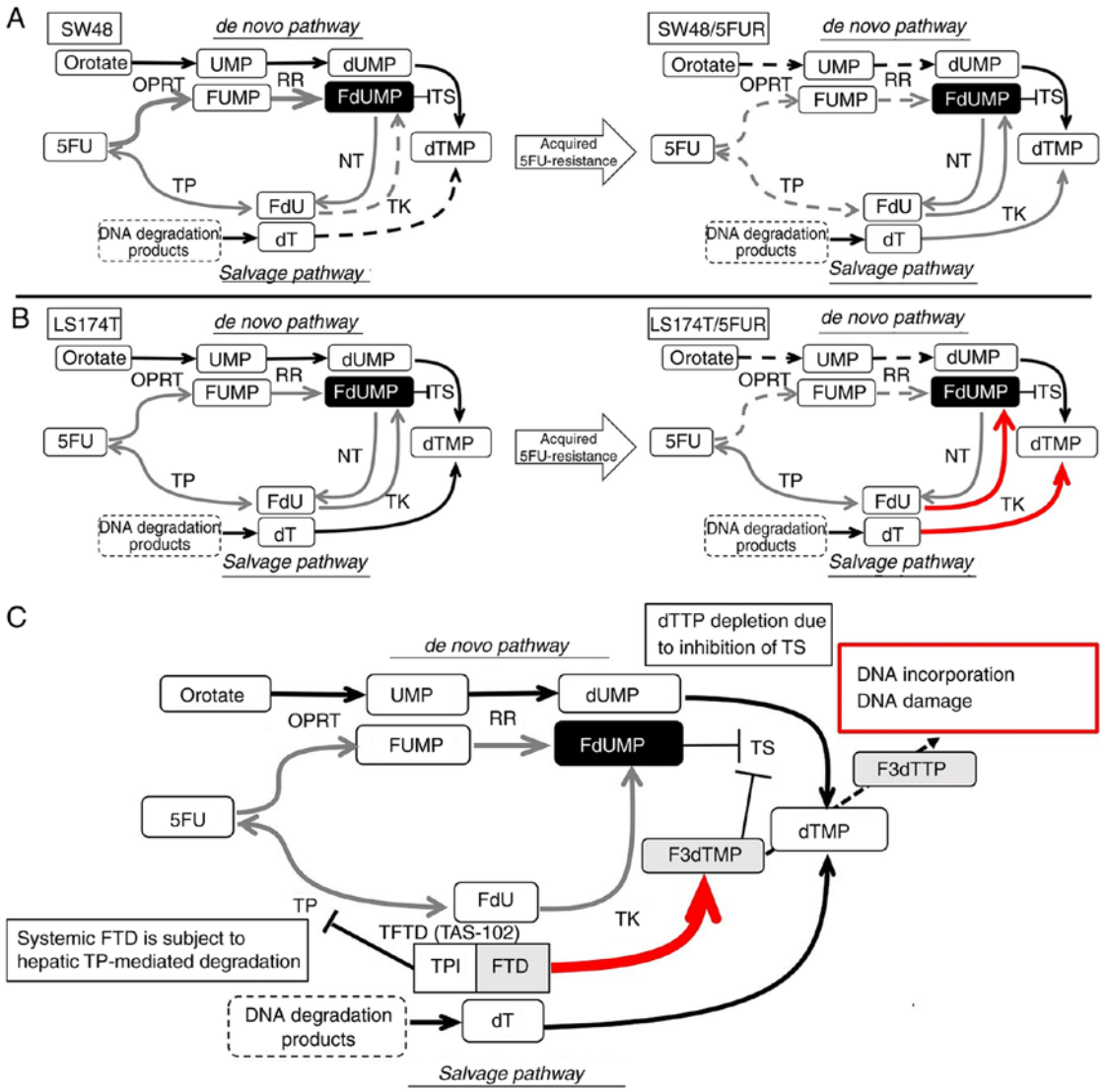

Figure 6. (A) Hypothesis of the acquired fluorouracil (5FU)-resistant mechanism in SW48 and SW48/5FUR cells. (B) Hypothesis of the acquired 5FU-resistant mechanism in LS174T and LS174T/5FUR cells. (C) Mechanism of action of TAS-102 and action point of FTD: Comparison with 5-FU; FTD is activated by TK. FdU, fluoro-deoxyuridine; FdUMP, fluoro-deoxyuridine monophosphate; FUMP, fluorouridine monophosphate; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; FTD, fluorouridine; TPI, thymidine phosphorylase inhibitor; F3dTMP, trifluoromethyl deoxyuridine 5'-monophosphate; F3dTTP, trifluoromethyl deoxyuridine 5'-triphosphate.

In the present study, we clarified that the mechanisms of acquired 5FU resistance differed in each cell line due to differences in 5FU metabolism, and thus, the strategies for overcoming 5FU resistance also varied from cell line to cell line. We hypothesize that some cells may change the enzymes for 5FU metabolism to reduce the synthesis of FdUMP whereas other cells may change the enzymes to reduce the sensitivity to FdUMP.

Many reports have shown a relationship between the efficacy of 5FU and the expression of metabolic enzymes for 5FU metabolism. OPRT has been reported as an important factor for 5FU resistance in cell lines, and there are many reports on the relationship between 5FU resistance and decreased OPRT levels in various cell lines (16,17). Moreover, some research has shown that TP is a predictive factor for sensitivity to 5FU, particularly in oral fluoropyrimidines (18,19). Furthermore, one report noted that tumors with increase expression of TK are likely to resist 5-FU-based chemotherapies (20).

However, in these studies, the acquired resistance and primary (*de novo*) resistance were not distinguished, and we assert that it is important to know what kind of changes occur when cancers acquire 5FU resistance. As acquired 5FU resistance, the changes of expression in metabolic enzymes lead to changes in the amount of FdUMP synthesis or the main route of FdUMP synthesis. In addition, focusing on changes of FdUMP sensitivity, it was confirmed by using two drugs; raltitrexed which directly inhibits TS and FdU which converts to FdUMP by TK as described in Fig. 4. To our knowledge, no study has investigated the mechanism of 5FU resistance focusing on both the amount of FdUMP synthesis and FdUMP sensitivity with accompanying changes in enzymes for 5FU metabolism. In the present study, although two cell lines exhibited similar levels of 5FU resistance, the expression of enzymes for 5FU metabolism, the amount of synthesized FdUMP and the sensitivity to FdUMP were different.

FTD is easily degraded by TP following oral administration, and the drug combination of FTD and TPI used in daily practice and known as TFTD (TAS-102) was found to significantly improve overall survival and progression-free survival of patients with metastatic CRC who were refractory to prior chemotherapy regimens including 5FU derivatives oxaliplatin and irinotecan (21,22). Once FTD is transported into the cytoplasm of tumor cells, it is phosphorylated to monophosphate (FTD-MP), diphosphate and triphosphate forms by TK, thymidylate kinase and nucleoside diphosphate kinase, respectively, exerting cytotoxic effects via their incorporation into DNA as shown in Fig. 6C (23-25). Therefore, TK is a predictive factor for the efficacy of FTD (26-28). In the present study, 5FU-resistant LS174T/5FUR cells showed decreased sensitivity to FdUMP, and it appears to be difficult to overcome this type of 5FU resistance by 5FU derivatives. However, these cells had increased TK expression and no cross-resistance to FTD. Therefore, the use of TFTD after chemotherapy including 5FU is a reasonable therapeutic strategy to overcome acquired 5FU resistance.

Several limitations associated with the present study warrant mention. In this study, the decrease of FdUMP sensitivity and FdUMP synthesis was observed only in limited 5FU-resistant cell lines. In addition, the difference in the proliferation speed of each cell line was not considered. Some reports have shown a slow cell proliferation tendency in 5FU-resistant CRC cell lines compared with parental cell lines (29,30). However, the relationship between DNA damage due to the cytotoxic drug and cell proliferation speed remains unclear. In addition, we could not develop the predictive factors for the acquisition of 5FU resistance to translate our results into daily practice.

In conclusion, we found that the changes in the expression levels of enzymes for 5FU metabolism, which lead to decreased amounts of FdUMP synthesis or decreased sensitivity to FdUMP, were associated with acquired 5FU resistance in colon cancer cell lines. We believe that clarifying the mechanism of acquired 5FU resistance can lead to the proposal of a novel strategy for overcoming 5FU resistance.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TS and RM conceived and designed the study. TS, RM, MFut, MFuk, HT, IY, YS, YI, TI, HI, YT, NO, NM, TT and KY acquired the data. TS and RM analyzed and interpreted the data and drafted the manuscript. TS, RM and KY critically revised the manuscript. KY supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

K. Yoshida has received grants, personal fees and nonfinancial support from Chugai Pharmaceutical Co., Ltd. during the conduction of the study; grants and personal fees from Taiho Pharmaceutical Co., Ltd.; grants and personal fees from Pfizer Inc.; grants and personal fees from Yakult Honsha Co., Ltd.; grants from Bristol-Myers Squibb; grants from Kyowa Hakko Kirin Co., Ltd., outside the submitted work; honoraria from Taiho Pharmaceutical Co., Ltd., Pfizer Inc., Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., and Yakult Honsha Co., Ltd.; and had a consultant or advisory relationship with Taiho Pharmaceutical Co., Ltd. and La Roche, Ltd. T. Takahashi has received honoraria for lectures from Takeda Pharmaceutical Co., Ltd. All of the other authors declare that they have no conflicts of interest.

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