

## Circumvention of 5-Fluorouracil Resistance in Human Stomach Cancer Cells by Uracil Phosphoribosyltransferase Gene Transduction

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A human stomach cancer cell line with acquired resistance to 5-fluorouracil (5-FU), NUGC-3/5FU/L, has been found to possess reduced ability to convert 5-FU into active metabolites. We attempted *in vitro* gene therapy for this 5-FU-resistant cell line. NUGC-3 and NUGC-3/5FU/L cells were infected with recombinant adenovirus (Ad) containing *Escherichia coli* uracil phosphoribosyltransferase (*UPRT*) gene driven by CAG promoter (CA), AdCA-*UPRT*, and changes in their 5-FU metabolism and sensitivity were investigated. Activities of orotate phosphoribosyltransferase increased from 10.2 and 1.56 (nmol/mg protein/30 min) in the uninfected cells of NUGC-3 and NUGC-3/5FU/L to 216 and 237, respectively, after the transfection of *UPRT* gene. The 5-FU nucleotide level in the acid-insoluble fraction increased from 7.32 to 15.9 (pmol/mg protein) in NUGC-3 cells on infection with AdCA-*UPRT*, and in NUGC-3/5FU/L cells it increased from 1.91 to 21.4. The 50% growth-inhibition concentration ( $IC_{50}$ ) was 12.7  $\mu$ mol/liter for NUGC-3 and much higher than 100  $\mu$ mol/liter for NUGC-3/5FU/L, indicating over 8-fold resistance. NUGC-3/5FU/L transfected with the *UPRT* gene showed very high sensitivity to 5-FU with an  $IC_{50}$  of 3.2  $\mu$ mol/liter. The high resistance in this metabolic activation-deficient cell line was thus completely reversed by transduction of an exogenous gene coding for a 5-FU-anabolizing enzyme.

Key words: 5-Fluorouracil — Uracil phosphoribosyltransferase — Drug resistance — Gene therapy — Adenoviral vector

It is widely known that 5-FU is effective against colorectal, stomach, head and neck, breast and other cancers; in particular it is the only drug useful for colorectal cancer. However, its efficacy is often hampered by the appearance of acquired resistance of tumor cells to 5-FU. The biochemical mechanisms of 5-FU resistance in human gastro-intestinal cancer cells which acquire resistance during repeated *in vitro* exposure to this drug include weaker inhibition of TS via decreased folyl-polyglutamate synthetase expression,<sup>1,2)</sup> *TS* gene amplification,<sup>3,4)</sup> and reduced activity of 5-FU-anabolizing enzymes.<sup>5,6)</sup> We also established 5-FU-resistant sublines

of three different human colon and stomach cancer cell lines, and found that poor conversion of 5-FU into its active metabolites due to reduced activity levels of enzymes involved in pyrimidine nucleotide synthesis is a major mechanism of resistance.<sup>7-9)</sup>

Reversal of drug resistance is an intriguing subject, and several successful approaches, at least experimentally, have been reported in the field of P-glycoprotein-associated multidrug resistance. However, few approaches to overcoming resistance to 5-FU have been reported, probably due to difficulty in modulating 5-FU-activating enzyme activity.

In recent years, various approaches to gene therapy for cancers have been reported.<sup>10)</sup> One major chemotherapeutic approach is conversion of a nontoxic prodrug into a toxic metabolite by the transfection of a so-called suicide enzyme gene, and two combination modalities, herpes simplex virus thymidine kinase with gancyclovir and *E. coli* cytosine deaminase with 5-fluorocytosine, have been widely tested. Studies on enhancing the sensitivity of human tumor cells to ara-C by transfer of deoxycytidine kinase gene have also been conducted.<sup>11, 12)</sup>

Very recently, we reported a novel approach for sensitizing various human cancer cell lines to 5-FU by transfection of the *E. coli UPRT* gene.<sup>13)</sup> This enzyme corresponds to OPRT in mammalian cells, and directly converts 5-FU into FUMP in the presence of PRPP as a

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Abbreviations used in this paper: AdCA-lacZ, adenovirus containing *lacZ* gene driven by CAG promoter; AdCA-*UPRT*, adenovirus containing *UPRT* gene driven by CAG promoter; ara-C, 1- $\beta$ -D-arabinofuranosylcytosine; *E. coli*, *Escherichia coli*; FdUMP, 5-fluoro-2'-deoxyuridylylate; 5-FU, 5-fluorouracil; FUTP, 5-fluorouridine-5'-triphosphate;  $\beta$ -gal,  $\beta$ -galactosidase;  $IC_{50}$ , 50% growth-inhibition concentration; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPRT, orotate phosphoribosyltransferase; PBS, phosphate-buffered saline; PRPP, 5-phosphoribosyl-1-pyrophosphate; TS, thymidylate synthase; *UPRT*, uracil phosphoribosyltransferase; FUMP, 5-fluorouridylylate.

co-substrate. FUMP is further metabolized into two major active metabolites, FdUMP and FUTP. In our previous report, we demonstrated that adenovirus-mediated transduction of the *UPRT* gene results in marked sensitization of colon, gastric, liver, and pancreas cancer cells to low concentrations of 5-FU.<sup>13</sup> It remained to be determined whether this gene therapy approach is also effective to overcome the 5-FU resistance of cancer cells. In the present study, we used a 5-FU-resistant subline of human stomach cancer NUGC-3 cells, which are deficient in metabolic activation of 5-FU due to relatively low activity levels of uridine phosphorylase and kinase and OPRT compared to the parent line,<sup>7</sup> and infected these cells with AdCA-UPRT in an attempt to reverse the 5-FU resistance by introducing a transgene of a 5-FU-anabolizing enzyme.

#### MATERIALS AND METHODS

**Chemicals** [<sup>3</sup>H]5-FU (462.5 GBq/mmol) was purchased from NEN Life Science Products (Wilmington, DE). MTT was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

**Cell lines** NUGC-3, a human stomach cancer cell line, was obtained from Japanese Cancer Research Resources Bank (Tokyo). Its 5-FU-resistant subline, NUGC-3/5-FU/L, was established by repeated 5-day exposures of NUGC-3 cells to stepwisely increasing concentrations of 5-FU, as previously reported.<sup>7</sup> Cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (M. A. Bioproducts, Walkersville, MD), 1% antibiotic antimycotic solution (Sigma) and 0.9 mg/ml amphotericin B (solubilized) (Sigma).

**Adenovirus infection *in vitro*** The recombinant adenovirus containing the *lacZ* or *UPRT* gene driven by the CAG promoter, AdCA-*lacZ* or AdCA-UPRT, was prepared as described in our previous report.<sup>13</sup> Infection was performed by incubating cells with AdCA-*lacZ* or AdCA-UPRT for 1 h and washing them with the complete culture medium.

**Assays for  $\beta$ -gal** Cells were plated in 96-well flat-bottomed plates so as to make 5,000 cells/100  $\mu$ l/well, incubated with AdCA-*lacZ* at an MOI of 10–100 for 1 h, and cultured for a further 48 h to allow adequate expression of the transduced gene. Cells were washed with PBS and fixed in PBS containing 0.5% glutaraldehyde for 10 min. The cells were rinsed in PBS containing 1 mmol/liter MgCl<sub>2</sub> and incubated for 1 h in PBS containing 5 mmol/liter K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mmol/liter K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 mmol/liter MgCl<sub>2</sub> and 1 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal; Wako Pure Chemical Industries, Ltd., Tokyo).<sup>14,15</sup> The cells were washed with PBS, and  $\beta$ -gal-positive cells per 100 cells were counted. Six hundred cells were counted in total.

**Assay of enzymatic conversion of 5-FU into its nucleotides**<sup>16,17</sup> Twenty-four hours after plating, cells were infected with AdCA-UPRT at an MOI of 100 for 1 h, and cultured in fresh medium for a further 48 h. Cytosol as an enzyme source was obtained from both uninfected and adenovirus-infected cells by sonicating them in homogenate buffer (50 mmol/liter Tris-HCl, 1 mmol/liter EDTA and 5 mmol/liter MgCl<sub>2</sub>; pH 7.4) at maximum output (Sonifier cell disrupter 350; Smith-Kline), and centrifuging the homogenate at 75,000g at 4°C for 20 min in a Beckman ultracentrifuge (model TL-100; Fullerton, CA). The reaction mixture for measuring direct conversion of 5-FU into FUMP by UPRT (including OPRT) consisted of 0.5 mmol/liter [<sup>3</sup>H]5-FU, 2 mmol/liter PRPP, 5 mmol/liter MgCl<sub>2</sub>, 15 mmol/liter 2-glycerophosphate, 0.6 mmol/liter  $\alpha,\beta$ -methyleneadenosine diphosphate and 50 mmol/liter Tris-HCl (pH 8.0). The reaction mixture for measuring the two-step conversion by uridine phosphorylase and kinase consisted of 0.5 mmol/liter [<sup>3</sup>H]5-FU, 5 mmol/liter ribose-1-phosphate, 5 mmol/liter ATP, 5 mmol/liter MgCl<sub>2</sub> and 15 mmol/liter 2-glycerophosphate. These mixtures were incubated at 37°C with 40–50  $\mu$ l of the cytosol in a total volume of 100  $\mu$ l for 30 min, and the reaction was stopped by heating them at 90–100°C. After centrifugation at 14,000 rpm for 2 min in a Tomy model MR-150 centrifuge (Tokyo), 20  $\mu$ l of the supernatant was charged with cold carrier on a PEI-cellulose thin-layer chromatography sheet (Merck, #1-05579, Frankfurt, Germany) and developed with water. The spots of substrate and product were distinguished under UV and excised. The radioactivity level in each was measured using a Beckman model LS 7500 scintillation counter. The protein content was measured using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

**Assay for intracellular 5-FU nucleotides** Twenty-four hours after 2.5 $\times$ 10<sup>5</sup> cells had been plated in 60 mm dishes, they were infected with either AdCA-*lacZ* or AdCA-UPRT at an MOI of 100 for 1 h. After 48 h culture, they were exposed to 1  $\mu$ mol/liter [<sup>3</sup>H]5-FU for 30 min. Cells were washed twice with PBS, and separated into acid-insoluble and -soluble fractions by treatment with 5% trichloroacetic acid. The acid-soluble fraction (20  $\mu$ l) was charged on a PEI-cellulose thin-layer chromatography sheet and developed with water. The spot of 5-FU nucleotides was distinguished under UV and excised. The acid-insoluble fraction was solubilized in 99% formic acid. According to our previous experience and also that of other researchers,<sup>17</sup> more than 90% of the 5-FU uptake in the acid-insoluble fraction during the early period (30 min) is incorporated into RNA. The radioactivity levels were measured using a scintillation counter. The protein content of both fractions was also measured.

**Drug sensitivity assays** Cells were plated at a density of 5 $\times$ 10<sup>3</sup>/100  $\mu$ l in 96-well plates. Twenty-four hours later,

the cells were infected with AdCA-lacZ or AdCA-UPRT at an MOI of 100 for 1 h and then cultured with various concentrations of 5-FU for 120 h. Cell growth was measured by means of a partially modified MTT assay<sup>18</sup>; cells were incubated in 100  $\mu$ l of MTT solution (50  $\mu$ g of MTT, 0.1 mg of glucose, 0.08  $\mu$ mol of  $MgSO_4$ , 225  $\mu$ g of  $NaHCO_3$ , 0.5  $\mu$ mol of HEPES/100  $\mu$ l of PBS(-)) for 1 h at 37°C, and shaken for 10 min after addition of 100  $\mu$ l of ethanol to solubilize the formazan formed. The optimal densities were read on an automated spectrophotometric plate reader at a single wavelength of 540 nm.

## RESULTS

The infection efficiency of recombinant adenovirus was examined by treating cells with AdCA-lacZ at various doses, followed by X-gal staining. As shown in Fig. 1, almost all cells treated with AdCA-lacZ at an MOI of 100 expressed  $\beta$ -gal activity in both NUGC-3 and NUGC-3/5FU/L lines. Based on this result, an MOI of 100 was determined as optimal. By using cytosol prepared from NUGC-3 and NUGC-3/5FU/L cells infected with mock or AdCA-UPRT as an enzyme source, the activity of the enzyme catalyzing the direct conversion of 5-FU into FUMP with PRPP as a co-substrate was compared between the control and AdCA-UPRT-infected cells. AdCA-lacZ-infected cells could not be used as a control because an extremely large amount of AdCA-lacZ would

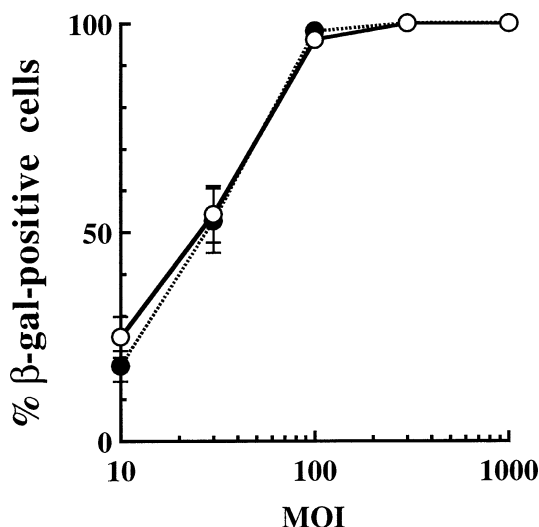


Fig. 1. *In vitro* gene transduction efficacy of recombinant adenovirus. NUGC-3 ( $\circ$ ) and NUGC-3/5FU/L ( $\bullet$ ) cells were infected with AdCA-lacZ at an MOI of 10–1,000. After X-gal staining, the  $\beta$ -gal-positive cells were enumerated. Each point is the mean of 6 determinations with the SD shown by a bar.

have been needed for this experiment. As is clearly demonstrated in Fig. 2A, high activity levels were observed with the infected cells of both lines: the degrees of elevation were 21- and 152-fold in NUGC-3 and NUGC-3/5FU/L cells, respectively. Activity of uridine phosphorylase plus uridine kinase was also measured using ribose-1-phosphate and ATP as co-substrates (Fig. 2B). No difference was found between the control and AdCA-UPRT-infected NUGC-3 cells, but the infected NUGC-3/5FU/L cells showed 4.7-fold more activity than the control.

Cells infected with AdCA-lacZ as a control or AdCA-UPRT were exposed to 1  $\mu$ mol/liter 5-FU for 30 min and intracellular levels of 5-FU nucleotides were compared. 5-FU nucleotides in the acid-insoluble fraction, which seem

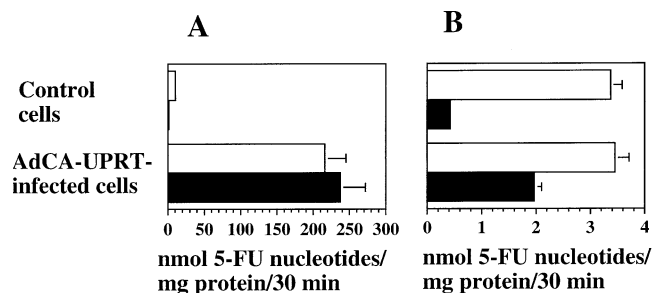


Fig. 2. Enzymatic conversion of 5-FU into its nucleotides by cytosol from control and AdCA-UPRT-infected cells. The cytosol and [<sup>3</sup>H]5-FU were incubated with PRPP (A) or ribose-1-phosphate and ATP (B) as co-substrates for 30 min. The open (NUGC-3) and solid black (NUGC-3/5FU/L) columns represent the mean enzymatic activities of 3 determinations with the SD values shown by bars.

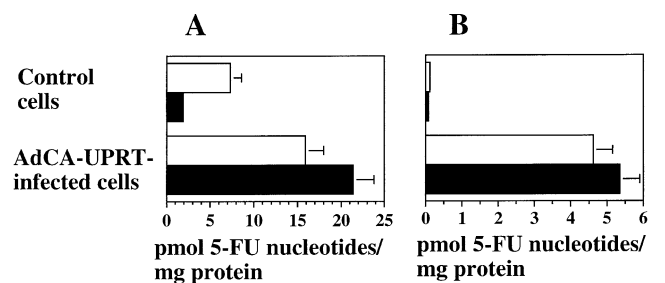


Fig. 3. Intracellular formation of 5-FU nucleotides from 5-FU in control and AdCA-UPRT-infected cells. AdCA-lacZ-infected cells were used as the control. Cells were incubated with 1  $\mu$ M [<sup>3</sup>H]5-FU for 30 min, and fractionated into acid-insoluble (A) and -soluble (B) fractions. The open (NUGC-3) and solid black (NUGC-3/5FU/L) columns represent the mean 5-FU nucleotide level of 3 determinations with the SD values shown by bars.

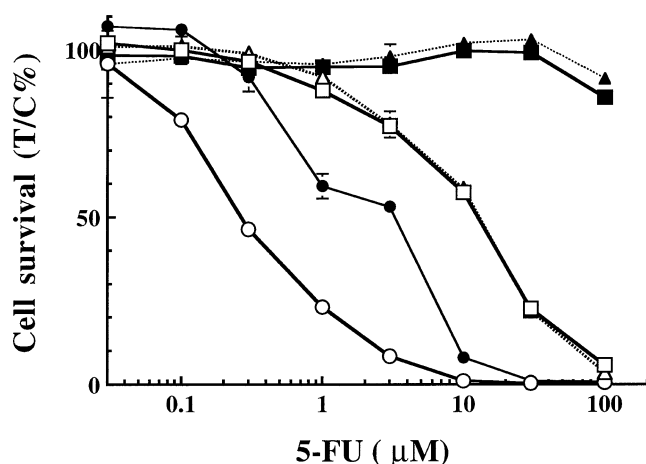


Fig. 4. Sensitivity to 5-FU of mock-, AdCA-UPRT- or AdCA-lacZ-infected cells. Cells were cultured with various concentrations of 5-FU for 120 h. The relative cell number was measured by MTT assay. Each point is the mean of 8 data with the SD shown by a bar. Open symbols, NUGC-3; filled symbols, NUGC-3/5FU/L. □ mock-, ○ AdCA-UPRT-, △ AdCA-lacZ-infected cells.

to include FdUMP consisting of covalent ternary complex of TS and FUTP incorporated into cellular RNA, amounted to  $7.32 \pm 1.26$  and  $1.91 \pm 0.20$  (pmol/mg protein) in the control NUGC-3 and NUGC-3/5FU/L cells, respectively (Fig. 3A). The infection with AdCA-UPRT increased the level of 5-FU nucleotides in the acid-insoluble fraction by 2.2- and 5.4-fold in NUGC-3 and NUGC-3/5FU/L cells, respectively. The level in NUGC-3/5FU/L cells was rather high compared to the parent NUGC-3 cells.

5-FU nucleotides in the acid-soluble fraction were also compared (Fig. 3B). Levels of acid-soluble 5-FU nucleotides, including free FdUMP and FUTP, in the AdCA-UPRT-infected cells showed 38.5- and 66.9-fold elevation compared with the control cells in NUGC-3 and NUGC-3/5FU/L, respectively. The ratios of total 5-FU nucleotide of the AdCA-UPRT-infected/control cells were 2.76 and 13.5 in NUGC-3 and NUGC-3/5FU/L, respectively.

Cells were infected with mock, AdCA-UPRT or AdCA-lacZ at an MOI of 100 for 1 h and then cultured with various concentrations of 5-FU for 120 h. As shown in Fig. 4, the  $IC_{50}$  values for uninfected NUGC-3 and NUGC-3/5FU/L cells were  $12.7 \mu\text{mol/liter}$  and much higher than  $100 \mu\text{mol/liter}$ , respectively; thus NUGC-3/5FU/L was at least 8-fold more resistant to 5-FU. However, when NUGC-3/5FU/L was treated with AdCA-UPRT, its  $IC_{50}$  shifted to  $3.2 \mu\text{mol/liter}$  which was significantly lower than that of uninfected NUGC-3 cells. It should be noted that not only NUGC-3/5FU/L but also NUGC-3 cells

were significantly sensitized. They were scarcely affected by infection with AdCA-lacZ.

## DISCUSSION

Defective anabolism into active nucleotides seems to play a major role in resistance to anticancer agents related to purine and pyrimidine. Reduced activity of deoxycytidine kinase has been reported to be a major mechanism of resistance to ara-C<sup>19)</sup> and gemcitabine.<sup>20)</sup> In the case of resistance to 5-FU, poor activity of several enzymes involved in the anabolism of 5-FU has also been demonstrated.<sup>5-9)</sup> Clinically, the relationship between response to 5-FU-based chemotherapy and content or activity of TS in tumor tissue has been extensively investigated. Most tumors with a relatively high content of TS are less responsive to 5-FU, though tumors with low TS content are not necessarily responsive.<sup>21, 22)</sup> This strongly suggests that, in addition to TS content, capacity for metabolism of 5-FU into its nucleotides in individual tumors is profoundly related to the clinical response to 5-FU.

Experimentally, only transfection of an exogenous gene coding for the corresponding anabolic enzyme is thought to be effective for reversing this type of resistance. In the case of ara-C resistance, Stegmann *et al.*<sup>23)</sup> attempted the transfection of deoxycytidine kinase gene into an ara-C-resistant rat leukemic cell line and succeeded in completely reversing a more than 9,000-fold resistance to ara-C. In the present study, we tried to transfect the *E. coli* UPRT gene into 5-FU-resistant human stomach cancer cells, which are deficient in 5-FU anabolism, with the aim of reversing 5-FU resistance *in vitro*.

Cytosol prepared from both NUGC-3 and NUGC-3/5FU/L cells infected with AdCA-UPRT showed surprisingly high activity levels of UPRT (including a relatively small amount of cellular intrinsic OPRT) as compared with the control cells (Fig. 2A). Intracellular acid-insoluble and -soluble 5-FU nucleotide levels were also increased by infection with AdCA-UPRT in both cell lines (Fig. 3), but the degree of elevation was not as large as for the UPRT activity. This indicates that intracellular formation of 5-FU nucleotides is limited by the intracellular PRPP level, even though an enormous amount of UPRT protein is expressed after the transfection. It should be noted that the lower 5-FU nucleotide levels in the acid-insoluble and soluble fractions in NUGC-3/5FU/L cells increased substantially relative to those in NUGC-3 cells after the infection with AdCA-UPRT (Fig. 3).

Based on these biochemical results, we assessed the sensitivity to 5-FU of uninfected and AdCA-UPRT-infected cells in a 120-h drug exposure assay (Fig. 4). NUGC-3/5FU/L cells were sensitized more than 31-fold by infection with AdCA-UPRT, and as a result became 4-fold more sensitive than the original NUGC-3 cells. This

clearly indicates that transfection of the *E. coli* UPRT gene completely reversed the high degree of 5-FU resistance in NUGC-3/5FU/L cells by increasing the intracellular level of active 5-FU nucleotides.

The parent NUGC-3 cells were also sensitized by infection with AdCA-UPRT, and the infected cells became 47-fold more sensitive than the uninfected ones (Fig. 4). It seems difficult to explain fully the marked sensitization induced by an about 2-fold increase in acid-insoluble levels of 5-FU nucleotide on infection with AdCA-UPRT (Fig. 3A). The results in Fig. 3 were obtained after only 30 min incubation of cells with 1  $\mu$ mol/liter 5-FU. Taking this into consideration, we speculate that the 5-FU nucleotide, particularly FdUMP, level in the acid-soluble rather than -insoluble fraction makes a major contribution to the prolonged inhibition of TS, because DNA-directed action seems predominant during long-term 5-FU exposure.<sup>1, 5, 24, 25)</sup>

Another point to be discussed is that even after infection with AdCA-UPRT, NUGC-3/5FU/L was more resistant than NUGC-3, although the degree of resistance in the infected cell lines is lower than in the uninfected ones. This could not be explained by the 5-FU nucleotide level

in either the acid-insoluble or -soluble fraction of the AdCA-UPRT-infected NUGC-3 and NUGC-3/5FU/L cells. Little is known about the molecular species in the acid-soluble fraction. If the FdUMP level in the acid-soluble fraction of NUGC-3 cells is significantly higher than that of NUGC-3/5FU/L cells, this might explain the difference in sensitivity between the AdCA-UPRT-infected NUGC-3 and NUGC-3/5FU/L cells. In this case, it is possible that ribonucleotide reductase is partially responsible for the high degree of resistance in NUGC-3/5FU/L cells, by diminishing the flow from FUMP to FdUMP.

In conclusion, the very high 5-FU-resistance in human stomach cancer NUGC-3/5FU/L cells, which have poor activities of uridine phosphorylase/kinase and OPRT, was completely reversed by adenovirus-mediated transduction of the *E. coli* UPRT gene. This methodology could be applicable to treat various 5-FU-resistant cancers. Further, the amount of 5-FU could be reduced to avoid undesirable side effects if the combination of 5-FU and this gene therapy were introduced for routine therapy.

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