Low cytosine triphosphate synthase 2 expression renders resistance to 5-fluorouracil in colorectal cancer

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Abbreviations: 5FU, 5-Fluorouracil; FUDR, Floxuridine; CRC, colorectal cancer; CTPS, cytosine triphosphate synthase 2; CTPS2, cytosine triphosphate synthase 2; PCR, polymerase, TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; TP, thymidine phosphorylase, UTP, uridine triphosphate; CTP, cytidine triphosphate.

Running title: Pathway Determinants of 5-Fluorouracil Sensitivity

Abstract

Understanding the determinants of resistance of 5-fluorouracil (5FU) is of significant value to optimising administration of the drug, and introducing novel agents and treatment strategies. Here, the expression of 92 genes involved in 5FU transport, metabolism, co-factor (folate) metabolism and downstream effects was measured by real-time PCR low density arrays in 14 patient-derived colorectal cancer xenografts characterised for 5FU resistance. Candidate gene function was tested by siRNA and uridine modulation, and immunoblotting, apoptosis and cell cycle analysis. Predictive significance was tested by immunohistochemistry of tumours from 125 stage III colorectal cancer patients treated with and without 5FU. Of 8 genes significantly differentially expressed between 5FU sensitive and resistant xenograft tumours, *CTPS2* was the gene with the highest probability of differential expression (*p=0.008*). Reduction of CTPS2 expression by siRNA increased the resistance of colorectal cancer cell lines DLD1 and LS174T to 5FU and its analogue, FUDR. CTPS2 siRNA significantly reduced cell S-phase accumulation and apoptosis following 5FU treatment. Exposure of cells to uridine, a precursor to the CTPS2 substrate uridine triphosphate, also increased 5FU resistance. Patients with low CTPS2 did not gain a survival benefit from 5FU treatment (p=0.072), while those with high expression did (p=0.003). Low *CTPS2* expression may be a rationally-based determinant of 5FU resistance.

Introduction

The development of molecular-targeted agents in cancer treatment has led to an accumulation of novel agents for testing in the clinic.[1, 2] The introduction of novel agents usually occurs, however, only in the context of failure of more established regimens.[3] In this context, the antimetabolite, 5-fluorouracil (5FU), is of significant interest. Today, 5FU remains one of the most commonly administered agents for cancer chemotherapy.[1, 4] In addition, it is a baseline component of many first-line regimens in many cancer types, including colorectal, gastric, lung, breast and skin cancer. Understanding the determinants of resistance of 5FU therefore holds significant value to optimising administration of the drug, and the introduction of novel agents and treatment strategies.

Over the years, numerous determinants of 5FU resistance have been identified, with the most prominent being genes and proteins involved in the metabolism and activity of the drug.[5-8] These have included thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), methylene tetrahydrofolate reductase and p53 for which many different features (gene variants, RNA and protein levels, and protein activity) of these enzymes have been found to associate with different patient outcomes from 5FU treatment in a manner consistent with their roles in 5FU metabolism. These findings are exemplified by a landmark report by Salonga *et al.*[9], in which patients resistant to 5FU treatment were observed to have high expression levels of at least one of *TS*, *TP* or *DPD*. This study highlighted the sensitivity of investigating pathway components of 5FU metabolism, and well as the relevance of RNA levels as 5FU determinants.

Genome-wide gene expression profiling studies have been performed in various cancer cell line[10-12] and tumour xenograft[13] models of 5FU sensitivity and resistance. More than 420 candidates have been identified by these studies, with the pathways implicated including those involved in DNA replication and repair, and protein processing and targeting. However, the candidate determinants identified in these studies have yet to gain any prominence, presumably due in part to the difficulty in rationalizing their relevance to 5FU activity. Another reason has been the lack of functional and clinical validation in independent samples series.

Cytotoxicity from 5FU is attributed to three mechanisms resulting from its anabolism: (1) inhibition of TS interfering with nucleotide pools, (2) incorporation into DNA, and (3) incorporation into RNA (Fig. 1).[4] Methylene tetrahydrofolate is an important co-factor in TS inhibition, implicating folate metabolism as an important factor in 5FU activity. Catabolism of 5FU is linked to its clearance, for which DPD is the initial and rate-limiting enzyme. All these processes involve numerous genes and proteins, of which only a minority have been investigated for their association with 5FU resistance.[14] To gain further insight into 5FU resistance, this study was undertaken to characterise determinants of 5FU resistance through real-time PCR analysis of 5FU pathway-based genes in colorectal tumour xenografts characterised for their resistance to 5FU.

Results

The overall expression of 5FU pathway genes associates with 5FU resistance in colorectal tumour xenografts

Unsupervised hierarchical clustering of the expression of the 92 5FU pathway genes in the 14 xenograft samples was performed to assess whether an association existed between 5FU pathway gene expression overall and 5FU resistance,. The clustering distinctly separated sensitive and resistant tumours (Fig. 2), supportive of an association. Three groups were identified, of which 1 group (III, consisting of 1 sample) appeared to be an outlier and was not considered further. The other two groups (I and II) differed significantly in the proportion of tumours resistant to 5FU (0/4, 0% vs 2/9, 22% respectively, $p<0.05$).

CTPS2 is the top candidate gene differentially expressed between 5FU resistant and sensitive tumour xenografts

In supervised analysis, expression of 8 (*CTPS2, SLC28A2, ITPA, NME6, ENTPD5, MLH1, PARP1, UCK1*) of the 92 genes examined were significantly different ($p<0.05$) between 5FU-sensitive and 5FU-resistant tumour xenografts (Supplementary Table S2). *SLC28A2* was higher in resistant tumours, while the other 7 genes were expressed at lower levels. Cytidine triphosphate synthase type 2 (*CTPS2*) had the highest probability to be differentially expressed ($p=0.008$), and hence was selected for further analysis.

CTPS2 suppression increases resistance to 5FU and FUDR

Thirteen colorectal cancer cell lines (listed in Supplementary Table S1) were screened for their IC_{50} to identify suitable models to test the effects of CTPS2 suppression on 5FU sensitivity. DLD1 and LS174T cells were the most sensitive (IC_{50} = 2.1 ± 0.2μM and 2.0 ± 0.3μM, respectively), and hence were chosen for subsequent experiments. DLD1 and LS174T also differ in p53 mutation status (mutant and wild-type respectively)[11] that is known to influence 5FU sensitivity. Basal gene and protein levels of CTPS2 in the cell line panel did not correlate with 5FU sensitivity (data not shown).

To verify the efficiency and characterise the timecourse of suppression of CTPS2 by CTPS2 siRNA, DLD1 cells were transfected with CTPS2 siRNA or controls and monitored for 120 hours. Doubling time were assessed and found not to be significantly different for negative control (20h) and CTPS2 silenced (22h) DLD1 cells ($p = 0.5$) (data not shown). Loss of CTPS2 protein expression was observed for 96 hours following exposure to CTPS2 siRNA but not controls. CTPS2 expression was restored in CTPS2 siRNA-transfected cells after 120 hours exposure (Fig. 3A).

DLD1 cells were then transfected with CTPS2 siRNA or controls and their IC_{50} values calculated 72 hours after 5FU exposure (96h post-siRNA transfection). A significant ($p<0.05$) 15 to 20-fold increase in the IC_{50} was observed in CTPS2-silenced cells (74 ± 38μM) compared to cells transfected with negative control siRNA (5.1 ± 1.2μM) and transfection reagent alone (3.8 ± 0.5μM) (Fig. 3B). CTPS2 siRNA also conferred an approximately 28-fold increased resistance to 5FU in the LS174T cell line compared to controls *($p < 0.05$)* (data not shown).

The effect of CTPS2 suppression on sensitivity to FUDR was also evaluated in DLD1 cells. CTPS2 siRNA-treated cells showed an increased IC_{50} for FUDR (15 ± 5.5μM) compared to cells transfected with negative control siRNA (3.0 ± 0.6μM) *(p=0.04)* and transfection reagent alone (2.3 ± 0.04μM) (*p=0.02*), although the effect was not as profound as for 5FU (Fig. 3C).

CTPS2 suppression reduces S-phase accumulation and apoptosis in cells after 5FU treatment

DLD1 cells treated with CTPS2 or negative control siRNA were exposed for 24 hours to 5μM or 70μM 5FU, corresponding to the IC_{50} of cells transfected with negative control or CTPS2 siRNA respectively. The higher concentration of 5FU resulted in marked S phase accumulation of cells transfected with the negative control, consistent with the mechanism of action of 5FU (Fig. 4A). In contrast, the cell cycle phase distribution of DLD1 cells transfected with CTPS2 siRNA was similar to that of controls. Exposure to 70μM 5FU for 24 hours also markedly increased the formation of nucleosomes in cells transfected with negative control compared to cells transfected with CTPS2 siRNA (Fig. 4B). Similar effects were not observed in cells treated with the lower concentration (5μM) of 5FU.

Excess extracellular uridine, mimicking reduced CTPS2, confers resistance to 5FU

CTPS2 is the rate-limiting enzyme in the synthesis of cytosine nucleotides, catalyzing the formation of CTP from UTP.[15] Incorporation of the 5FU anabolite, FUTP, into RNA is a major mechanism of 5FU cytotoxicity.[4] We therefore hypothesized that the association between low CTPS2 levels and 5FU resistance may be due to the accumulation of UTP

that competes and reduces FUTP incorporation into RNA. We therefore mimicked reduced CTPS2 levels in DLD1 cells by culturing them in the presence of 1mM uridine (precursor of UTP) prior to the addition of 5FU. In support of the hypothesis, cells exposed to 1mM uridine showed an approximately 6-fold increase in the IC_{50} for 5FU (32.8 ± 1.2μM) compared to cells grown in the absence of this nucleotide (5.0 ± 0.9 μM) (*p= 0.04*).

CTPS2 siRNA does not sensitise DLD1 cells to a selective TS inhibitor, raltitrexed

To further establish the role of CTPS2 in promoting 5FU-mediated cell death through the RNA incorporation pathway, the IC_{50} of a selective TS inhibitor raltitrexed[16 17] was measured in DLD1 cells transfected with or without CTPS2 siRNA. There was no significant change in the IC_{50} of raltitrexed in the presence of CTPS2 siRNA (1.2 ± 0.055μM) or negative control siRNA (1.6 ± 0.073μM) (*p = 0.9*).

Patients with high CTPS2 levels gained a survival benefit from 5FU treatment, while those with low levels did not

CTPS2 was expressed at 1+, 2+ and 3+ intensity in the cytoplasm of 239 (54%), 30 (7%), 7 (2%) of 444 colorectal cancer samples respectively that were intact in the tissue array sections. Positive CTPS2 expression did not correlate with gender, age, ethnicity, stage, tumour site, tumour size, histological differentiation, lymphatic invasion, perineural invasion or vascular invasion (Supplementary Table S3). To investigate the predictive significance of CTPS2, the differences in survival between patients treated with and without 5FU in subgroups of CTPS2 positive and negative stage III colorectal cancer patients were examined. In CTPS2 positive patients, 5FU treatment was associated with a

significantly better survival (*p=0.003*), while there was no significant difference in survival according to 5FU treatment in CTPS2 negative (*p=0.072*) patients (Fig. 5). In an examination of the clinicopathological factors listed above, only age significantly differed between treated and non-treated groups in both CTPS2 positive and negative subgroups (both *p<0.05*), with the treated arm containing more younger patients (56%) than the untreated arm (9%).

Discussion

In this study, we examined the expression of 92 genes involved in 5FU metabolism, transport, activity and folate metabolism in 14 patient-derived colorectal tumour xenografts by real-time PCR LDA in order to gain further insight into 5FU resistance. The pathway-based approach was chosen to optimize the number of genes assessed and the likelihood that candidates identified could be rationalized for their function. The pathways examined were selected on the basis that most of the prominent determinants of 5FU resistance are components of these pathways.[14] The choice to analyze gene expression derived from the numerous reports linking gene expression to 5FU resistance.[5, 9, 18, 19] Analysis by real-time PCR LDA was selected to maximize the precision and dynamic range of analysis,[20] and obviated the need for real-time PCR validation required in hybridization array studies. In support of this approach, the overall expression patterns of the pathway genes analyzed in this study distinctly separated xenograft tumours that were sensitive and resistant to 5FU in unsupervised clustering (Fig. 1).

Of the 92 genes examined, *CTPS2* had the highest probability (*p=0.008*) of being differentially expressed between sensitive and resistant tumours, and hence was selected as the optimal candidate for further investigation. In functional studies, suppression of *CTPS2* by siRNA markedly increased resistance to 5FU in IC_{50} studies (Fig. 3), consistent with the association of low *CTPS2* expression with 5FU resistance, observed in xenograft models. The increase was observed in two cell lines, suggesting the changes were not cell-specific. The cell lines also differed in their *TP53* mutation status (Supplementary Table S1), reducing the likelihood these mutations were a major factor in the changes in resistance. Suppression of *CTPS2* also increased resistance to the 5FU

analogue, FUDR, indicating the association was not restricted to 5FU, and was applicable to other fluoropyrimdines.

To further verify the functional link between CTPS2 and 5FU, the effect of CTPS2 suppression was examined on the known hallmarks of 5FU cytotoxicity of cell cycle S-phase accumulation and apoptosis.[4] Suppression of *CTPS2* by siRNA reduced S-phase accumulation and diminished apoptosis (Fig. 4), consistent with the association of low CTPS2 levels and 5FU resistance. These results, taken together with the IC_{50} findings, support both a phenotypic and mechanistic role for CTPS2 expression in determining 5FU cytotoxicity.

CTPS2 encodes an isoform of CTP synthetase (CTPS), which is the rate limiting enzyme in the synthesis of cytosine nucleotides required for RNA and DNA synthesis.[21] In humans, an equally active isoform is encoded by *CTPS*, although the dominant isoform in normal and malignant cells is unclear.[22] In rationalizing the link between low CTPS2 levels and 5FU resistance, one possible explanation was that the reduced rate of CTPS2 results in accumulation of UTP. This in turn leads to competition with the 5FU anabolite, FUTP, for incorporation into RNA, thereby reducing biochemical errors that would normally cause cells to accumulate in S-phase and commit them to apoptosis. This hypothesis was tested in this study by simulating UTP accumulation through the addition of excess extracellular uridine. In support of the hypothesis, the addition of uridine rendered sensitive DLD1 cells resistant to 5FU, at the same time reinforcing the contribution of RNA incorporation in 5FU cytoxoticity. Indeed, Peters *et al* showed that 1mM uridine inhibited the incorporation 5FU into RNA, thus reversing the growth inhibitory effect of 5FU, in WiDR colorectal cancer cells.[23] Decreased expression of

UMP kinase was also reported to increase 5FU resistance in colorectal cancer cells due to impaired incorporation of FUTP into RNA.[24] Furthermore, silencing CTPS2 in DLD1 cells had no apparent effect on sensitivity of raltitrexed, which does not influence the RNA-mediated cytotoxicity, thereby providing additional support of involvement of the RNA pathway.

A lack of reports on the relationship between 5FU resistance and *CTPS2* makes it difficult to gain further insight into mechanisms of actions. Whelan *et al.* showed that treatment of CHO cells with UV radiation and ethylmethanesulfonate generated mutations in *CTPS* that altered its synthase activity and increased the intracellular pools of CTP and dCTP.[25] Interestingly, these mutations in highly conserved regions of *CTPS* were also associated with resistance to 5FU. However, these results are on *CTPS* mutations and not *CTPS2* expression, and are in a different cellular context, so caution needs to be exercised in their interpretation.

To verify the clinical relevance of CTPS2 expression, we examined tissue samples from 444 colorectal cancer patients treated with and without 5FU chemotherapy for the predictive significance of CTPS2 stained immunohistochemically. Consistent with the xenograft and in-vitro findings associating low *CTPS2* expression and 5FU resistance, patients with high CTPS2 expression gained a survival benefit from 5FU ($p=0.003$), while those with low levels did not ($p=0.072$). Nevertheless, these results should be treated with caution given the latter association almost reached statistical significance. Moreover, an imbalance in age between patients treated and not treated with 5FU chemotherapy in this non-trial sample series suggests these observations should only be considered to be of a preliminary nature.

In conclusion, the results of this study have shown *CTPS2* expression may be a significant phenotypic, mechanistic and clinically relevant determinant of 5FU cytotoxicity in an investigation of 92 5FU pathway genes. The identification of *CTPS2*, and its role in UTP equilibration, has also highlighted the contribution of the less-explored mechanism of 5FU incorporation into RNA. The results suggest CTPS2 expression could be a useful marker for guiding appropriate patient selection for 5FU treatment, as well as a potentially useful target for modulation in fluoropyrimidine optimization strategies. Indeed, increased CTPS activity has been reported in hepatic cancer cells,[15] and leukaemia, liver, lung and colon tumours.[26] Further characterization of the tumour expression and predictive significance of CTPS2 in independent samples series will help to clarify its clinical potential.

Materials and Methods

Tumour Xenografts

Samples of 14 colorectal tumour xenografts characterised previously for 5FU resistance[27] were obtained from the Max-Delbrück-Center for Molecular Medicine (Berlin-Buch, Germany). With resistance defined as less than 50% tumour shrinkage after drug treatment for 40 days, 6 xenografts were sensitive and 8 resistant to 5FU.

Cell lines

Thirteen colorectal cancer cell lines (listed in Supplementary Table S1) were obtained from American Type Culture Collection (ATCC, Rockville, MD), or from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). Cells were routinely cultured in DMEM or RPMI1740 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 50,000 units penicillin and 50mg streptomycin (Sigma, St Louis, MO) at 37°C in a humidified atmosphere containing 5% CO_2.

Primary Tumour Samples

Tissue array blocks constructed previously,[28] containing cores from 501 consecutive, stage I-IV primary colorectal cancer cases at the National University Hospital, Singapore between 1990 and 1999 inclusive were used for immunohistochemistry (IHC). Corresponding clinical information, including patient gender, age, ethnicity, treatment and survival and tumour stage, site, size, histological differentiation, lymphatic invasion, perineural invasion, and vascular invasion were also obtained. Analysis of the samples and data was approved by the Institutional Review Board of National University of Singapore (NUS-277).

Compounds

The cytotoxic drugs 5FU, FUDR and raltitrexed were obtained commercially (Sigma). All stock solutions were prepared in DMSO (MP Biomedical, Solon, OH) at a final concentration in culture media of 0.25% (v/v).

Gene expression analysis

A total of 92 genes (displayed in Fig. 1 and listed in Supplementary Table S2) involved in the metabolism, transport, activity and co-factor (folate) metabolism of 5FU were identified from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database (http://www.genome.ad.jp/kegg/pathway.html) and literature review.[5, 14, 29] An additional 4 genes involved in cell housekeeping were also included (*18S*, *ACTB*, *B2M* and *GAPDH*). Real-time RT-PCR low density arrays preloaded with these 96 corresponding inventoried TaqMan® Gene Expression Assays were then ordered from Applied Biosystems (Foster City, CA).

Total RNA was extracted from tumour xenografts using TRI reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA yields and purity were determined spectrophotometrically at 260 and 280nm using the Nanodrop 2000 (Thermo Scientific, Wilmington, DE). RNA quantification from low density arrays was performed by real-time PCR analysis according to manufacturer's instructions. Briefly, 1µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. The reaction mixtures were incubated at 25°C for 10 min, followed by 37°C for 120 min and 95°C for 5 min. Subsequently, 9µl of cDNA sample containing 400ng of total RNA along with 41µl of nuclease-free water and 50µl of PCR master mix (2x) were loaded into each sample port on the low density array. The array

was centrifuged twice for 1 min at 331 x g at room temperature and then sealed. Real-time PCR was performed using an ABI PRISM 7900 HT Sequence Detection System. Relative quantification was performed according to the ΔΔCt method,[30] with *GAPDH* used as the reference gene to normalize for loading, and the COLO201 cell line as a calibrator sample.

Inhibition by small interfering RNA (siRNA)

Pre-designed Silencer Select siRNA (CTPS2, sense strand: 5'-GGU UCG AGG UAA ACC CUA ATT-3' and antisense strand: 5'-UUA GGG UUU ACC UCG AAC CGA-3'; Negative Controls, sense strand: 5'-UAA CGA CGC GAC GAC GUA ATT-3' and antisense strand: 5'-UUA CGU CGU CGC GUC GUU ATT-3') were purchased from Applied Biosystems. To minimize off-target effects, all sequences were BLAST-searched against the human genome to ensure ≤ 75% homology matches to other genes. Cells were transfected with CTPS2 siRNA (5nM) or negative controls (10nM) using siPORT NeoFX transfection agent (Applied Biosystems) at 50% cell confluence and incubated in serum-free Opti-MEM I medium (Invitrogen) according to the manufacturers' instructions.

Immunoblot Analysis

Adherent cells were grown to 30-50% confluence and then exposed to siRNA or controls at various concentrations and times as indicated. Cells were then harvested and prepared in denaturing lysis buffer for immunoblotting as previously described.[31] The antibody to CTPS2 for immunoblotting was obtained from Sigma, and anti-mouse secondary antibody from Cell Signaling Technology (Danvers, MA).

Inhibition of proliferation assay

Cells in 90µl medium were seeded (3000 cells/well) onto 96-well microtitre plates (Nunc, Rochester, NY). After 24 hours, 10µl of medium containing 5FU or FUDR with graded concentrations ranging from 0.1µM to 1000µM was added to the wells. Effect on cell numbers was assessed using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) CellTiter 96® (Promega, Madison, WI) assay at 72h post-treatment. The IC_{50} was calculated as the drug concentration that inhibited cell proliferation by 50% inhibition compared with vehicle controls as described previously.[32]

Assessment of Cell Cycle Phase Distribution

Exponentially growing cells were seeded into 25cm^2 tissue culture flasks at 1x10^6 cells per flask and allowed to attach for 24 hours before the addition of siRNA and 5FU. Following incubation for 24 hours, the cultures were washed and any detached cells discarded. The remaining cells attached to the culture dish were removed by trypsinisation, resuspended in 200µL of cold phosphate-buffered saline (PBS) and then fixed with 2mL of ice-cold 70% ethanol. Following centrifugation (900g x 5 minutes) the pellet was resuspended in 800µL PBS containing 100µL of 1mg/mL RNase A and 100µL of 400µg/mL propidium iodide (both from Sigma) and stored overnight at 4^{o}C. Samples were analysed on a LSRII Flow Cytometer (BD, Franklin Lanes, NJ) equipped with an argon laser tuned to 488nm and the red fluorescence collected at 630nm. The data was analysed using WinMDI v2.8 and DNA histograms were gated on a display of DNA peak signal against DNA area to exclude clumps or debris.

Apoptosis Measurements

The Cell Death ELISA® (Roche, Mannheim, Germany) kit was used for the measurement of apoptosis. Cells were plated in 96-well plates (3000 cells/well) and the following day were treated with drug or solvent in a volume adjusted to 200μL with 10% FCS/RPMI. After 24 hours, nucleosomes were quantified according to the manufacturer's instructions.

Uridine exposure

Exponentially growing cells were seeded into 25cm^2 tissue culture flasks at 1x10^6 cells per flask and allowed to attach for 24 hours before the addition of 1mM uridine (Sigma) for 24 hours as previously described.[23] The media was changed after 24 hours before 5FU addition and MTS assay carried out at 72 hours.

Immunohistochemistry

Four μm sections were cut from tissue array blocks, dewaxed and subjected to automated antigen retrieval with citrate buffer (pH 6.0) and immunostaining in the Bond-MAX (Leica, Wetzlar, Germany) according to manufacturer's recommendations. Reagents were obtained from the Bond Compact Polymer Refine Detection kit (Leica). Mouse monoclonal anti-CTPS2 antibody was obtained from Abnova (Taipei City, Taiwan) and applied at a concentration of 1 in 1000. Slides were scored for their intensity and extent (%cell) of tumour cell staining by a pathologist (BT) who had no knowledge of patient information. Cases with any cytoplasmic staining in tumour cells were considered positive.

Statistical analysis

Unsupervised hierarchical clustering analysis was carried out with the *heatmap.2* function in the *gplots* library to examine gene expression relationships, and to identify subgroups in the xenograft tumours. The Mann-Whitney U test was used to identify differentially expressed genes between resistant and sensitive tumours. A one-tailed t-test was used to identify differences in IC_{50} between cells transfected with siRNA or negative controls. Differences in survival were tested by the log-rank test and Kaplan-Meier plots were graphed. All statistical tests were performed at the 5% significance level. All analyses were performed in the statistical software R package version 2.7.1 (R Foundation for Statistical Computing, Vienna, Austria) except survival analysis, for which PASW Statistics 18 (SPSS, Chicago, IL) was used.

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Figure Legends

Figure 1. - Genes analyzed in this study and their roles in the 5FU metabolism, transport and activity, and co-factor (folate) metabolism.

Figure 2. - Unsupervised hierarchical clustering of xenograft samples based on the expression of 96 genes. Red indicates low expression, and green indicates high expression. The colours above the heatmap indicate tumours that were sensitive (red) and resistant (blue) to 5FU. The numbers below the heatmap indicate the three groups (I-III) identified by unsupervised clustering.

Figure 3. - (A) Immunoblot analysis for CTPS2 protein expression following transfection of DLD1 colorectal cancer cells with CTPS2 siRNA or controls for up to 120 hours. GAPDH was used as a loading control. Representative immunoblots of 2 independent experiments are shown. Inhibition of proliferation curves for 5FU (B), and FUDR (C) following transfection with *CTPS2* siRNA or controls. The assay was performed at 96 hours (72 hours post-treatment) post-transfection. Data represents $n \geq 6$ independent experiments.

Figure 4. - Effect of 5FU on cell cycle phase distribution (A) and apoptosis (B) in DLD1 cells with or without CTPS2 siRNA. Cells were exposed to IC_{50} concentrations of 5FU for transfected (70μM) or negative control transfected (5μM) cells for 24 hours (48 hours post-transfection). Control cells received DMSO. Negative control cells received scrambled siRNA. Apoptosis data displays mean ± SD (n = 3) and are normalised to control cell values.

Figure 5. - Representative CTPS2 immunohistochemical stains of a colorectal cancer with 1+ (A) and 3+ (B) intensity at 40x magnification. Kaplan Meier curves of the

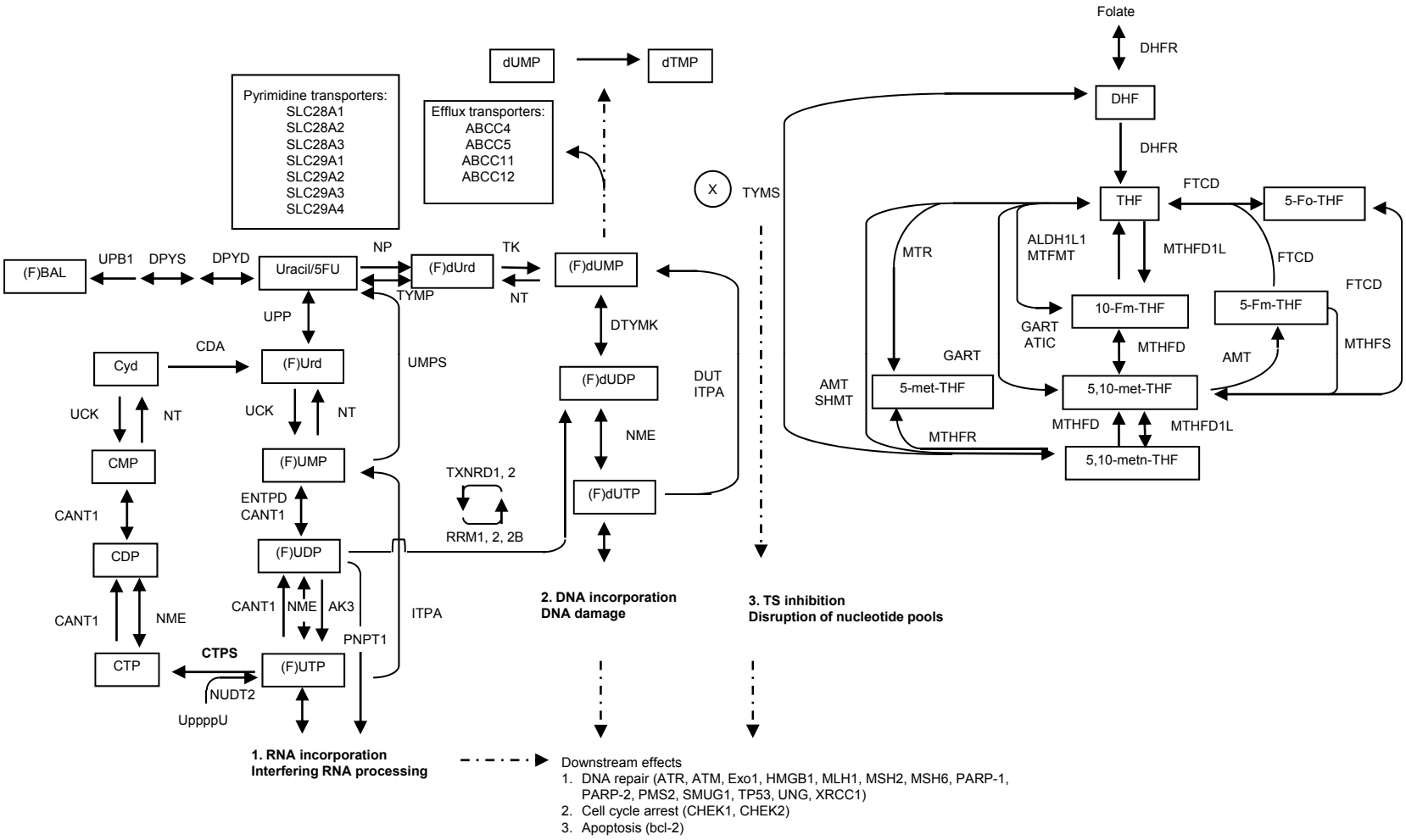
survival of patients treated with 5FU chemotherapy (dashed lines) and not receiving chemotherapy (unbroken lines) in CTPS2 negative (C) and CTPS2 positive (D) patients.

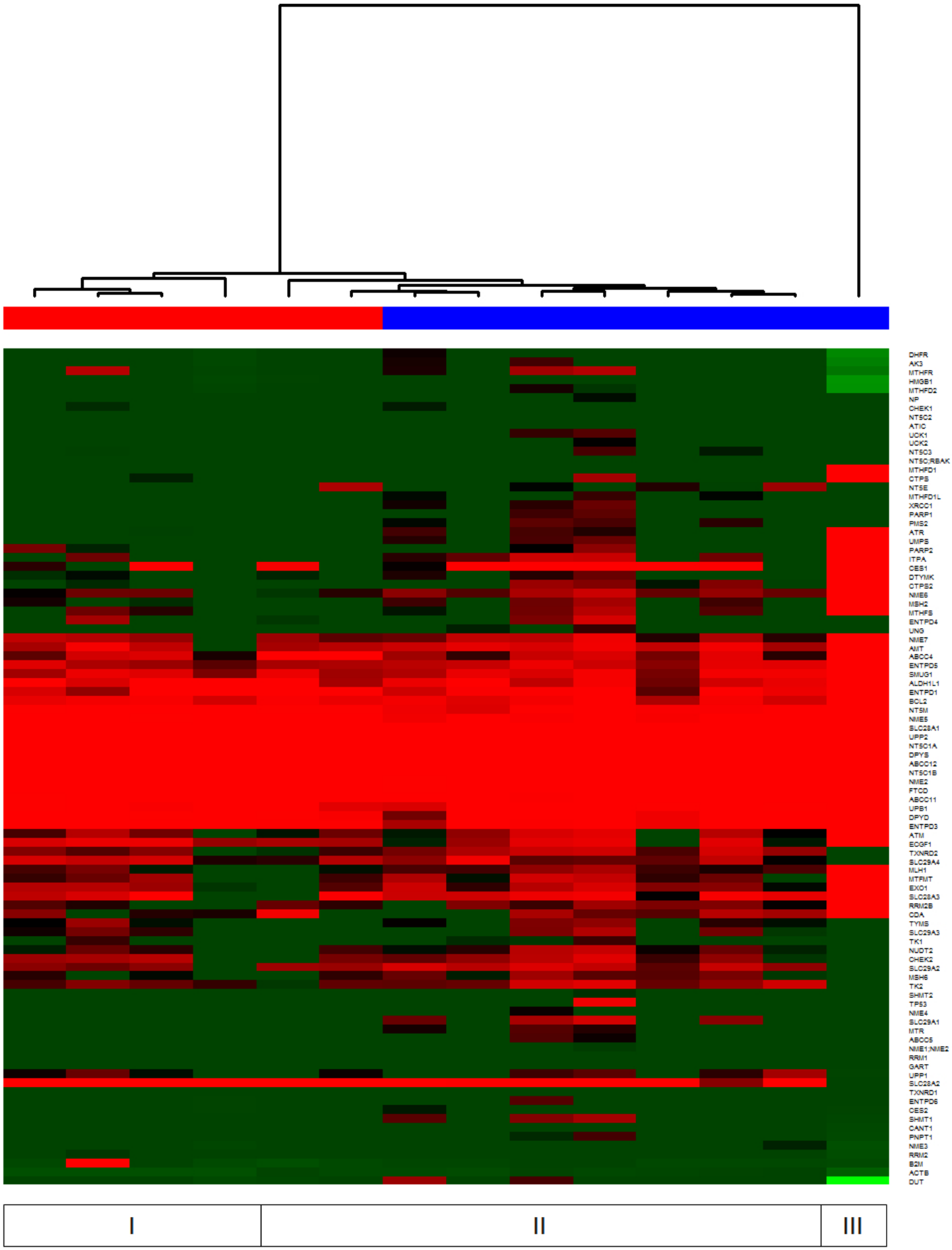
Supplementary Table Legends

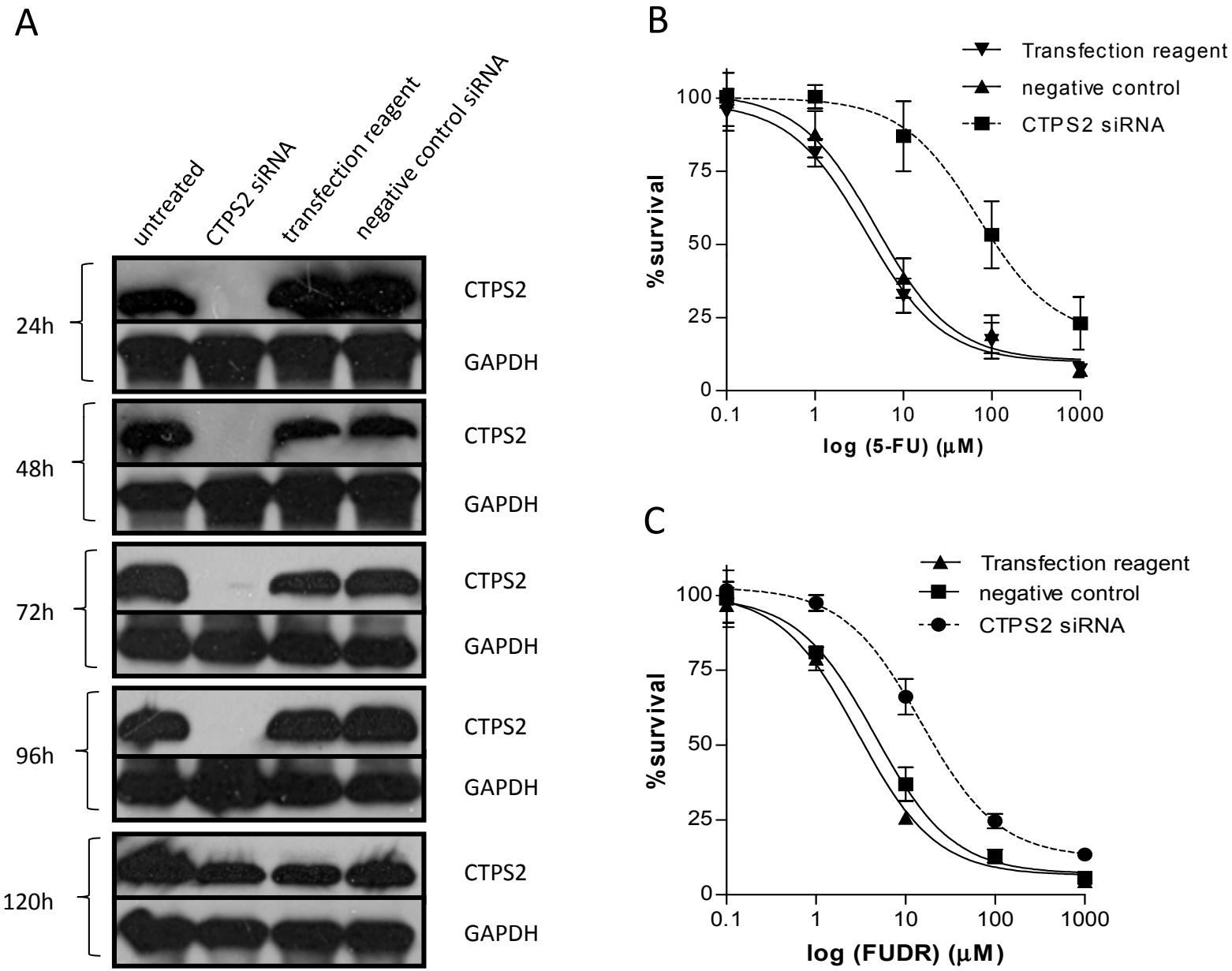
Supplementary Table S1 - IC_{50} values (μM) for 5FU and FUDR in the 13 colorectal cancer cell lines tested

Supplementary Table S2 - Median expression of 92 5FU pathway genes in 6 sensitive and 8 resistant colorectal tumour xenografts.

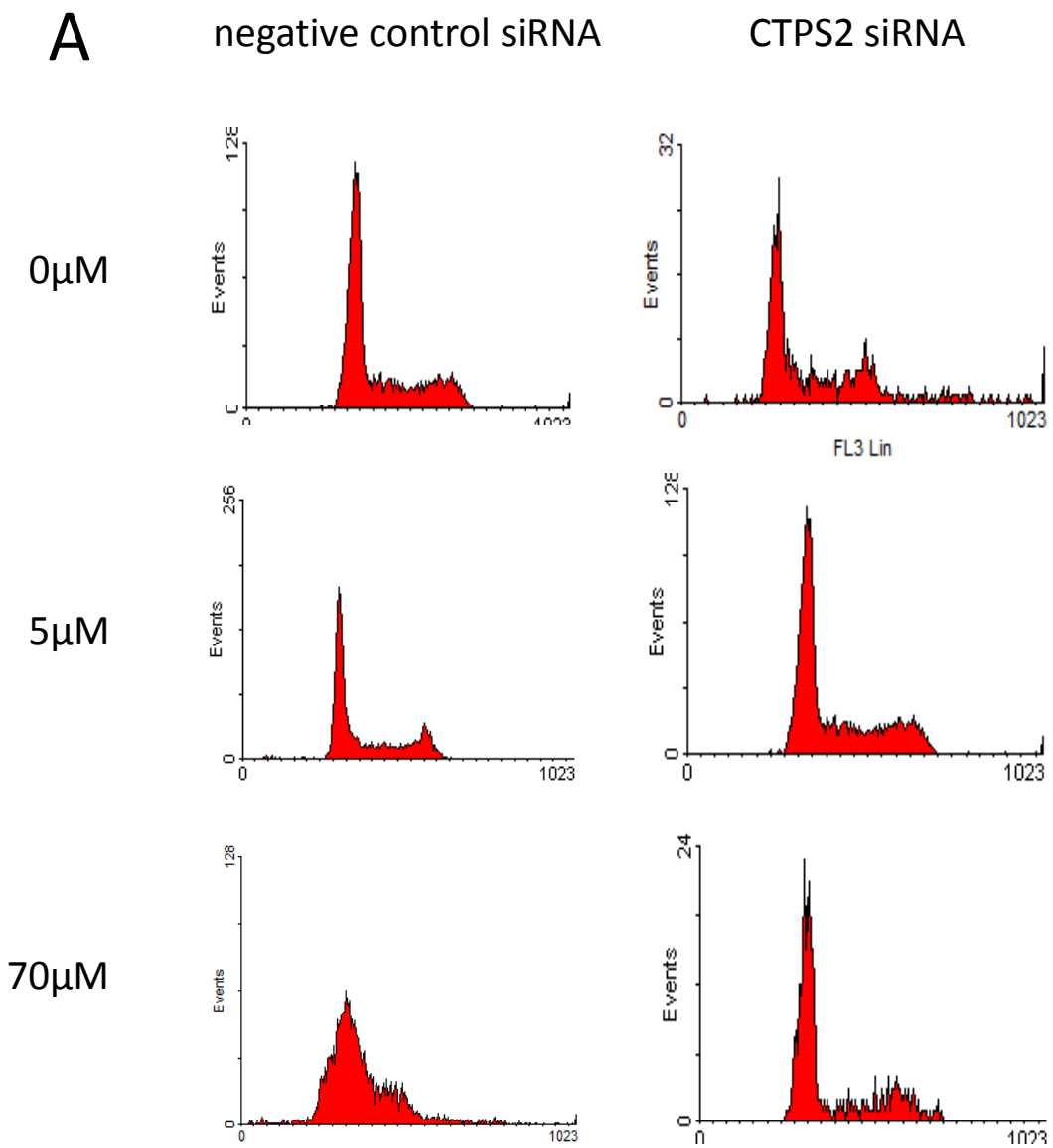
Supplementary Table S3 - Association of CTPS2 expression and clinicopathological factors in colorectal cancer patients


Pyrimidine transporters:
SLC28A1
SLC28A2
SLC28A3
SLC29A1
SLC29A2
SLC29A3
SLC29A4
Efflux transporters:
ABCC4
ABCC5
ABCC11
ABCC12
dUMP
dTMP
X
TYMS
Folate
DHFR
DHF
DHFR
THF
FTCD
5-Fo-THF
ALDH1L1
MTFMT
MTHFD1L
FTCD
MTR
FTCD
10-Fm-THF
5-Fm-THF
GART
ATIC
MTHFD
AMT
MTHFS
GART
AMT
SHMT
5-met-THF
5,10-met-THF
MTHFD
MTHFD1L
MTHFR
5,10-metn-THF
(F)BAL
UPB1
DPYS
DPYD
Uracil/5FU
NP
(F)dUrd
TK
(F)dUMP
TYMP
NT
DTYMK
UPP
CDA
Cyd
(F)Urd
UMPS
(F)dUDP
DUT
ITPA
UCK
NT
UCK
NT
NME
CMP
(F)UMP
TXNRD1, 2
ENTPD
CANT1
(F)dUTP
CANT1
RRM1, 2, 2B
CDP
(F)UDP
CANT1
NME
AK3
ITPA
PNPT1
CANT1
NME
CTP
CTPS
(F)UTP
NUDT2
UppppU
2. DNA incorporation
DNA damage
3. TS inhibition
Disruption of nucleotide pools
1. RNA incorporation
Interfering RNA processing
Downstream effects
1. DNA repair (ATR, ATM, Exo1, HMGB1, MLH1, MSH2, MSH6, PARP-1, PARP-2, PMS2, SMUG1, TP53, UNG, XRCC1)
2. Cell cycle arrest (CHEK1, CHEK2)
3. Apoptosis (bcl-2)

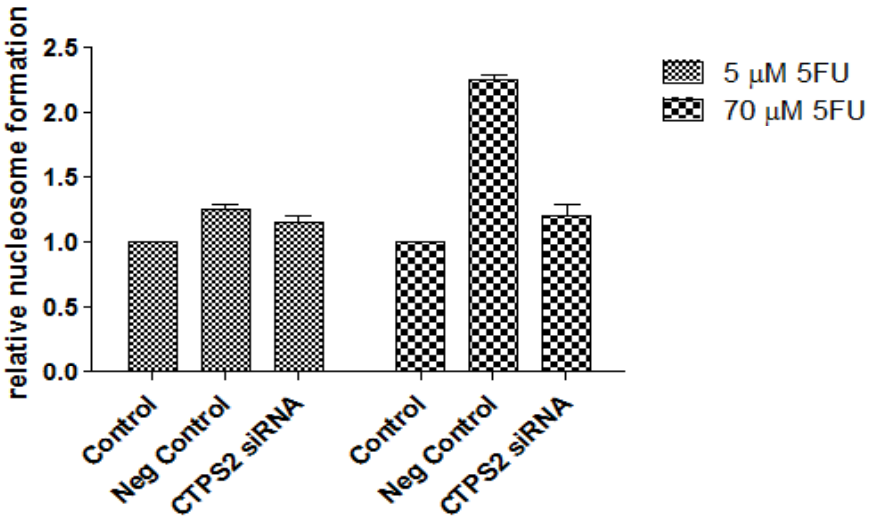

DHFR
AK3
MTHFR
HMGB1
MTHFD2
NP
CHEK1
NT5C2
ATIC
UCK1
UCK2
NT5C3
NT5C;RBAK
MTHFD1
CTPS
NT5E
MTHFD1L
XRCC1
PARP1
PMS2
ATR
UMPS
PARP2
ITPA
CES1
DTYMK
CTPS2
NME6
MSH2
MTHFS
ENTPD4
UNG
NME7
AMT
ABCC4
ENTPD5
SMUG1
ALDH1L1
ENTPD1
BCL2
NT5M
NME5
SLC29A1
UPP2
NT5C1A
DPYS
ABCC12
NT5C1B
NME2
FTCD
ABCC11
UPB1
DPYD
ENTPD3
ATM
EGFI
TXNRD2
SLC29A4
MLH1
MTFMT
EXO1
SLC28A3
RRM2B
CDA
TYMS
SLC29A3
TK1
NUDT2
CHEK2
SLC29A2
MSH6
TK2
SHMT2
TP53
NME4
SLC29A1
MTR
ABCC5
NME1;NME2
RRM1
GART
UPP1
SLC28A2
TXNRD1
ENTPD6
CES2
SHMT1
CANT1
PNPT1
NME3
RRM2
B2M
ACTB
DUT
I
II
III

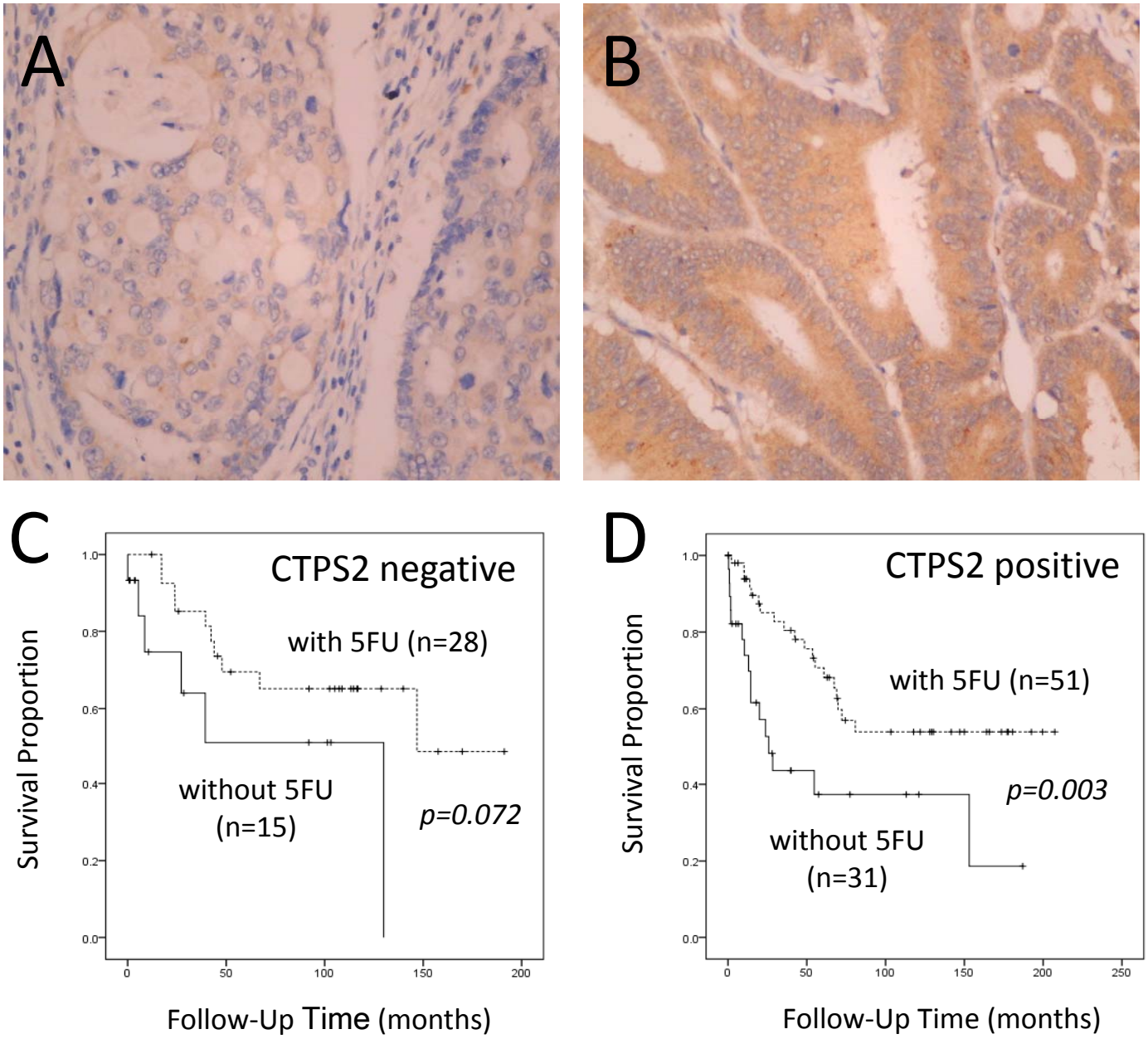

A
untreated
CTPS2 siRNA
transfection reagent
negative control siRNA
24h
48h
72h
96h
120h
CTPS2
GAPDH
B
Transfection reagent
negative control
CTPS2 siRNA
%survival
log (5-FU) (μM)
C
Transfection reagent
negative control
CTPS2 siRNA
%survival
log (FUDR) (μM)

A


negative control siRNA
CTPS2 siRNA
0μM
5μM
70μM
Events
FL3 Lin
0
1023

B


relative nucleosome formation
2.5
2.0
1.5
1.0
0.5
0.0
5 μM 5FU
70 μM 5FU
Control
Neg Control
CTPS2 siRNA
Control
Neg Control
CTPS2 siRNA


A
B
C
CTPS2 negative
with 5FU (n=28)
without 5FU
(n=15)
p=0.072
Survival Proportion
Follow-Up Time (months)
D
CTPS2 positive
with 5FU (n=51)
p=0.003
without 5FU
(n=31)
Survival Proportion
Follow-Up Time (months)

Cell Line	*TP53* status*	5FU	FUDR
LS174T	Wildtype	2.0 ± 0.3	3.5 ± 0.9
DLD1	Mutant	2.1 ± 0.2	2.4 ± 1.5
OUMS23	Unknown	2.3 ± 1.4	4.0 ± 0.4
CCK81	Mutant**	5.4 ± 0.5	9.2 ± 0.7
COLO320	Mutant	7.0 ± 3.2	2.1 ± 0.8
HT29	Mutant	7.5 ± 0.8	9.0 ± 1.5
LS513	Wildtype***	7.5 ± 0.9	6.1 ± 0.8
WiDR	Mutant	8.7 ± 1.7	6.8 ± 0.8
COLO205	Mutant	13 ± 3.6	11 ± 3.6
HCT116	Wildtype	16 ± 6.7	16 ± 0.5
HCC56	Unknown	19 ± 0.8	11 ± 1.5
RKO	Wildtype	23 ± 2.6	15 ± 1.1
SW403	Wildtype	30 ± 2.6	12 ± 0.9

* According to Mariadason et al. (2001) Cancer Res. 63:8791

** http://cellbank.nbio.go.jp

*** Fisher et al. (1999) Cancer Res. 59:331

Gene	Gene Name	5FU Pathway Group	Sensitive	Resistant	Higher in	p-value*
CTPS2	CTP synthase II	Metabolism (pyrimidine)	0.024155	0.008665	sensitive	0.0081
SLC28A2	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	Transporters	0.000000	0.000045	resistant	0.0087
ITPA	Inosine triphosphatase	Metabolism (pyrimidine)	0.026345	0.006565	sensitive	0.0127
NME6	Non-metastatic cells 6	Metabolism (pyrimidine)	0.010095	0.004835	sensitive	0.0200
ENTPD5	Ectonucleoside triphosphate diphosphohydrolase 5	Metabolism (pyrimidine)	0.003680	0.001750	sensitive	0.0426
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2	DNA repair/apoptosis/cell cycle regulation	0.011920	0.007825	sensitive	0.0426
PARP1	Poly (ADP-ribose) polymerase 1	DNA repair/apoptosis/cell cycle regulation	0.032265	0.021435	sensitive	0.0426
UCK1	Uridine-cytidine kinase 1	Metabolism (pyrimidine)	0.047615	0.021515	sensitive	0.0426
MTHFD1	Methylenetetrahydrofolate dehydrogenase	Metabolism (folate)	0.065935	0.034295	sensitive	0.0593
ATIC	5′-aminoimidazole-4-carboxamide ribonucleotide formyltransferase	Metabolism (folate)	0.083895	0.046995	sensitive	0.0813
ENTPD6	Ectonucleoside triphosphate diphosphohydrolase 6	Metabolism (pyrimidine)	0.107045	0.048350	sensitive	0.0813
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1	DNA repair/apoptosis/cell cycle regulation	0.016305	0.008340	sensitive	0.0813
RRM2B	Ribonucleotide reductase M2 B	Metabolism (pyrimidine)	0.009350	0.005555	sensitive	0.1062
TK2	Thymidine kinase 2	Metabolism (pyrimidine)	0.007450	0.005620	sensitive	0.1062
ACTB	Actin, beta	Housekeeping genes	3.427820	1.238835	sensitive	0.1079
AK3	Adenylate kinase 3	Metabolism (pyrimidine)	0.083245	0.042565	sensitive	0.1079
AMT	Aminomethyltransferase	Metabolism (folate)	0.003425	0.001395	sensitive	0.1079
ATR	Ataxia telangiectasia and Rad3 related	DNA repair/apoptosis/cell cycle regulation	0.022875	0.012585	sensitive	0.1079
CANT1	Calcium activated nucleotidase I	Metabolism (pyrimidine)	0.071235	0.037780	sensitive	0.1079
NME4	Non-metastatic cells 4	Metabolism (pyrimidine)	0.084740	0.038245	sensitive	0.1079
TXNRD2	Thioredoxin reductase 2	Metabolism (pyrimidine)	0.007935	0.004010	sensitive	0.1079
CES1	Carboxylesterase 1	Metabolism (pyrimidine)	0.012865	0.000045	sensitive	0.1355
MSH6	mutS homolog 6	DNA repair/apoptosis/cell cycle regulation	0.012685	0.007240	sensitive	0.1419
NT5C	5′,3′-nucleotidase, cytosolic	Metabolism (pyrimidine)	0.048265	0.037095	sensitive	0.1419
UMPS	Uridine monophosphate synthetase	Metabolism (pyrimidine)	0.019020	0.013065	sensitive	0.1419
MTFMT	Mitochondrial methionyl-tRNA formylтransferase	Metabolism (folate)	0.008575	0.004980	sensitive	0.1812
NME3	Non-metastatic cells 3	Metabolism (pyrimidine)	0.091140	0.044965	sensitive	0.1812
SLC29A2	Solute carrier family 29 (nucleoside transporters), member 2	Transporters	0.005035	0.002860	sensitive	0.1812
UCK2	Uridine-cytidine kinase 2	Metabolism (pyrimidine)	0.044350	0.029900	sensitive	0.1812
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	Metabolism (folate)	0.023170	0.015190	sensitive	0.2284
NME1	Non-metastatic cells 1	Metabolism (pyrimidine)	0.093885	0.057180	sensitive	0.2284
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase 1	DNA repair/apoptosis/cell cycle regulation	0.002665	0.000720	sensitive	0.2284
EXO1	Exonuclease 1	DNA repair/apoptosis/cell cycle regulation	0.005870	0.004160	sensitive	0.2448
18S	18S ribosomal RNA	Housekeeping genes	1577.000000	721.000000	sensitive	0.2824
CES2	Carboxylesterase 2	Metabolism (pyrimidine)	0.158870	0.067860	sensitive	0.2824
HMGB1	High-mobility group box 1	DNA repair/apoptosis/cell cycle regulation	0.126005	0.050660	sensitive	0.2824
MTHFD1L	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) I-like	Metabolism (folate)	0.038600	0.022890	sensitive	0.2824
MTHFS	5,10-methenyltetrahydrofolate synthetase	Metabolism (folate)	0.015735	0.009670	sensitive	0.2824
PMS2	Postmeiotic segregation increased 2	DNA repair/apoptosis/cell cycle regulation	0.023075	0.014625	sensitive	0.2824
NT5M	5′,3′-nucleotidase, mitochondrial	Metabolism (pyrimidine)	0.000070	0.000135	resistant	0.2996
NT5C1A	5′-nucleotidase, cytosolic IA	Metabolism (pyrimidine)	0.000000	0.000000	resistant	0.3123
NME5	Non-metastatic cells 5	Metabolism (pyrimidine)	0.000000	0.000000	no difference	0.3319
ATM	Ataxia telangiectasia mutated homolog	DNA repair/apoptosis/cell cycle regulation	0.007190	0.003955	sensitive	0.3450
CTPS	CTP synthase	Metabolism (pyrimidine)	0.041895	0.038275	sensitive	0.3450
NT5C2	5′-nucleotidase, cytosolic II	Metabolism (pyrimidine)	0.052670	0.044505	sensitive	0.3450
NT5C3	5′-nucleotidase, cytosolic III	Metabolism (pyrimidine)	0.021125	0.016180	sensitive	0.3450
NT5E	5′-nucleotidase, ecto	Metabolism (pyrimidine)	0.032785	0.014315	sensitive	0.3450
RRM1	Ribonucleotide reductase M1 polypeptide	Metabolism (pyrimidine)	0.067605	0.056640	sensitive	0.3450
CDA	Cytidine deaminase	Metabolism (pyrimidine)	0.009655	0.005305	sensitive	0.4136
DHFR	Dihydrofolate reductase	Metabolism (folate)	0.084180	0.054905	sensitive	0.4136
DTYMK	Deoxythymidylate kinase	Metabolism (pyrimidine)	0.014430	0.012865	sensitive	0.4136
SLC29A4	Solute carrier family 29 (nucleoside transporters), member 4	Transporters	0.002905	0.006770	resistant	0.4136
DPYS	Dihydropyrimidinase	Metabolism (pyrimidine)	0.000000	0.000000	no difference	0.4705
DPYD	Dihydropyrimidine dehydrogenase	Metabolism (pyrimidine)	0.000065	0.000015	sensitive	0.4723
ALDH1L1	Aldehyde dehydrogenase I family, member L1	Metabolism (folate)	0.000085	0.000885	resistant	0.4728
DUT	dUTP pyrophosphatase	Metabolism (pyrimidine)	0.055040	0.066185	resistant	0.4908
GART	Phosphoribosylglycinamide formyltransferase	Metabolism (folate)	0.107385	0.063860	sensitive	0.4908
NME7	Non-metastatic cells 7	Metabolism (pyrimidine)	0.004390	0.003225	sensitive	0.4908
SLC29A1	Solute carrier family 29 (nucleoside transporters), member 1	Transporters	0.023170	0.014715	sensitive	0.4908
TXNRD1	Thioredoxin reductase 1	Metabolism (pyrimidine)	0.104650	0.081275	sensitive	0.4908
UNG	Uracil-DNA glycosylase	DNA repair/apoptosis/cell cycle regulation	0.020505	0.017505	sensitive	0.4908
UPB1	Ureidopropinase, beta	Metabolism (pyrimidine)	0.000085	0.000005	sensitive	0.5012
SLC28A3	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	Transporters	0.002240	0.000855	sensitive	0.5181
SLC28A1	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 1	Transporters	0.000015	0.000005	sensitive	0.5444
ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3	Metabolism (pyrimidine)	0.000045	0.000040	sensitive	0.5565
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	Metabolism (pyrimidine)	0.000080	0.000170	resistant	0.5595
B2M	Beta-2-microglobulin	Housekeeping genes	1.747910	1.239310	sensitive	0.5728
TYMP	Thymidine phosphorylase	Metabolism (pyrimidine)	0.002675	0.002780	resistant	0.5728
MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent)	Metabolism (folate)	0.049950	0.046530	sensitive	0.5728
PNPT1	Polyribonucleotide nucleotidyltransferase 1	Metabolism (pyrimidine)	0.030980	0.029950	sensitive	0.5728
SHMT1	Serine hydroxymethyltransferase 1 (soluble)	Metabolism (folate)	0.022060	0.020710	sensitive	0.5728
TP53	Tumor protein p53	DNA repair/apoptosis/cell cycle regulation	0.049035	0.034860	sensitive	0.5728
ABCC11	ATP-binding cassette, sub-family C (CFTR/MRP), member 11	Transporters	0.000000	0.000020	resistant	0.6294
ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	Transporters	0.032575	0.020935	sensitive	0.6620
CHEK1	CHK1 checkpoint homolog	DNA repair/apoptosis/cell cycle regulation	0.035380	0.035350	sensitive	0.6620
NUDT2	Nudix (nucleoside diphosphate linked moiety X)-type motif 2	Metabolism (pyrimidine)	0.010735	0.009960	sensitive	0.6620
RRM2	Ribonucleotide reductase M2 polypeptide	Metabolism (pyrimidine)	0.042510	0.033525	sensitive	0.6620
TK1	Thymidine kinase 1	Metabolism (pyrimidine)	0.016225	0.014425	sensitive	0.6620
TYMS	Thymidylate synthase	Metabolism (pyrimidine)	0.012875	0.011230	sensitive	0.6620
ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	Transporters	0.001685	0.003240	resistant	0.7546
ENTPD4	Ectonucleoside triphosphate diphosphohydrolase 4	Metabolism (pyrimidine)	0.017445	0.025310	resistant	0.7546
PARP2	Poly (ADP-ribose) polymerase 2	DNA repair/apoptosis/cell cycle regulation	0.015515	0.020025	resistant	0.7546
FTCD	Formiminotransferase cyclodeaminase	Metabolism (folate)	0.000000	0.000000	no difference	0.7876
NME2	Non-metastatic cells 2	Metabolism (pyrimidine)	0.000000	0.000000	no difference	0.8214
UPP2	Uridine phosphorylase 2	Metabolism (pyrimidine)	0.000000	0.000000	no difference	0.8322
MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)	Metabolism (folate)	0.025335	0.023045	sensitive	0.8518
SHMT2	Serine hydroxymethyltransferase 2	Metabolism (folate)	0.052590	0.050295	sensitive	0.8518
BCL2	B-cell CLL/lymphoma 2	DNA repair/apoptosis/cell cycle regulation	0.000695	0.000450	sensitive	0.8972
NP	Nucleoside phosphorylase	Metabolism (pyrimidine)	0.056805	0.066200	resistant	0.9497
SLC29A3	Solute carrier family 29 (nucleoside transporters), member 3	Transporters	0.014900	0.015370	resistant	0.9497
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1	DNA repair/apoptosis/cell cycle regulation	0.022450	0.023745	resistant	0.9497
CHEK2	CHK2 checkpoint homolog	DNA repair/apoptosis/cell cycle regulation	0.005095	0.005850	resistant	1.0000
UPP1	Uridine phosphorylase 1	Metabolism (pyrimidine)	0.011070	0.017130	resistant	1.0000
ABCC12	ATP-binding cassette protein C12; ATP-binding cassette transporter sub-family C member 12	Transporters	0.000000	0.000000	no difference	ND
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping genes	1.000000	1.000000	no difference	ND
NT5C1B	5′-nucleotidase, cytosolic IB	Metabolism (pyrimidine)	0.000000	0.000000	no difference	ND

* p-value from Mann-Whitney test; ND = not determinable

	CTPS2 Negative	CTPS2 Positive	p-value*
Gender			
Male	73/168 (43%)	136/276 (49%)	
Female	95/168 (57%)	140/276 (51%)	0.241
Age			
<60 years old	45/168 (27%)	84/276 (30%)	
>=60 years old	123/168 (73%)	192/276 (70%)	0.451
Ethnicity			
Chinese	143/168 (85%)	247/276 (90%)	
Non-Chinese	25/168 (15%)	29/276 (11%)	0.180
Stage			
I-II	86/168 (51%)	123/276 (45%)	
III-IV	82/168 (49%)	153/276 (55%)	0.203
Tumour Site			
Colon	139/168 (83%)	206/276 (75%)	
Rectum	29/168 (17%)	70/276 (25%)	0.060
Tumour Size			
<5cm	88/162 (54%)	164/267 (61%)	
>=5cm	74/162 (46%)	103/267 (39%)	0.158
Histological Differentiation			
Poor	21/162 (13%)	23/275 (8%)	
Moderate-Well	141/162 (87%)	252/275 (92%)	0.139
Lymphatic Invasion			
Absent	151/168 (90%)	253/276 (92%)	
Present	17/168 (10%)	23/276 (8%)	0.609
Perineural Invasion			
Absent	163/168 (97%)	262/276 (95%)	
Present	5/168 (3%)	14/276 (5%)	0.342
Vascular Invasion			
Absent	145/168 (86%)	252/276 (92%)	
Present	23/168 (14%)	23/276 (8%)	0.079

* p-value from Fisher's Exact test