Genomic analysis of *uridine monophosphate synthetase* reveals novel mRNA isoforms associated with fluorouracil resistance in colorectal cancer

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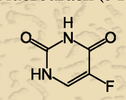
1. Abstract

The drug fluorouracil (5-FU) is an anti-metabolite chemotherapy commonly used in the treatment of many cancer types including colorectal, head and neck, pancreatic, breast, and stomach. Acquired or intrinsic resistance to 5-FU is also common. Several genes including those involved in the metabolism of 5-FU are candidates for predicting response to 5-FU. In particular, the gene *uridine monophosphate synthetase* (*UMPS;* also known as *OPRT*) is thought to be primarily responsible for conversion of 5-FU to active anti-metabolites in tumor cells. Germline or somatic mutations which result in loss of *UMPS* activity are predicted to confer resistance to 5-FU treatment. We developed two genome-wide assays capable of measuring expression of alternatively spliced mRNA isoforms. Using these assays we observed reciprocal differential expression of two *UMPS* isoforms in a colorectal cancer cell line with acquired 5-FU resistance relative to the 5-FU sensitive cell line from which it was derived. While the canonical *UMPS* isoform was down-regulated in resistant cells, a novel isoform arising as a consequence of exon-skipping was up-regulated. The underlying mechanism responsible for this shift in isoform expression was determined to be a heterozygous splice site mutation acquired in the resistant cell line. We catalogued the diversity of *UMPS* mRNA isoforms by generating and sequencing clones representing a total of ten alternative isoforms. We developed sequencing and expression assays to specifically detect alternative *UMPS* isoforms and used these to determine that *UMPS* was recurrently disrupted by mutations and aberrant splicing in additional 5-FU resistant colorectal cancer cell lines. Detecting and measuring the amount of aberrant *UMPS* isoforms with the assays we report may prove useful in predicting clinical response to 5-FU.

Fluorouracil (5-FU)



5-FU is a uracil analog classified as an anti-metabolite. 5-FU was developed in the 1950's and is now administered to ~2 million cancer patients per year worldwide (Rich et al. 2004). 5-FU, like uracil is preferentially utilized by actively dividing cells. In the treatment of colorectal cancer it is generally administered as an adjuvant therapy and often used in combination with other drugs, such as Cisplatin, Oxaliplatin, Leucovorin, and Irinotecan.

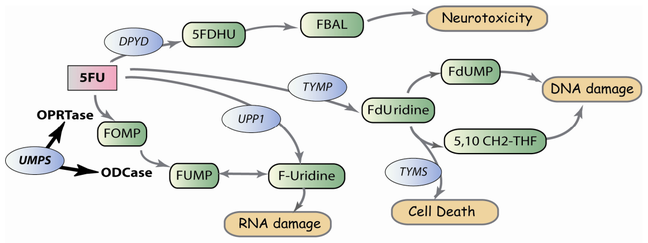
5-FU is also known as: Adrucil; Efudex; Carac; Fluoroplex; 5-fluoro-1H-pyrimidine-2,4-dione.

2. Introduction

The drug 5-FU and its analogs, pro-drugs and oral versions, particularly Capecitabine are widely used in the treatment of colorectal and other cancers. Despite their wide usage, primary or acquired resistance to 5-FU, is a major limiting factor in the treatment of cancer. Response rates for 5-FU vary from 6% to 53% depending on dose, schedule and combination with modifiers (Bleiberg et al. 2002). While 5-FU is now relatively inexpensive, new versions with improved response rates can cost thousands of dollars per month of therapy. For example, in the last fiscal year, the BC Cancer Agency regional cancer centres treated 1,871 cancer patients (primarily gastrointestinal and breast) using chemotherapy protocols involving 5-FU and/or Capecitabine at a total cost of 1.33 million dollars. Due to the high usage and low-to-moderate response rates for 5-FU, the cost associated with 5-FU resistance is considerable. Developing methods to predict and ultimately overcome this resistance is therefore an important area of research. Although the precise mechanism of action is still a subject of debate in the literature, 5-FU is thought to act by at least three mechanisms, each requiring metabolic activation of 5-FU (**Figure 1**). Varying response to 5-FU is thought to be mediated primarily by differences in the metabolic pathways of 5-FU activation and degradation (Pinedo et al. 1988).

A number of genes including *uridine monophosphate synthetase* (*UMPS* aka *OPRT*), *dihydropyrimidine dehydrogenase* (*DPYD* aka *DPD*), *thymidine phosphorylase* (*TYMP* aka *TP*), *uridine phosphorylase 1* (*UPP1* aka *UP*), *thymidine synthetase* (*TYMS)* and others are known to be involved in the metabolism of 5-FU (**Figure 1**). Mutations which alter the expression level or structure of these genes and affect the activity of the enzymes they encode may contribute to 5-FU resistance and poor clinical outcome in some patients. One of the most studied genes related to 5-FU action is *DPYD,* for which a clinical test for 5-FU toxicity exists (Myriad Genetics Inc.; DNAVision SA; and GenPath Diagnostics Inc.). *DPYD* deficiency is associated with increased probability of severe adverse response to 5-FU including multi-organ toxicity, especially neurotoxicity (Han et al. 2008). Drugs such as S-1, Tegafur-Uracil (UFT) and Eniluracil have been developed to improve response to 5-FU primarily by inhibiting *DPYD* and thereby reducing catabolism of 5-FU in the liver and increasing the amount of drug reaching the tumor. Although *DPYD* is believed to account for a large percentage (as much as 80%) of the catabolism of 5-FU in the liver (Allegra et al. 1999), expression of additional 5-FU metabolism genes (including *UMPS*) within the tumor is required for conversion of 5-FU to active anti-metabolites (Maring et al. 2005).

Figure 1. Simplified 5-FU metabolism pathway*



*Pathway is a modification of that reported by PharmGKB

UMPS enzymatic domains
OPRTase (EC 2.4.2.10): orotate + 5-phospho-a-D-ribose 1-diphosphate = orotidine 5'-phosphate + diphosphate
ODCase (EC 4.1.1.23): orotidine 5'-phosphate = UMP (uridine 5'-phophate) + CO2

Recent studies have emphasized the potential role for *UMPS* in mediating 5-FU resistance and proposed that this gene may serve as a clinical biomarker of resistance in cancer patients, but there is considerable disagreement in the literature as to the relative importance of genes predicted to be involved in 5-FU action. Determining those genes which are critical to this process and identifying gene variants that may confer drug resistance can be accomplished by genome-wide analyses which compare drug-sensitive and resistant cell populations. The use of genomic methods to identify the molecular causes of disease phenotypes is an area of rapid development (Griffith and Marra, 2007). We recently developed two novel genome-wide approaches to profile alternatively processed transcripts. The first method uses splicing microarrays for 'alternative expression analysis' ('ALEXA') (Griffith et al. 2008) and the second uses an Illumina DNA sequencer for massively parallel transcriptome shotgun sequencing (manuscript in preparation). We applied these methods to the study of 5-FU resistance in colorectal cancer (CRC) cell lines and observed reciprocal differential expression of *UMPS* isoforms in CRC cells with acquired 5-FU resistance relative to the sensitive cells from which they were derived. We then conducted an RT-PCR and sequencing validation of this event in additional cell lines, determined the underlying genomic mechanism, and catalogued the diversity of *UMPS* transcript variation

Uridine monophosphate synthetase (UMPS)

UMPS, also known as *orotate phosphoribosyl transferase* (*OPRT*) is a highly conserved gene involved in pyrimidine metabolism. The most commonly observed mRNA of *UMPS* consists of 6 exons (2658 bp in length) and generates a protein of 481 amino acids containing two enzymatic domains (*OPRTase* and *ODCase*) and a dimerization interface.

Since 5-FU is a pyrimidine analog, *UMPS* is believed to be involved in 5-FU metabolism. The expression level of *UMPS* has been correlated with 5-FU efficacy in cell lines (Sakomoto et al. 2007) and metastatic colorectal cancer (Ichikawa 2003). Polymorphisms of *UMPS* have been associated with toxicity to 5-FU chemotherapy.

References

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3. Results - isoform expression

We applied custom designed alternative expression analysis ('ALEXA') microarrays to profile polyA+ RNA isolated from MIP101 (5-FU sensitive) and MIP/5FU (5-FU resistant) colorectal cancer cells (Griffith et al. 2008). Analysis of these data revealed an apparent reciprocal differential expression of two isoforms of *UMPS*, one containing six exons and the other skipping exon 2 (see **Figure 2A-D**). We subsequently observed the same differential expression of alternative isoforms by massively parallel transcriptome shotgun sequence analysis of the same polyA+ RNAs (**Figure 2E**). RT-PCR was used to profile the expression of *UMPS* isoform A and B in three 5-FU sensitive/resistant cell line pairs. Resistant derivatives of MIP101, RKO and HCT116 (MIP/5FU, RKO/5FU and HCT/5FU respectively) were created as previously described (Tai et al. 2005). *UMPS* Isoform A was expressed in all six cell lines. Both MIP/5FU and HCT/5FU exhibited an over-expression of *UMPS* Isoform B relative to the sensitive cell line from which they were derived (**Figure 3**). Semi-quantitative PCR indicated a significant decrease in Isoform A in MIP/5FU cells relative to MIP101 and a significant increase of Isoform B in both MIP/5FU and HCT/5FU cells (**Table 1**).

Figure 2 A-D. Differential expression of alternative *UMPS* isoforms in 5-FU sensitive and resistant cells

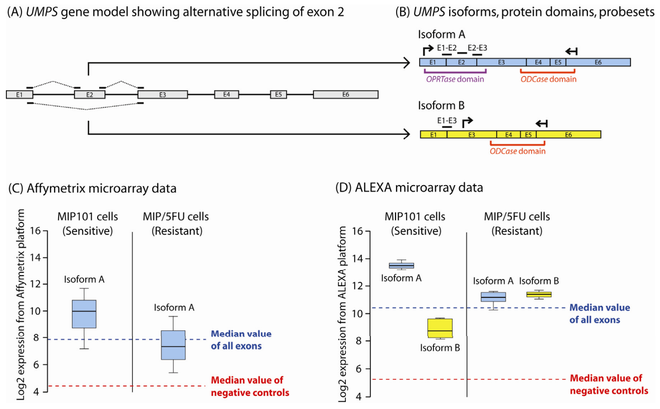


Figure 2-E. Whole transcriptome shotgun sequence data corresponding to the *UMPS* locus

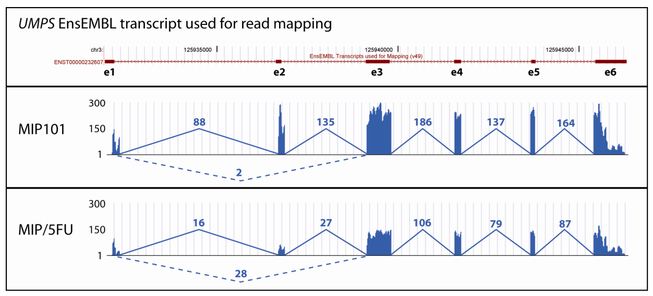


Figure 3. RT-PCR detection of *UMPS* isoforms A and B in six 5-FU sensitive or resistant CRC cell lines

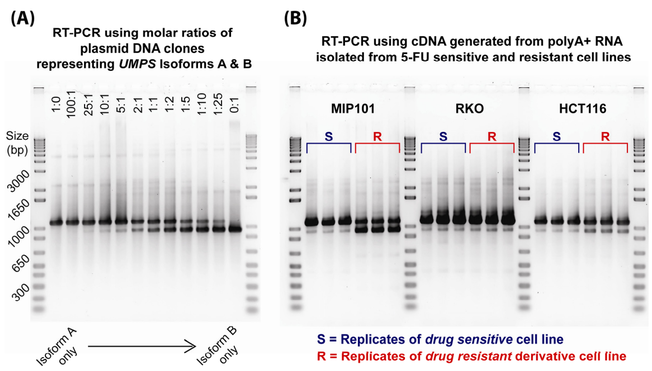


Table 1. Semi-quantitative RT-PCR validation of *UMPS* isoform A and B expression in 5-FU sensitive & resistant cell lines

Cell Line Comparison	Isoform A fold change	Isoform B fold change
MIP101 (sensitive) minus MIP/5FU (resistant)	-4.92 ± 1.40 †	23.45 ± 1.05 †
RKO (sensitive) minus RKO/5FU (resistant)	1.14 ± 1.23	1.34 ± 1.29
HCT116 (sensitive) minus HCT/5FU (resistant)	-1.24 ± 1.20	6.27 ± 1.40 †

4. Results – isoform sequence and structure

Although the observed reciprocal differential expression of *UMPS* isoforms A and B appeared to be the result of a change in splicing patterns we sought to confirm the underlying mechanism. We hypothesized that one or more mutations were acquired in the *UMPS* locus during the selection of 5-FU resistance and these were responsible for the altered splicing pattern. To investigate this hypothesis, we sequenced genomic DNA extracted from each of the 5-FU sensitive/resistant cell line pairs (**Figure 4**).

Approximately 300 *UMPS* cDNA clones were generated by RT-PCR of polyA+ RNA using an oligo-dT primer for ss-cDNA synthesis followed by amplification with primers designed to flank the *UMPS* ORF. We successfully generated clones representing both the 'canonical' *UMPS* isoform A, the predicted *UMPS* isoform B as well as several other novel isoforms. Represented within our clone collection are a total of 10 distinct spliced *UMPS* isoforms (denoted as A-J in **Figure 5**).

Figure 4. Graphical overview of SNPs and mutations found by genomic sequencing

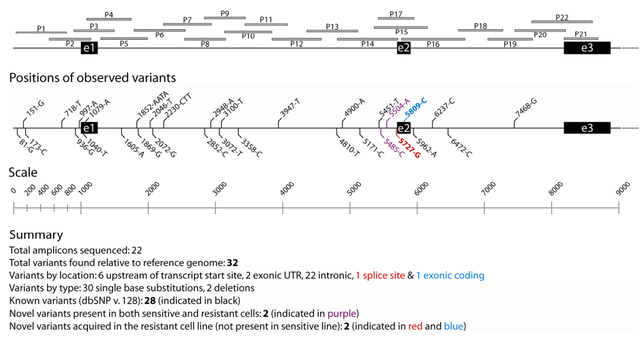
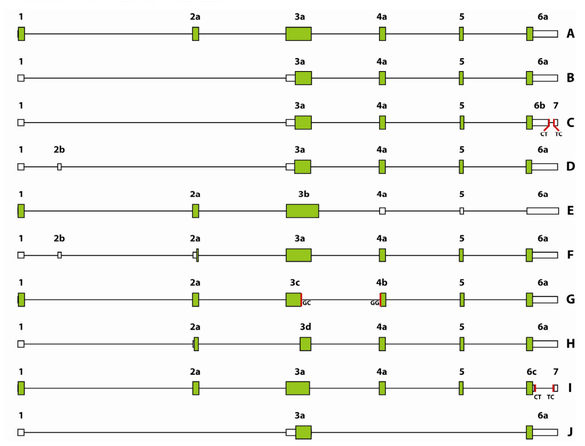
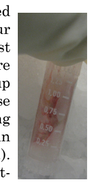


Figure 5. Cloning and sequencing of alternative *UMPS* isoforms from six colorectal cancer cell lines



5. Clinical validation

Twenty six fresh frozen colorectal tumor samples with matched adjacent normal samples were obtained from the Ontario Tumour Bank. To be included in our retrospective cohort, the patient must have received adjuvant or neoadjuvant 5-FU. Nine 'responders' were defined as patients with no disease progression reported (follow-up was 5-22 months). Fifteen 'non-responders' were defined as those patients whose clinical records noted at least one of the following criteria: local or distant recurrence; disease progression resulting in death; adverse drug response (e.g. neutropenia, neuropathy, etc.). Analysis of these samples and requests for additional pre- and post-treatment samples is underway.



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