

METABOLOMICS AND PHARMACOGENETICS BASED 5-FLUOROURACIL MONITORING IN COLORECTAL CANCER PATIENTS

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ABSTRACT

Objective: To provide quick and accurate clinical diagnostic tools those are currently not available leading to improper management of colorectal cancer (CRC) patients.

Methods: The metabolomic profiles of 10 CRC patients treated with 5-fluorouracil and 24 healthy volunteers were analysed. The subjects were genotyped for *UGT1A1*28*, *DPYD 1896 T>C* and *DPYD*5*.

Results: Our results show alterations in the metabolism of bile acid, glycolysis and fatty acid in patients. The distinctive metabolite profiles established using PLSDA identify several biomarkers for diagnostic use in clinical settings. The predictive PLSDA model revealed 100% accuracy of metabolites differentiating CRC patients and healthy volunteers. In addition, the metabolic profiles associated with different genotypes of *DPYD* and *UGT1A1* explains the impact of genetic variation on differential drug responses.

Conclusion: Pharmacogenetics and metabolomics profiles are potential platforms for more comprehensive monitoring of patient's disease progress and drug response. Further study is however needed to validate the use of biomarkers identified.

Keywords: Metabolomics, Pharmacogenetics, Polymorphism, Colorectal cancer (CRC), 5-fluorouracil (5-FU).

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant diseases among men and women in Malaysia as well as other developed countries [1]. Generally, surgery is the first line of treatment for patients with colorectal cancer especially if the tumour is at its early stage [2]. However, patients with advanced stages who fail to recover from surgery or have recurrences; chemotherapy or adjuvant radiotherapy is used. 5-fluorouracil (5-FU) is the standard treatment for colorectal cancer and various other types of cancers including breast, head and neck [3]. The use of 5-FU is often complicated with serious toxicity due to high-grade haematological and non-haematological complications such as neutropenia, thrombocytopenia, diarrhoea, hand-foot syndrome, and stomatitis [4]. Large inter-individual differences in patient's clinical outcomes following 5-FU treatment is the major clinical problem facing the clinicians [5].

Assessment of 5-FU responses among different patients is complicated. One of the variables is the highly polymorphic dihydropyrimidinedehydrogenase (DPD) enzyme which is responsible for more than 80% of the elimination of 5-FU (Johnson and Diasio 2004) [6]. Single nucleotide polymorphisms (SNPs) located in the key regions of dihydropyrimidine dehydrogenase (DPD) enzyme have been correlated with low levels of DPD activity and predispose patients to toxicity [7]. In addition, high bilirubin levels have been associated with high risk of cancer recurrence or non-responsiveness to 5-FU as it causes resistance to apoptosis [8]. Metabolism of bilirubin is held responsible by the polymorphic UDP glucuronosyltransferase 1A1 (*UGT1A1*); therefore increased of bilirubin levels due to reduced *UGT1A1* enzyme activity has been reported. *UGT1A1*28* which is the most prevalent genetic variants was reported to cause approximately 70% reduction of UDP glucuronosyltransferase 1A1 activity [9-10]. We therefore genotype the subjects with respect to polymorphism of *DPYD* and *UGT1A1*28* and study the differential expression of metabolites based on phenotypes predicted by genotypes. Quick and accurate clinical diagnostic tools are currently not available to monitor effectiveness of therapy. Systematic and comprehensive approaches are therefore desired. Pharmacogenetics hold promises in personalized medicine by giving accurate dose based on the genetic backgrounds. As the observed clinical outcomes of patients depends not only on the genetic factors but also on

environmental interaction, we believe that integration of multiple platforms would provide the clinician a holistic approach in understanding patient's clinical status. We therefore report here the findings of pharmacogenetics and metabolomics analysis performed in a cohort of colorectal cancer patients in a local hospital. This integrated approach is believed to provide the clinician with better understanding about the patients' clinical status and hopefully to achieve better quality of patients care and outcomes.

MATERIALS AND METHODS

Materials

All solvents and chemicals were of high performance liquid chromatography (HPLC) grade except where otherwise stated. Acetonitrile and methanol were obtained from Merck (KGaA, Darmstadt, Germany). Ultra-pure (18 Ω m Mili-Q) distilled water (Millipore, MA, USA) was used. The mobile phase for denaturing high performance liquid chromatography (dHPLC) consists of i. 100 mM triethylammonium acetate (TEAA), 0.1 mM EDTA (Helix Buffer Packs) and ii. 100 mM triethylammonium acetate (TEAA), 0.1 mM EDTA with 25% (v/v) acetonitrile (Helix Buffer Packs).

Subjects

The study was approved by the Human Research Ethics Committee of Universiti Teknologi MARA and Research Ethics Committee of the Malaysian Ministry of Health (NMRR-09-29-3303). A total of 10 colorectal cancer patients treated with 5-fluorouracil (CRC-5-FU) chemotherapy (FOLFOX regimen) and 24 healthy volunteers were included in the metabolomic analysis.

The demographic of the subjects are presented in Table1. The subjects were requested to fast for 10 hours before blood sampling the next day.

Collection of blood samples

Ten (10) millilitres of blood were collected by venepuncture of the antecubital veins. Five millilitres were used for DNA extraction. The remaining was transferred to another tube and left to coagulate for 30 min, followed by centrifugation at 3500 g for 15 min. The sera were then stored immediately at -80°C for metabolomics analysis.

Table 1: Demographic data

	Genotyping Patients (N = 26)	Metabolomics Patients (N = 10)	Genotyping+Metab Healthy controls (N= 24)
Age (years)			
Median + SD	58 + 9.4	58 + 7.6	19 +3.6
Range	33 to 70	47 to 70	18 to 30
Gender, n			
Male	10	10	8
Female	16	0	16
Body surface area (m²)			
Mean + SD	1.59 + 0.18	1.55 + 0.21	-
Range	1.26 to 2.1	1.26 to 2.1	-
Weight (kg)			
Mean + SD	57.7 + 11.7	59.1 + 12.6	56.5 + 11.7
Range	39 to 90	39 to 90	43 to 90
Body mass index (BMI)			
Mean + SD	22.8 + 4.0	23.1 + 4.3	23.7 + 2.6
Range	15.9 to 31.1	15.9 to 31.1	19.6 to 28.5
Disease status, n (%)			
Metastasis (M1)	17 (65.3%)	4 (40%)	-
Non-metastasis (M0)	9 (34.6%)	6 (60%)	-
Toxicity, n (%)			
Neutropenia	11 (42.3%)	7 (70%)	-
Non neutropenia	15 (57.7%)	3 (30%)	-

Sample preparation for genotyping of *DPYD*5* (rs1801159), *DPYD 1896 T>C* (rs17376848) and *UGT1A1*28* (rs8175347)

Genomic DNA was extracted using alkaline lysis method as described previously [11]. Genotyping was performed using allele specific polymerase chain reaction (ASPCR) methods for *DPYD*5* (rs1801159) and *DPYD 1896 T>C* (rs17376848). On the other hand, detection of *UGT1A1*28* (rs8175347) were done using denaturing high performance liquid chromatography (dHPLC). Samples were selected randomly for validation using direct sequencing method [12].

Work flow for metabolomics study

Equal volume of distilled water (pH 10) was added into 100 µL of serum sample and vortexed briefly. Protein precipitation was performed by adding 200 µL of cold acetonitrile. Sample was vortexed briefly (10 seconds) and centrifuged (10,350 g, 10 min at 4°C). The supernatant was transferred to a 1.5 ml microcentrifuge tube and the steps were repeated 3 times. The supernatant was dried using Vacuum Concentrator (5301, Brinkmann, Eppendorf). Then, the samples were reconstituted with 50 µL of mobile phase. Two (2) µL of the samples were injected to LC/MS. The workflow for metabolomic study to generate a predictive model is summarized in Figure 1

Sample analysis was performed on Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS (Agilent Technologies, Santa Clara, CA, USA). The column used was a Zorbax Eclipse Plus C18 with column ID of 1.8 µm (2.1 x 100 mm) and was maintained at 40°C during the run. The mobile phase (A) consisted of 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile and was run at flow rate of 0.25 mL/min. A linear gradient was developed over 36 minutes from 5% to 95% of mobile phase (B). Total run time was 48 minutes for each analysis. ESI source settings were as follows: V Cap 4000 V, skimmer 65 V and fragmentor 125 V. The nebulizer was set at 45 psig and the nitrogen drying gas was set at a flow rate of 12 L/min. Drying gas temperature was maintained at 350°C. Calibration of the mass spectrometry was performed before each batch of sample analysis to ensure accurate mass detection. The mass spectrometer was tuned to allow detection of compounds to accuracy of ± 2 ppm. Internal reference ions (m/z121.0509 and 922.0098) were injected to correct mass throughout the sample analysis (Figure 2).

The data was processed by Agilent MassHunter Qualitative Analysis software and Mass Profiler Professionals (Agilent Technologies, Santa Clara, CA, USA).

These steps include molecular feature extraction, background subtraction, data filtering, statistical analysis by ANOVA and PCA,

followed by database searching (Figure 1). Identification of endogenous and exogenous metabolites was done using metabolite identification software, METLIN Personal Metabolite Database (Agilent Technologies, Santa Clara, CA, USA).

The algorithm used groups the ions into one or more features (compounds) that have related m/z values. Noise was removed by using absolute height parameter which was set at 200. Filtering by Analysis of Variance (ANOVA) and fold change analysis were the next steps. Fold change of 2 and above was used as well. The metabolites were analyzed by Principal Component Analysis (PCA) and Partial Least Square Discrimination analysis (PLSDA).

Statistical analysis and visualization

For genotyping analysis, data is presented as genotype and allele frequencies with the 95% confidence interval [$p + \sqrt{1.96 p (1-p)/n}$]. Allele frequencies of *UGT1A1*28* (rs8175347), *DPYD 1896 T>C* (rs17376848) and *DPYD*5* (rs1801159) with their respective Hardy Weinberg Equilibriums were calculated. For metabolomics analysis, un-paired T-test and Analysis of Variance (ANOVA) with Benjamini-Hochberg multiples testing correction were used.

RESULTS

Differential metabolites profiles of colorectal cancer patients treated with 5-fluorouracil (CRC-5-FU) and healthy volunteers

Analysis of the metabolites showed distinctive separation between the patients and healthy volunteers. Entities were filtered based on their ANOVA *p* values 0.01 and fold change cut-off of 2. The predictive model with PLSDA revealed 100% accuracy of this training set (Figure 3).

The differential metabolite profiles were investigated based on cancer versus non cancer state where age plays a minimum role. Therefore, the healthy volunteers were not age-matched. Several classes of metabolites that were shown to be down-regulated in CRC-5-FU patients compared to healthy volunteers include dicarboxylic acid (DA), polyunsaturated fatty acids (PUFA), amino acids and their conjugates. Conversely, some metabolites were found to be up-regulated in CRC-5-FU such as uremic toxins, bilirubin, fatty acids, bile acid and its conjugates. In subsequent hierarchy clustering analysis, 49 metabolites were differentially expressed in the healthy volunteers and CRC-5-FU group (Figure 4). All metabolites were classified according to their class of compounds based on information in various metabolomic database including Lipidmaps (<http://www.lipidmaps.org>), Human Metabolome Database (<http://www.hmdb.ca>) [13] and ChEMB (<http://www.ebi.ac.uk>).

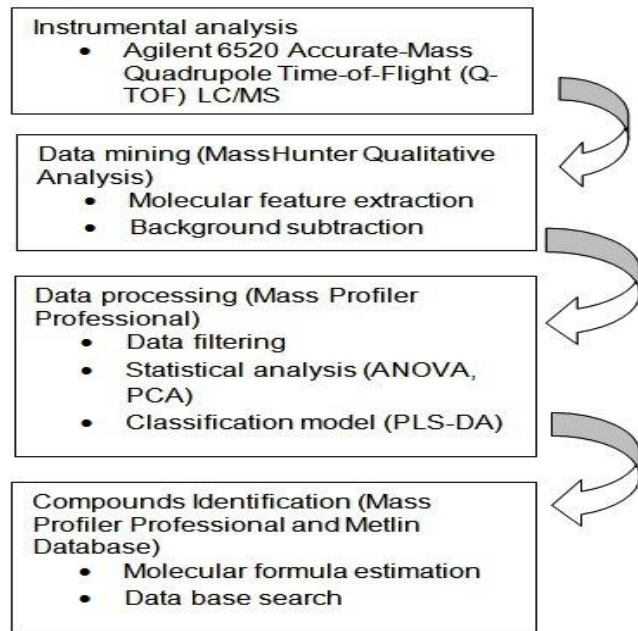


Fig. 1: Workflow for metabolomic study to generate a predictive model.

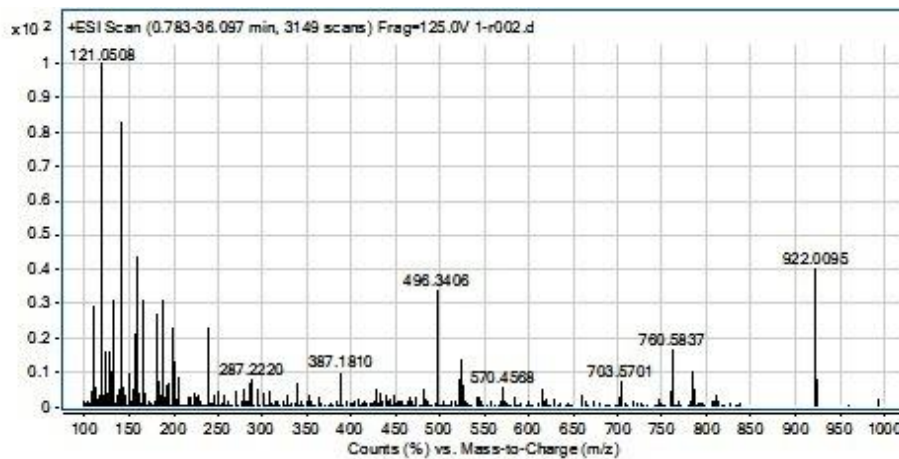


Fig. 2: ESI spectrum shows Internal reference ions (m/z121.0509 and 922.0098) used to correct mass accuracy.

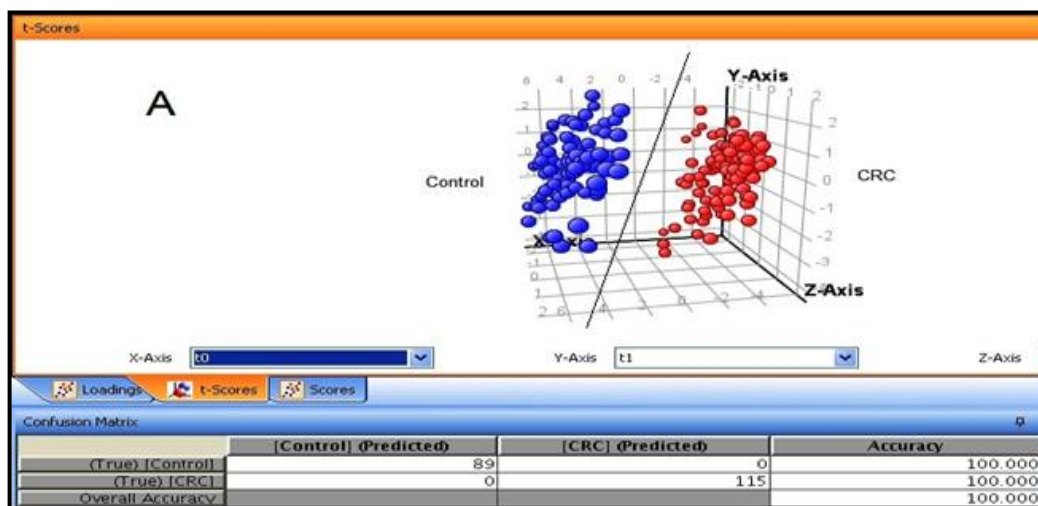


Fig. 3: PLS-DA Analysis and training set showing clustering and predictive accuracy.

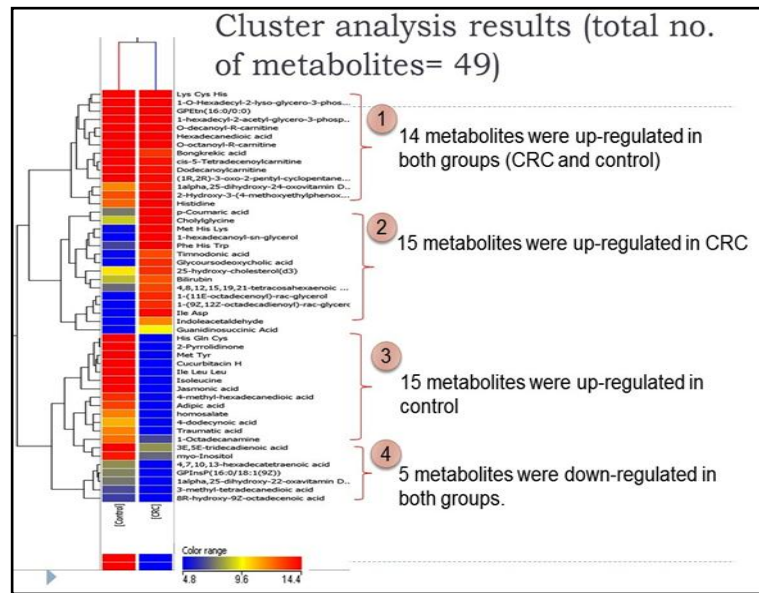


Fig. 4: Dendrogram tree shows 49 differentially expressed metabolites in healthy controls and CRC-5-FU groups.

Metabolites profiling based on genotypes of DPYD*5 (rs1801159), DPYD 1896 T>C (rs17376848) and UGT1A1*28 (rs8175347)

DPD deficient patients with DPYD*5 (rs1801159) variants showed low abundance of acyl carnitine metabolites compared to patients with normal DPD. These metabolites are known as O-decanoyl-R-carnitine, palmitoylcarnitine and cis-5-tetradecenoylcarnitine.

Inosine was significantly higher in patients with DPYD 1896 T>C (rs17376848) variant as compared to homozygous wild-type. On the other hand, L-urobilin (a by-product of bilirubin) was shown to be highly abundant in patients with homozygous mutant UGT1A1*28 (rs8175347).

A list of metabolites profile between different genotypes is summarized in Table 2 (i) and (ii).

Stage of cancer (metastasis versus non-metastasis)

PCA analysis and Unpaired t-test analysis (p <0.05) showed differently regulated metabolite profiles between stages. 32 entities

were filtered which satisfied the corrected p-value of 0.02 and 2 fold changes. Unpaired t-test (FC >=2.0) performed on [Stage II] Vs [Stage III] revealed that only 2 metabolites were differentially expressed; while 8 metabolites were different for [Stage II] Vs [Stage IV] (FC >=2.0). PLSDA showed that the overall predictive accuracies of these metabolites sets are 64.28% and 100% for [Stage II] Vs [Stage III] and [Stage II] Vs [Stage IV] respectively. The low predictive capability in differentiating [Stage II] Vs [Stage III] could be due to high similarity of the metabolite profiles in patients at stage II and stage III cancer. However, stage II patient can be differentiated from stage IV using the model. Metabolites which are differently expressed according to TNM staging are listed in table 2 (iii).

Metabolic profiles of patients with toxicity versus no toxicity: Neutropenia ; A total of 6 metabolites were found to be associated with the occurrence of neutropenia among patients treated with 5-FU. These include several carnitine metabolites which were found to be higher in neutropenic patients as compared to non-neutropenic group [Table 2 (iiii)].

Table 2: List of metabolites in CRC-5-FU patients

(i) Group	DPYD variant (Normal DPD versus deficient DPD)		p-value	Regulation	
	Metabolites	Description		Normal	Deficient
DPYD 1896 T>C	Inosine	It is an intermediate in the degradation of purines and purine nucleosides to uric acid.	0.002	Down	Up
	2-methyl-tridecanedioic acid	Dicarboxylic acids.	0.002	Down	Up
	Tyr Glu	Dipeptide.	0.002	Down	Up
	Sphingosine-1-phosphate	Potent bioactive actions in the sphingolipid metabolism, calcium signaling pathway and neuroactive ligand-receptor interaction.	0.000	Up	Down
DPYD*5	4,8,12,15,19,21-tetracosahexaenoic acid	Unsaturated fatty acids.	0.000	Down	Up
	Sphingosine	Component of glycosphingolipid metabolism.	0.000	Down	Up
	1-(9Z,12Z-octadecadienyl)-rac-glycerol	Energy source and membrane component.	0.000	Down	Up
	2-methyl-tridecanedioic acid	Dicarboxylic acids.	0.000	Down	Up
	cis-5-tetradecenoylcarnitine	A metabolic marker of very long chain acyl-dehydrogenase deficiency.	0.006	Up	Down
	Hexadecanedioic acid	A dicarboxylic acids which has an antitumour activity.	0.016	Down	Up
	O-decanoyl-R-carnitine (HMDB00791)	Detected in medium-chain acyl-CoA dehydrogenase deficiency.	0.000	Up	Down
Palmitoylcarnitine	Facilitates the transfer of long-chain fatty acids from cytoplasm into mitochondria during the oxidation of fatty acids.	0.016	Down	Up	

(ii) UGT1A1*28 (Homozygous wild-type versus homozygous mutant)					
Group	Metabolite	Description	p-value	Regulation Wild-type	Mutant
UGT1A1*28	L-urobilin	A by-product of bilirubin degradation.	0.010	Down	Up
	O-octanoyl-R-carnitine	Detected in medium-chain acyl-CoA dehydrogenase (MCAD) deficiency..	0.001	Down	Up
	Threonate	Short chain hydroxyl acids derived from glycated proteins or from degradation of ascorbic acid.	0.009	Up	Down
(iii) Disease status (Metastasis versus non-metastasis)					
Group	Metabolites	Description	p-value	Regulation Metastasis	Non-metastasis
Progression of cancer	24-Nor-5beta-cholane-3alpha,7alpha,12alpha,23-tetrol	Bile acid and derivatives.	0.000	Down	Up
	β-eleostearic acid	Role in growth inhibition and apoptosis.	0.000	Down	Up
	Eicosapentaenoic acid (EPA)		0.000	Down	Up
	N-Acetylsphingosine	Ceramide-induced apoptosis.	0.000	Down	Up
(iv) Toxicity (Neutropenia versus non-neutropenia)					
Group	Metabolites	Description	p-value	Regulation Neutropenia	Non-neutropenia
Toxicity	4,8,12,15,19,21-tetracosahexaenoic acid	Fatty acids.	0.000	Down	Up
	Val Gly	Dipeptide.	0.000	Down	Up
	17-phenyl-trinor-PGF2alpha isopropyl ester	Stimulate neutrophil chemotaxis.	0.006	Down	Up
	Stearoylcarnitine	Lipid catabolism, fatty acid transport and energy production.	0.000	Up	Down
	Palmitoylcarnitine	Facilitates the transfer of long-chain fatty acids from cytoplasm into mitochondria during the oxidation of fatty acids.	0.001	Up	Down
	Ile CysAsn	Tripeptide.	0.000	Up	Down

Statistical test for each group:

- (i) Un-paired T-test; *p*-value less than 0.01 is regarded as significant
- (ii) Un-paired T-test; *p*-value less than 0.05 is regarded as significant.
- (iii) Un-paired T-test; *p*-value less than 0.05 is regarded as significant.
- (iv) Un-paired T-test; *p*-value less than 0.05 is regarded as significant.

DISCUSSION

Differential metabolites profiles of (CRC-5-FU) treated patients and healthy volunteers

Metabolic alterations in chronic diseases such as cancer and bowel obstruction have been associated with poor nutritional status and cachexia [14]. In this study, we observed a significantly lower myo-inositol (a sugar alcohol) in CRC-5-FU patients. This finding is in accordance with "Warburg hypothesis" that proposes high consumption of glucose during proliferation of cancer cells. This led to increased production of lactic acid via glycolysis pathway and subsequent depletion of glucose from the surrounding tissues as reported by previous study [15]. A study by Reeves and Cammarata showed that low levels of myo-inositol cause accumulation of polyol (an oxidative stress marker) which indicates cellular oxidative stress that may lead to cell injury, inflammation and eventually, cancers [16].

In addition to myo-inositol, some metabolites involved in muscle metabolism and gluconeogenesis were found to be down-regulated in CRC-5-FU patients compared to healthy volunteers. Significantly lower isoleucine levels in CRC-5-FU patients was observed indicating impairment in branched-chain amino acid (BCAA) pathways which could be due to cytotoxic effects of 5-FU. 5-FU is known to inhibit the phosphorylation of thiamine pyrophosphate (TPP) that is required for oxidative decarboxylation of branched-chain amino acids (BCAA). Depletion of acylcarnitine metabolites was a secondary effect of low abundance of isoleucine and other amino acids that are involved in branched-chain amino acid (BCAA) pathway [17]. Therefore, acylcarnitines (cis-5-tetradecenoylcarnitine,

docosanoylcarnitine, O-octanoyl-R-carnitine), isoleucine and myo-inositol could be important in assessing patients' clinical status, progress of the cancer and 5-FU induced adverse effects.

Conversely, guanidinosuccinic acid (GSA), a metabolite of uremic toxins was detected in higher abundance among the CRC-5-FU patients. Increased production of GSA could be due to an increased oxidation of argininosuccinic acid (ASA) by free radicals [18]. Hepatic accumulation of hydroxyl radicals could be the result of the inhibition of nitric oxide (NO) formation by 5-FU as previously reported by Jung et al., [19]. Presence of GSA would interfere with the transmethylation pathway whereby homocysteine would be converted to cystathionine through the transsulfuration pathway instead of methionine [20]. An increased cystathionine level may promote oxidative damage, inflammation and endothelial dysfunction and vascular damage. Therefore, GSA could be another good biomarker for the monitoring of adverse effects of 5-FU.

Similarly, various free fatty acids (phosphocholine, glycerophosphocholine) and glycine-conjugated bile acids were observed to be higher in CRC-5-FU patients compared to healthy volunteers. Phosphocholine and other choline related metabolites were closely linked to the activation of inflammatory pathways [15]; while glycine-conjugated bile acids have been reported to be risk factors for colorectal cancer as they induce reactive oxygen species (ROS) in the synaptosomal membrane system [22].

One study reported that a total of 20 colon cancer patients who were diagnosed with liver metastases and severe bilirubinemia had poor survival rates [23]. Therefore, higher abundance of bilirubin in CRC-5-FU patients compared to the volunteers may indicate poor

response towards chemotherapy. On the other hand, cucurbitacins was found to be low in CRC-5-FU compared to volunteers. This compound has been reported to affect the binding of albumin and bilirubin. The albumin-bilirubin complex may in turn inhibit lipid peroxidation and subsequently protect cells from damage [24].

Metabolites profiling based on genotypes of *DPYD*5* (rs1801159), *DPYD 1896 T>C* (rs17376848) and *UGT1A1*28* (rs8175347)

Cachexia is an incidence of progressive weight loss as well as deterioration of skeletal muscle and adipose tissue that occurs due to poor nutritional status in chronic diseases. Studies had demonstrated that cancer patients with low levels of acylcarnitines are cachexic [25]. In this study, DPD deficient patients with *DPYD*5* variants showed low abundance of acylcarnitine metabolites (O-decanoyl-R-carnitine, palmitoylcarnitine and *cis*-5-tetradecenoylcarnitine) compared to normal DPD. Low levels of acylcarnitine metabolites in these patients could be due to diarrhoea or diuresis that were induced by 5-FU.

Inosine was significantly higher in patients with *DPYD 1896 T>C* variant as compared to homozygous wild-type. This may provide clues for the mechanism of serious adverse effects or 5-FU resistance in patients with genotype of *DPYD 1896 T>C*, indicating a

relationship between the variant and serious 5-FU- adverse effects or resistance. As reported by Yamamoto et al., [26], inosine inhibits uridine uptake in nucleoside transport pathway resulting in the uridine being retained and accumulated in the plasma [38]. Normally, in purine nucleoside pathway, inosine is converted to hypoxanthine and in the process, produces ribose 1-phosphate (R1-P) as an intermediate, which is a key intermediate metabolite in the uracil salvage pathway. It acts as phosphate donor for uracil to be converted to uridine and subsequently transformed to uracil nucleotides. Since uracil shared the same metabolism pathway with 5-FU, the utilization of R1-P in uracil salvage pathway is the same as activation of 5-FU via its anabolism pathway. Therefore, more 5FUrd and 5-FUTP, the activated 5FU will be made available for its cytotoxic effects (Figure 5). The involvement of inosine in uracil salvage pathway should be taken into consideration when inosine is administered as a supplement or for therapeutic purposes especially in DPD deficient patients where the risk of 5-FU related toxicity is high. Thus patients having this genotype with high inosine levels should be monitored closely for 5FU adverse effects. A long period of trial and error in search for optimum therapy can be avoided and better patient's quality of life can be achieved. This study highlights the importance of randomised studies of nutritional intervention in these patients as appropriate nutrients in correct amount may be recommended to the patients.

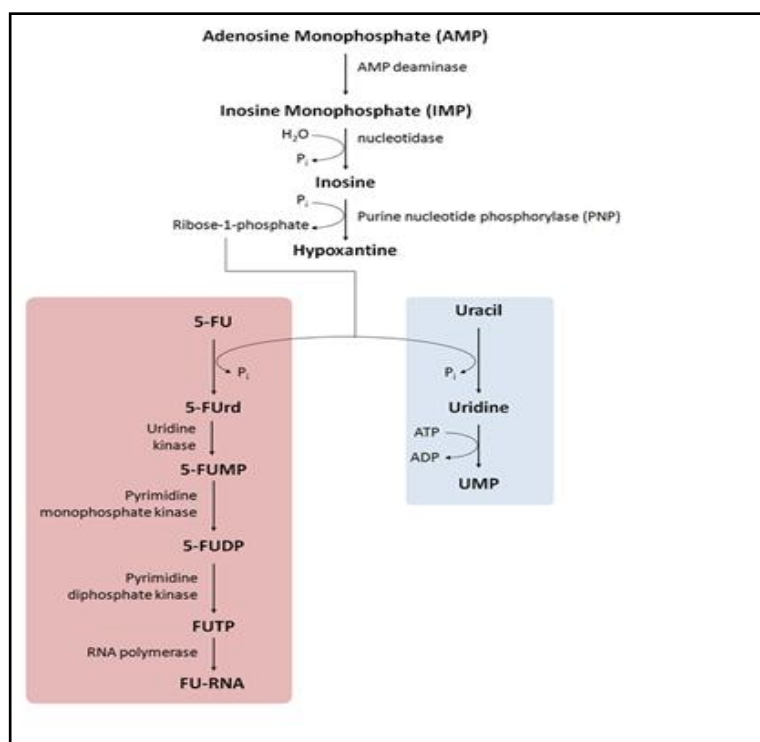


Fig. 5: Utilization of R1-P in uracil salvage pathway and anabolism pathway of 5-FU.

On the other hand, *L*-urobilin (a by-product of bilirubin) was shown to be highly abundant in patients with homozygous mutation of *UGT1A1*28*. This result is in accordance with other studies reporting that *UGT1A1*28* leads to reduction of glucuronidation activity in the liver and causes an increase of bilirubin in patients [8]. High levels of bilirubin posed an interest in this study as the risk of recurrence or non-responsiveness to the chemotherapy was increased as bilirubin has been associated with apoptosis resistance [27]. Currently, patients' bilirubin levels are not routinely monitored. We therefore recommend monitoring of bilirubin levels especially in patients who carry *UGT1A1*28* variant.

Stage of cancer (metastasis versus non-metastasis)

Results of this study show low abundance of β -eleostearic, eicosapentaenoic acid (EPA) and N-acetylsphingosine in the M1 group in comparison to M0. β -eleostearic may affect the progression

of cancer and this was supported by previous studies which showed its inhibitory effects on the growth of tumour cells *in vitro* [28]. It is therefore a biomarker that can be used to provide clues to the metastasis state of patients.

Patients with metastasis were also discovered to have low abundance of eicosapentaenoic acid (EPA), a metabolite serving as an anti-inflammatory and anti-cancer agent. The increase of EPA levels inhibits pro-inflammatory metabolites including leukotrienes (LTs), prostaglandins (PGs) and cytokines from inflammatory cells [29]. EPA has been shown to inhibit the proliferation as well as metastasis of breast cancer cells. Jordan and Stein [30] reported that EPA improves cytotoxic effect of 5-FU and other cytotoxic drugs in various cancer cell lines including colon. This suggests that EPA could influence the prognosis of the disease and outcome of the chemotherapy.

A third metabolite, N-acetylsphingosine was noted to be of low concentration in metastasis patients as well. It could be linked to dysregulation of apoptosis via Bcl-2 regulation pathway. Bcl-2 is usually expressed in normal human keratinocytes (NHK) and overexpression of Bcl-2 was linked to a sign of dysplasia [31]. N-acetylsphingosine therefore has the potential to be used in monitoring progression of cancer and metastasis state of patients.

Metabolic profiles of patients with toxicity versus no toxicity: Neutropenia

Neutropenia is a sign of serious hematologic toxicity in chemotherapy. It is usually associated with life-threatening infections, dose reduction or withdrawal of chemotherapeutic agents. The observed elevated level of carnitine metabolites in patients with neutropenia can be related to a study done by Fan *et al.*, [32]. They found that carnitine plays a crucial role in the apoptosis pathway by inducing up-regulation of caspase-9 and caspase-3. Therefore, influence of carnitine levels towards neutrophils apoptosis should be further investigated in order to understand the pathophysiology of neutropenia in patients treated with 5-FU.

CONCLUSIONS

Our results confirmed that alterations of the metabolic profiles in colon carcinoma are associated with perturbation in the metabolism of bile acid, glycolysis and fatty acid. The distinctive metabolite profiles of CRC-5-FU patients and healthy volunteers established using the PLSDA predictive model provides clues of several biomarkers or patterns which can be developed for diagnostic use in clinical settings. In addition, the differential metabolic profiles associated with different genotypes of *DPYD* explain the impact of genetic variation on differential drug responses. The roles and impact of the differentially expressed metabolites discussed in this study have been supported by other studies. However, no study has looked at these metabolites together with patient's genotypes. This will provide important clinical information to clinicians and will allow aggressive treatment strategy to be undertaken.

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