https://scilett-fsg.uitm.edu.my/
Vol. 15(2) JUNE 2021

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v15i2.13810

Extracellular Metabolites Profile of Different Stages Colorectal Cancer Cell Lines

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Received: 1 Feb 2021; Accepted: 22 April 2021; Published: 15 June 2021

ABSTRACT

Metabolic footprinting involves the determination of metabolites excreted or secreted by the cells. This study aimed to identify the differential extracellular metabolites in colorectal cancer (CRC) cells for the determination of molecular changes that occur as CRC progresses. CRC cells at different stages ie; SW 1116 (stage A), HT 29 and SW 480 (stage B), HCT 15 and DLD-1 (stage C), and HCT 116 (stage D) were grown in culture. The media in which the cells were grown are subjected to metabolomics profiling using Liquid Chromatography Mass Spectrometry-Quadrupole Time of Flight (LC/MS Q-TOF). Statistical and metabolic pathway analysis was performed using Metaboanalyst software and identification of metabolites was determined by the METLIN database. A total of 27 differential extracellular metabolites were identified in CRC cells

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of different stages compared to stage A cells. Data from the Partial least squares-discriminant analysis (PLS-DA) score plot shows a clear separation between CRC cells of different stages with a few overlaps between stage B and C. Further analysis using variable importance in projection (VIP) revealed 14 differential extracellular metabolites that were most significant in differentiating CRC cells of the advanced stages from stage A which are 5-hydroxy-L-tryptophan, indoleacetaldehyde, 4,5-dimethylthiazole, 8-oxodiacetoxyscirpenol, bisnorbiotin, 5-amino-6-(5'phosphoribosylamino) uracil, glyceryl 5-hydroxydecanoate, sphinganine, 8,8-diethoxy-2,6-dimethyl-2-octanol, l-cystine, thiamine acetic acid, phytosphingosine, PE (20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), N-(2R-hydroxypentacosano-yl)-2S-amino-1,3S,4R-octadecanetriol. The different expressions of metabolites may indicate altered metabolic pathways in the more advanced CRC cells compared to stage A. This study highlights the importance of conducting both metabolomics profiling of extracellular and intracellular to generate a more complete understanding on the molecular changes that occur as CRC progresses.

Keywords: *Metabolomics, extracellular, metabolites, colorectal cancer, stages*

INTRODUCTION

Colorectal cancer (CRC) is one of the most common type of cancer in the world with continuous increasing incidence in developing countries [1-3]. In developing countries, increased in detection of CRC among the younger population (less than 50 years old) are detected probably due to improvement in screening and diagnostic tools [4]. Early detection enables more effective treatment to be given. However, the molecular changes associated with the progression of the disease is still unclear. Therefore, the use of 'Omics' technologies to unravel the molecular events leading to the advancement of the disease state has been carried out. Metabolomics, the study of the metabolite profiles, has been suggested to offer advantages over the other Omics techniques as it reflects the cell phenotype and is affected not only by the biological factors but exogenous factors as well [5].

There are three types of metabolomics analysis: metabolic profiling, metabolic fingerprinting and metabolic footprinting [6]. Metabolic profiling describes the metabolites present at the time sample is taken. Metabolic fingerprinting analyses the differences in intracellular metabolites between diseased and healthy states. Normally for metabolic fingerprinting, tissues or cells are used as samples. Metabolic footprinting determines the metabolites that are secreted or excreted. Additionally, in cell metabolomics, the nutrients consumed or failed to be taken up by cells could also be determined [7]. Thus, determining metabolic fingerprinting and footprinting enables a more complete picture of cellular metabolism and its derangement to be identified.

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Metabolic fingerprinting and metabolic footprinting have been extensively carried out in human biological samples [8-12]. However, the results from different studies showed differences in the biomarkers identified and the main metabolic pathways affected [13,14]. Similar attempts were observed in studying the molecular changes as CRC advances using human tissues yielded different biomarkers and main affected pathways [14].

Recently, we have published our findings on metabolic fingerprinting using CRC cells of different stages, classified using the Dukes criteria [15]. The use of cell metabolomics provides opportunities to determine the effect of the disease on cellular metabolism without the confounding factors [16]. In this study, we aim to determine the differential extracellular metabolites profile in CRC cells of different stages. Therefore, the untargeted metabolomics was used to characterize the extracellular metabolic profile in CRC cells of different stages from Dukes' types A, B, C and D. Moreover, from the findings, we also aim to determine the associations of the metabolic extracellular profiles with the metabolic intracellular profiles from our previous study [15]. From this study, the findings will reveal a better understanding on the metabolic changes taking place as CRC advances.

EXPERIMENTAL

Cell Culture

CRC cells SW 1116, Dukes' type A (stage A), HT 29 and SW480, Dukes' type B (stage B), HCT 15 and DLD-1, Dukes' type C (stage C), and HCT 116, Dukes' type D (stage D) were purchased from AddexBio, USA. The CRC Dukes' stages were classified by the American Type Culture Collection (ATTC) as well as other studies [17,18]. Cells were grown in DMEM, high glucose (Gibco, USA) supplemented with fetal bovine serum (Gibco, USA), 10 % and penicillin-streptomycin (Gibco, USA), 1 %. Cells were seeded for $1x10^6$ cells/mL into a 6-well culture plate and incubated overnight in a 5 % CO^2 incubator (Binder, Germany), 37 °C.

Extraction of Extracellular Metabolites

Extracellular metabolites in the culture media were extracted using extraction solvent of methanol:water (8:1, -20 °C). Briefly, after an overnight incubation, 50 µL of media in which cells were grown were mixed with 450 µL of extraction solvent. The extraction solution were centrifuged at 16 000 rpm for 5 minutes at 40 °C, and the supernatant was transferred to a microcentrifuge tube. The samples were concentrated using a concentrator, modes V-AQ (aqua

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solution), 30 °C, brake-on (Invitrogen, USA) and stored at -80 °C until further analysis. Fresh media was used and served as a blank.

Extracellular Metabolites Profiling By LC/MS Q-TOF

LC/MS Q-TOF (Agilent Technologies 6520, USA) equipment with an electrospray ionization (ESI) source was used to analyse the samples together with a 1200 Rapid Resolution system (Agilent Technologies, USA). ESI source with positive and negative ion modes were used to include all negative and positively charged compounds to enable a large number of molecules to be detected. Prior to samples injection into LC/MS Q-TOF system (Agilent Technologies 6520, USA), the dried extracted samples were dissolved with 30 µL of mobile phase acetonitrile:water (1:1) for positive mode and ammonium formate:water (1:1) for negative mode analysis and centrifuged at 10 000 rpm at 4 °C for 10 minutes. The supernatant was aliquoted and transferred to the HPLC vial for untargeted metabolomics analysis. The acquired LC/MS mass spectrometer data was processed according to previous studies [15,19]. The analysis was performed in three technical replicates and three biological replicates for each positive and negative modes.

Data Processing and Analysis

The acquired data from LC/MS was processed by MassHunter Qualitative Analysis with DA Reprocessor (Agilent Technologies, USA) and transferred into Microsoft Excel. The data of each compound presented was normalised by blank which corrected the intensity data of samples with intensity data of fresh media to eliminate if there are matrix components that could interfere with the ability of the test method to measure the analyte of interest. The METLIN Personal Metabolite database was used to identify the metabolite compounds. Statistical analysis and visualisation were performed by Metaboanalyst 4.0 [20]. One-way ANOVA was used to determine the significant difference in the abundance of the compound between all different groups ($p < 0.001$, fold change; 2). Partial least squares-discriminant analysis (PLS-DA) score plot, and variable importance in projection (VIP) score was determined. VIP score close to or greater than one can be considered important in a given model. Workflow analysis for metabolomics profiles was showed at Appendix.

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RESULTS AND DISCUSSION

Extracellular Metabolic Profile Analysis of CRC Cells of Different Stages

The extracellular metabolites in CRC cells of the different stages were determined by LC/MS-QTOF and the differential extracellular metabolites were determined by comparing the metabolic profile of each stage of CRC cells versus stage A. The findings showed that there were 27 differential extracellular metabolites were identified in CRC cells of the more advanced stages compared with stage A (Table 1). The results of PLS-DA score plot showed that the CRC cells could be separated based on the differential extracellular metabolites identified with overlaps observed between stage B and C (Figure 1). PLS-DA score plot shows a clear separation between CRC cells of different stages with a few overlaps between stage B and C which indicates that levels of identified metabolites were differently expressed between stages.

Most Important Extracellular Metabolites Profiles and Metabolic Pathways Affected

Following the PLS-DA scores plot, VIP scores were determined (Figure 2). The result showed 14 differential extracellular metabolites that were most important in differentiating CRC cells of the more advanced stages from stage A. These metabolites are 8-oxodiacetoxyscirpenol, 5,6-dihydroxyindole, T2 triol, thiamine acetic acid, 1-beta-D-glucopyranosyloxy-3-octanone, 5-hydroxy-l-tryptophan, isopetasoside, 3-O-methylniveusin A, indoleacetaldehyde, PE(20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), butyl butyrate, bis(glutathionyl)spermine, beta-D-glucuronoside, and glyceryl 5-hydroxydecanoate.

Until now, the differential extracellular metabolites presented above (Figure 2) were not reported in CRC of metabolic footprinting study before. Thus, the mechanism of these metabolites in CRC remains unclear. However, only butyl butyrate has been reported by Uchiyama et al. [8]. In the present study, extracellular metabolite of butyl butyrate was decreased in CRC cells of different stages. This finding was consistent with Uchiyama et al. [8], where the authors reported that serum butyrate was decreased in CRC of different stages. In fact, butyl butyrate is involved in lipid metabolism [21]. Lipid metabolism is a cellular process which consists of synthesis or breakdown of lipid molecules for energy or storage, respectively. Hence, decreased level of butyl butyrate in secreted CRC cells may reflect decreased secretion. Alternatively, it may be due to increase in uptake of this metabolite by the more advanced CRC cells to meet the increased energy demand for cancer progression.

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Table 1: Differential extracellular metabolites in CRC cells of stage B, C, and D compared to stage A

Metabolites	Log FC relative to stage A			P value
	B	C	D	
(25S)-26-Hydroxy-24-methylenecycloartan-3-one	-7.96	-7.46	-8.83	3.35×10^{-84}
Butyl butyrate	-8.15	-7.66	-9.02	8.31×10^{-83}
Bis(glutathionyl)spermine	-13.61	-12.76	-13.65	1.01×10^{-57}
Isopetasoside	-3.46	-7.69	-9.06	1.01×10^{-21}
3-O-Methylniveusin A	1.12	-11.04	-11.93	9.65×10^{-16}
beta-D-Glucuronoside	-3.50	-4.79	-12.90	1.67×10^{-15}
T2 Triol	-1.38	-7.77	-9.14	8.37×10^{-11}
5-Hydroxy-L-tryptophan	3.15	3.46	3.51	4.18×10^{-11}
Indoleacetaldehyde	9.60	9.80	9.58	1.68×10^{-11}
Gingerglycolipid A	-1.70	-7.48	-7.56	2.24×10^{-10}
4,5-Dimethylthiazole	12.34	10.31	12.18	1.02×10^{-10}
8-Oxodiacetoxyscirpenol	3.77	6.43	7.42	6.54×10^{-9}
Bisnorbiotin	10.65	8.63	10.36	6.37×10^{-9}
5-Amino-6-(5' phosphoribosylamino) uracil	13.10	11.10	12.80	4.96×10^{-9}
1-beta-D-Glucopyranosyloxy-3-octanone	-1.17	-7.58	-8.94	4.04×10^{-9}
Glyceryl 5-hydroxydecanoate	5.45	4.72	7.52	2.75×10^{-9}
Sphinganine	3.30	2.93	3.98	1.18×10^{-9}
8,8-Diethoxy-2,6-dimethyl-2-octanol	3.30	2.79	3.26	4.09×10^{-8}
Tetradecanoylcarnitine	-11.13	-10.13	-11.02	2.18×10^{-8}
L-cystine	2.20	1.24	2.24	9.53×10^{-7}
PE(20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	5.16	6.09	7.30	3.57×10^{-7}
LysoPE(0:0/20:4(5Z,8Z,11Z,14Z))	2.77	-3.90	-4.76	4.68×10^{-6}
Thiamine acetic acid	2.27	2.95	3.43	9.43×10^{-5}
5,6-Dihydroxyindole	-1.42	-2.38	-3.41	4.10×10^{-5}
Nigellic acid	-1.86	-1.32	-1.33	3.99×10^{-5}
N-(2R-Hydroxypentacosanoyl)-2S-amino-1,3S,4R-octadecanetriol	10.69	8.77	10.66	3.30×10^{-5}
Phytosphingosine	2.49	1.69	3.19	1.33×10^{-5}

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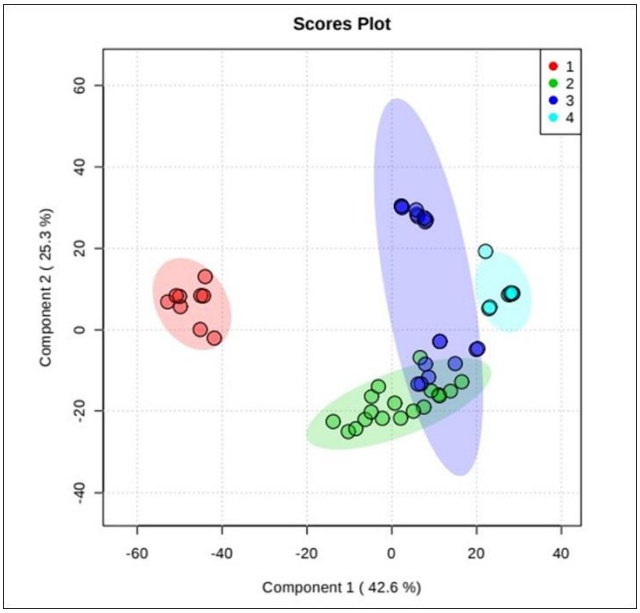

Figure 1: PLS-DA scores plot discriminating the extracellular metabolites in CRC cells of different stages. Red plots (1) represent stage A, green plot (2) represent stage B, blue plot (3) represent stage C, turquoise plot (4) represent stage D. PLS-DA score plot with percentage variance for component 1 (42.6%) and component 2 (25.3%).

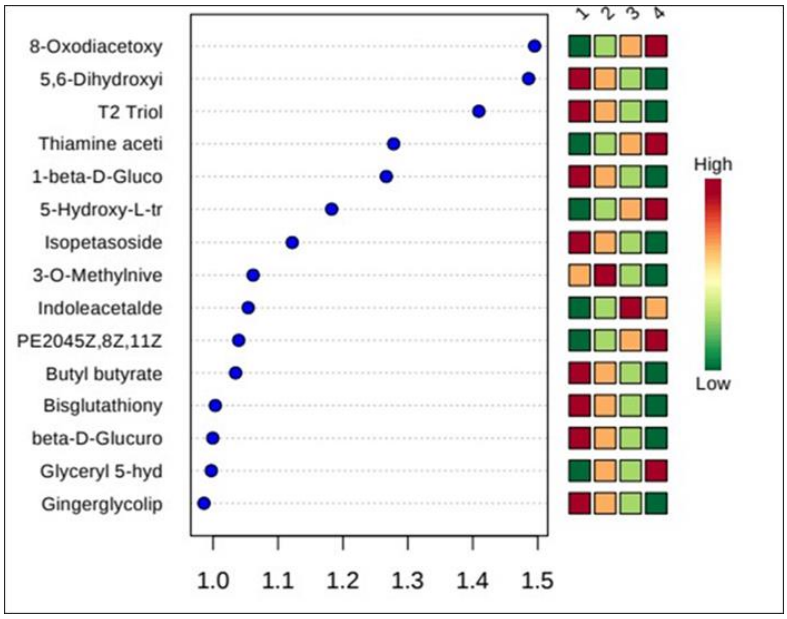

Figure 2: VIP scores of extracellular metabolites in CRC cells of different stages.
1 represents stage A, 2 represents stage B, 3 represents stage C, and 4 represents stage D.

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Based on VIP score, there are several other differential extracellular metabolites which are also involved in lipid metabolism. T2 triol, 1-beta-D-glucopyranosyloxy-3-octanone, isopetasoside, and 3-O-methylniveusin A levels decreased, while 8-oxodiacetoxyscirpenol, glyceryl 5-hydroxydecanoate and PE(20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) levels increased in CRC cells of the more advanced stages grown. Although the exact role of each metabolite is unclear, their involvement in lipid metabolism may suggest the importance of this metabolic pathway to generate the energy needed for CRC cell growth and metastasis as the disease progresses.

Thiamine acetic acid level was found to be increased in the advanced staged CRC cells. The increased secretion of thiamine acetic acid by the more advanced CRC cells may reflect increased intracellular level of this substance. Thiamine acetic acid is involved in thiamine metabolism [21] and is metabolized to thymine pyrophosphate (TPP), a cofactor in carbohydrate metabolism [22]. Additionally, an *in vitro* study on breast cancer cells suggest that thiamine may play a role in cancer cell growth as thiaminase, a thiamine-degrading enzyme, was observed to delay the growth of these cancer cells [23].

The levels of 5-hydroxy-l-tryptophan and indoleacetaldehyde were also observed to increase in the advanced staged of CRC cells. In fact, 5-hydroxy-l-tryptophan and indoleacetaldehyde are reported to be involved in tryptophan metabolism [24] and are the immediate precursors of serotonin [21]. 5-Hydroxy-L-tryptophan is a type of amino acid and synthesized by l-tryptophan [21]. Previous studies have reported that tumor growth is associated to tryptophan metabolism by suppression of antitumor immune response and eventually increase the malignant properties of cancer cells [25, 26]. Additionally, previous studies also indicated that tryptophan metabolites are able to promote cancer cell motility and metastasis due to overexpression of tryptophan-2,3-dioxygenase and indoleamine 2,3-dioxygenase 1 in cancer cells [27-29]. Therefore, in this study, increased level of these metabolites could be due to demand of CRC cells to growth and metastasis as the disease progresses.

Bis(glutathionyl)spermine level was decreased in the advanced staged cells. Bis(glutathionyl)spermine is involved in glutathione metabolism and may reflect alterations in glutathione metabolism [24]. The energy requirement for growth of cancer cells is higher, thus increase in energy metabolism leads to produce high level of the reactive oxygen species. Glutathione maintains the oxidative status of the cells by scavenging free radicals. Hence, the decreased level of Bis(glutathionyl)spermine in the more advanced cancer cells reflect the increased requirements for glutathione to protect cells against oxidative stress [30].

Beta-D-glucuronoside level was decreased in the advanced staged cells and may reflect involvement of the pentose and glucuronate interconversions pathway [24]. Pentose and glucuronate interconversions pathway are closely associated with glycolysis and gluconeogenesis pathways and has been reported to be significantly dysregulated in several types of cancers including CRC [31,32]. Previous study has reported that dysregulation of pentose and glucuronate

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ISSN: 1675-7785
eISSN: 2682-8626

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interconversions pathway in cancer is due to elevation of several enzymes such as phosphoglucomutase, UDP-glucose pyrophosphorylase 2, UDP-glucose-6 dehydrogenase and UDP-glucuronosyltransferase [33].

The data of the 27 differential metabolites were further analysed to determine the metabolic pathways affected using the Metaboanalyst software. The findings revealed several metabolic pathways were significantly affected in secreted CRC cells of different stages and the result showed that tryptophan metabolism was the most significant pathway that affected as CRC progress (Figure 3). As mentioned above, tryptophan metabolism was affected due to demand of cancer cells to growth and metastasis as the disease progresses.

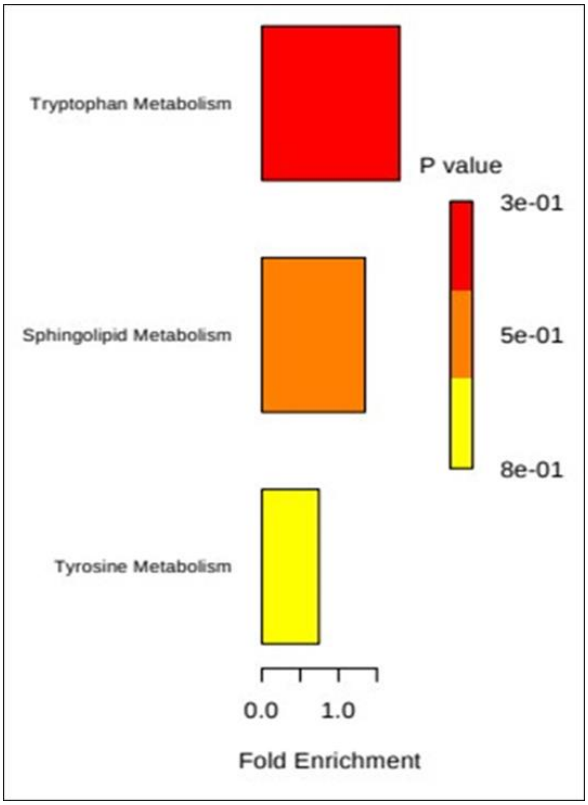

Figure 3: Metabolic pathways affected in extracellular CRC cells of different stages. Metabolites set enrichment analysis (MSEA) results indicated the most perturbated metabolites sets.

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In our previous study on metabolic fingerprinting of the same cells, we have reported 7 differential intracellular metabolites that were most significant to differentiate the more advanced stage cells from stage A [15]. Findings in the present study showed that the identified differential extracellular metabolites were different from those identified in the metabolic fingerprinting.

There are several possibilities for the differences in metabolites profiles and metabolic pathways affected between the metabolic fingerprinting and footprinting. One of the possibilities is the metabolites determined via metabolic footprinting may be present in the growth media due to accumulation and overflow of intracellular metabolites i.e. metabolites are transported out to the environment [34]. However, in the present study, the probability of the metabolites identified in the media was due to overflow is low as they are not associated with increased intracellular metabolites levels. Thus, there may be preferential transport mechanisms of these metabolites out of cells. For example, hypoxanthine has been reported to be transported out of cells to reduce oxidative status as a result of increased oxidative free radicals generated by the increased metabolic rates of cancer cells [35].

This metabolic footprinting study illustrates the preference for cells to take up certain substances while excreting some other substances to support their growth. Such an observation is not new as during cells growth, cells have been shown to consume glucose and glutamine and secrete lactate and glutamate into the medium [36]. The metabolites leak into the microenvironment may be necessary to facilitate growth of cells through cell-cell interactions mediated by the secreted metabolites [37].

The affected metabolic pathways identified in the present study confirms the metabolic derangements identified earlier [15] and the additional information could be obtained by conducting both types of studies. A schematic representation of changes in metabolic pathways as CRC progresses from stage A is shown in Figure 4. Based on the findings of previous study [15], upregulation of glycolysis and pentose phosphate pathway (PPP) were observed suggesting increased need for energy as cancer cells grow. Amino acids are the major source of nutrients for cancer cells. Thus, the increased rate of amino acids metabolism may occur to maintain cancer cells growth and rapid proliferation. Moreover, alteration in riboflavin metabolism leads to increase rate in ATP production, and altered in nucleotide metabolism, which are necessary metabolic pathways for cell growth. There was also increased rate of lipid metabolism observed which is required for numerous cellular processes e.g., cell growth, proliferation, differentiation, and structural components of cell membranes. In the present study, some metabolites involved in tyrosine, tryptophan and sphingolipid metabolisms are preferentially secreted by CRC cells of later stages which may act as a protective mechanism for example against oxidative free radicals. Obviously, this study highlights the importance of doing both techniques to generate a more complete understanding on the molecular changes that occur as cancer advances.

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CONCLUSION

In conclusion, the differential extracellular metabolites identified are different from the ones identified during metabolomic fingerprinting. Moreover, this study concludes that there is important of doing both techniques to generate a more complete understanding on the molecular changes that occur as CRC advances. This study also has provided some directions for future study, where the characterization is important in the development of *in vitro* models for drug discovery and therapeutics strategies study.

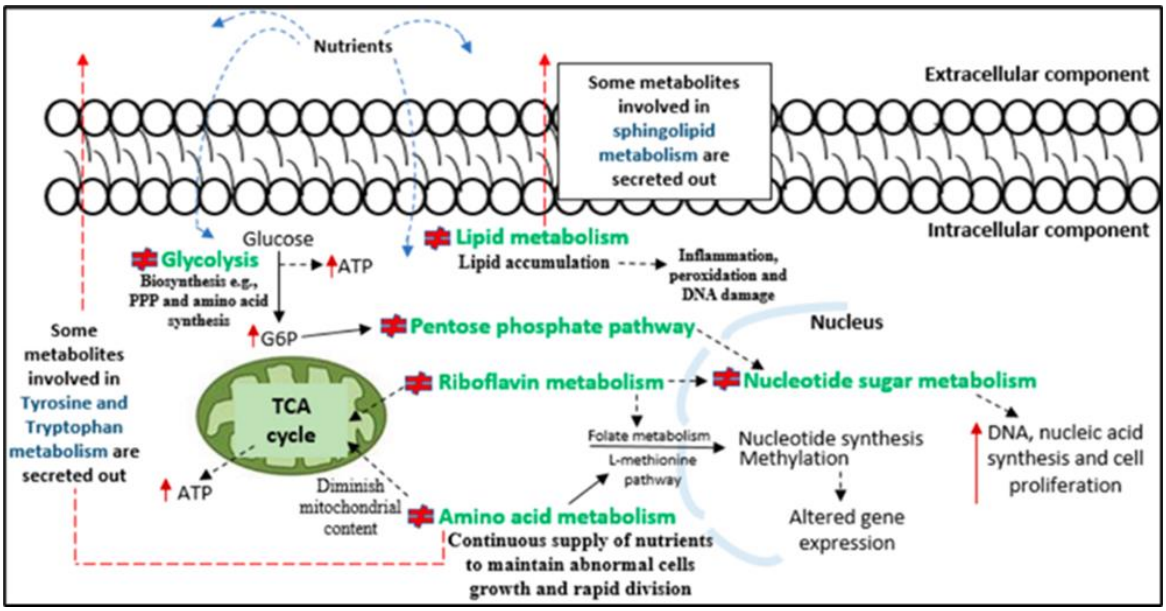

Figure 4: Schematic of Altered Metabolic Pathways in CRC cells as the Disease Progresses
Notes: Altered ⇌, increase ↑

ACKNOWLEDGMENTS

The authors would like to acknowledge the support of Universiti Teknologi Mara (UiTM), Cawangan Negeri Sembilan, Kampus Kuala Pilah and Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia for providing the facilities and financial support on this research.

CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare absence of conflicting interests with the funders.

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eISSN: 2682-8626

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Vol. 15(2) JUNE 2021

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v15i2.13810

APPENDIX

Information on cells used in the study

Cells	Disease	Tissue	Morphology	Patient
SW 1116	Colorectal adenocarcinoma; Dukes' type A	Colon	Epithelial	Male, Caucasian, 73-year-old
HT 29	Colorectal adenocarcinoma; Dukes' type B	Colon	Epithelial	Female, Caucasian, 44-year-old
SW 480	Colorectal adenocarcinoma; Dukes' type B	Colon	Epithelial	Male, Caucasian, 50-year-old
HCT 15	Colorectal adenocarcinoma; Dukes' type C	Colon	Epithelial	Male; adult
DLD-1	Colorectal adenocarcinoma; Dukes' type C	Colon	Epithelial	Male; adult
HCT 116	Colorectal carcinoma	Colon	Epithelial	Male; adult

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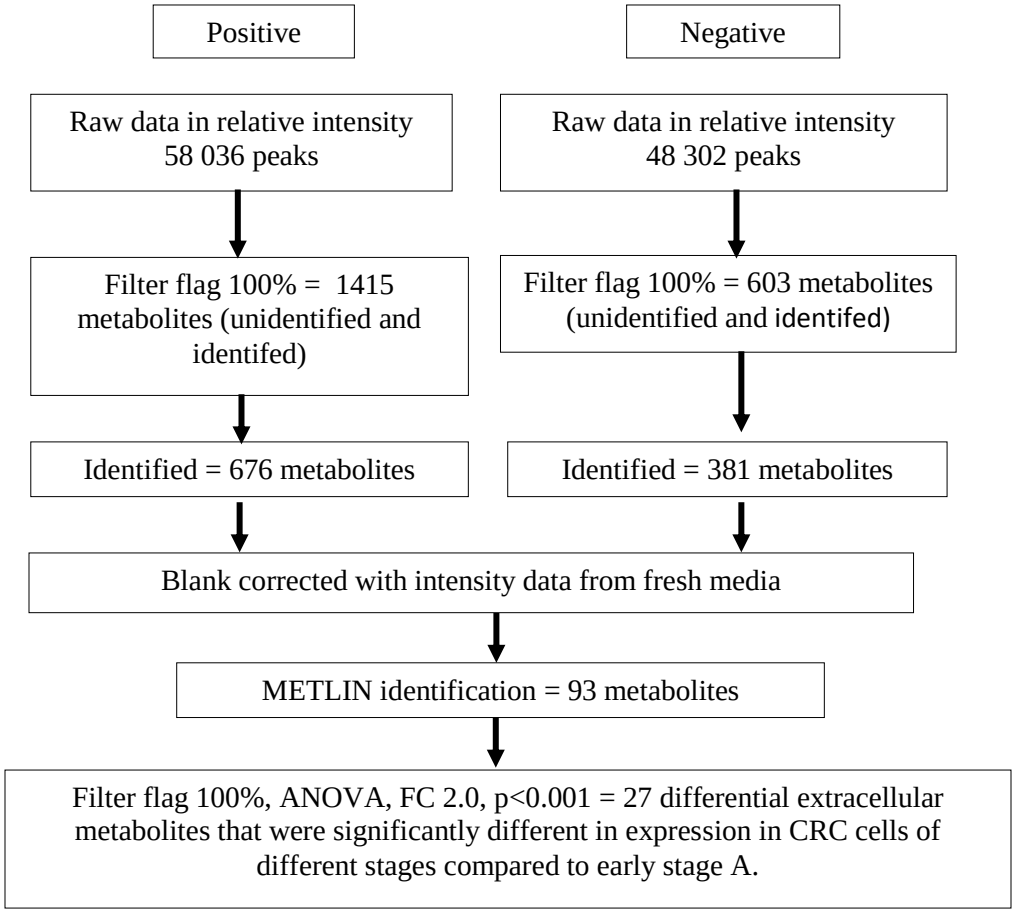

Workflow analysis of metabolomics profiles in secreted CRC cells of different stages