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Investigation of extracellular metabolites in cancer and healthy colon cells in a time-dependent manner

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Abstract

Metabolite analysis is critical in the cancer field because of provides information about the metabolic status of cells. The profiling of extracellular metabolites presents technical advantages over intracellular metabolites, such as easier access to extracellular metabolites without a quenching method and the growth medium containing high biomass. This study aimed to investigate the extracellular level of metabolites in colon cancer cells in a time-dependent manner. 1 x 10^6 cells were seeded in 4 Petri dishes and glucose, pyruvate, citric acid (TCA) cycle metabolites, and D2-Hydroxyglutarate (D2-HG) in the conditioned medium were determined by 3,5-Dinitro Salicylic Acid (DNS) and Ultra Performance Liquid Chromatography (UPLC) method and pyruvate assay for 24-96th hours. The results showed that glucose is consumed, and pyruvate and TCA cycle intermediates are released in decreasing amounts in all cell lines. It was also observed that glucose was more consumed, and TCA cycle metabolites were less released in metastatic colon cancer cells (SW620) than in primary colon adenocarcinoma cells (Caco-2). Most importantly, D2-HG oncometabolite was released more into the growth medium of colon cancer cells than normal colon cells for four days. In conclusion, the D2-HG is highly produced and released to the growth medium of colon cancer cell lines in a cancer-type-specific manner.

Keywords: Colon cancer, exametabolome analysis, metabolite, UPLC

1. Introduction

During carcinogenesis, cells undergo metabolic and behavioral changes in a multistage process due to mutations. Metabolic changes include reprogramming of intracellular metabolism that controls inappropriate cell proliferation and adapts to the tumor microenvironment [1]. Cancer has become the most common disease after significant developments in treating heart diseases [2]. There are more than 200 types of cancer; the third most common type in men and women is colon cancer. Because of its mortality and high incidence, approximately 2 x 10^6 new colon cancer cases were observed in 2020 in stages III and IV [3,4]. The results highlighted that we need new therapeutic methods to increase the 5-year survival rate for late-stage patients. Recently, metabolic reprogramming has become a field of increasing interest [5,6].

Metabolism consists of many vital reactions in which small molecules known as metabolites are produced [7,8]. Cells are like factories that produce a wide variety of chemicals including metabolites. The analysis of metabolite levels is critical as it will provide information about the metabolic status of the cells and determines the risk and frequency of diseases [9–12]. Metabolites produced and accumulated inside the cell are secreted to the extracellular medium by specific metabolic overflow [13]. Analysis of the correct level of intracellular metabolites is essential because the reactions occur inside the cell. However, it is challenging due to finding a reliable quenching method that does not damage the cell membrane and there is a low biomass ratio in the intracellular medium. On the other hand, extracellular metabolites can be accessed more easily without a quenching method. In addition, profiling the extracellular metabolite provides information about the environmental conditions and the metabolic status of cells [14]. In recent years, the comprehensive profiling of extracellular metabolites known as exametabolome analysis to assess or compare the metabolic status of cancer cells has gained importance. In recent years, as a result of the valuable developments in the metabolomics

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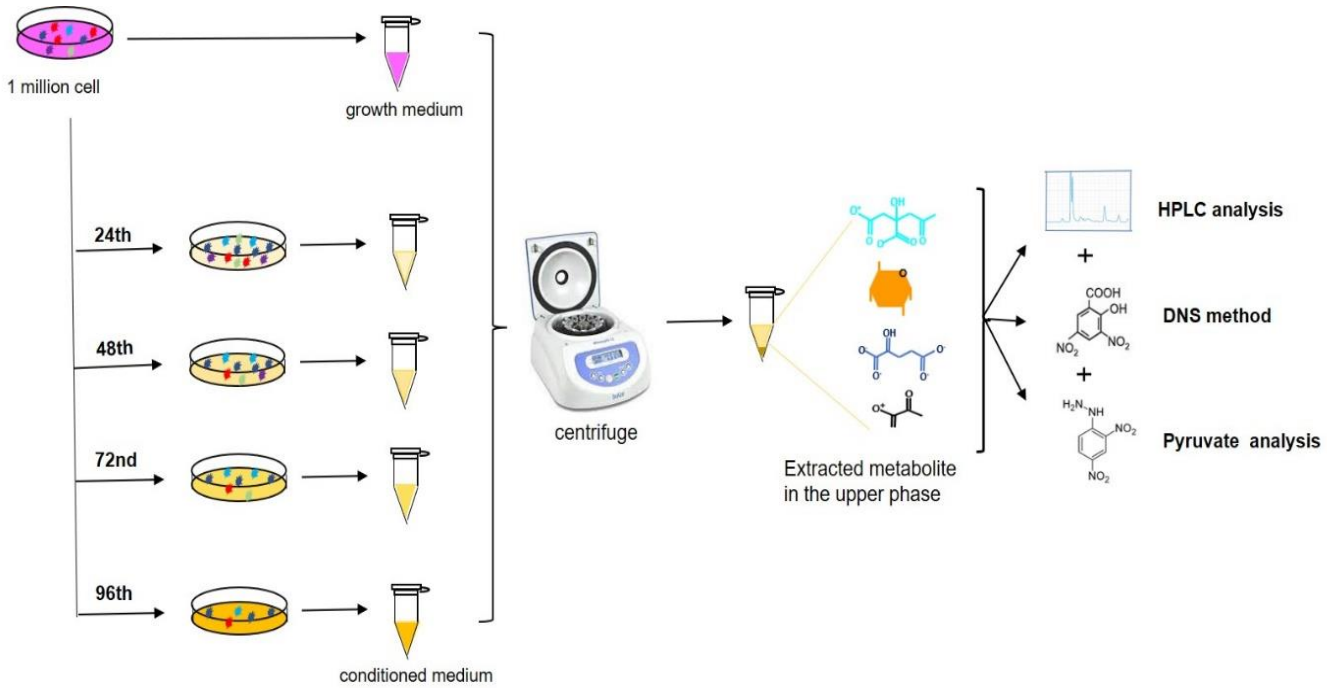

Figure 1. The extracellular metabolite extraction procedure. 1 x 106 cells were seeded in 4 Petri dishes. Approximately 300 µl conditioned medium was obtained from the Petri dishes and the cell number was determined using Thoma lame after 24, 48, 72, and 96 hours. The conditioned media were centrifuged at 17,000 g and +4 °C for 15 minutes and the supernatants were used for further analysis. Glucose, pyruvate, TCA cycle metabolites, and D2-HG in the conditioned medium were determined by DNS and HPLC method and pyruvate assay for 24-96th hours

field, metabolite analysis has become the more affordable, reliable, and reproducible postgenomic method [15].

Metabolic reprogramming is one of the hallmarks of cancer cells, and some central metabolic pathways such as Glycolysis, TCA cycle, and pentose phosphate are aberrantly regulated during cancer progression [16]. The TCA cycle has been known as a signalling hub in cell metabolism because it connects many metabolic pathways. The TCA cycle plays a role in energy production and the biosynthesis of building blocks in the cell [17]. Recently it has been observed that the abnormal level of TCA cycle metabolites is related to different diseases such as obesity, diabetes, heart failure, and cancer [9,10,18,19]. Succinate, fumarate, and 2-hydroxyglutarate are the TCA cycle-related metabolites and they affect processes of cancer development and progression [20–22]. Until now, the level of TCA cycle-related metabolites has been investigated during colon cancer progression [23–25]. However, it is not known how the extracellular metabolite levels change while colon cancer cells gain metastatic properties.

The present study used primary and metastatic colon cancer cell lines, and the extracellular metabolite level changes were investigated when cells gained metastatic properties. The levels of extracellular metabolite in the TCA cycle and glycolysis were determined by the pyruvate assay and DNS and HPLC methods in healthy and cancer cell lines for four days, and colon cancer-specific metabolite was investigated.

2. Materials and Methods

2.1. Cell Lines

Normal colon (CCD-18Co), colon adenocarcinoma (Caco-2), and metastatic colon cancer (SW620) cell lines were grown in DMEM, MEM, and RPMI, respectively. The content of all growth media and incubation conditions of cells have been described in a previous study [26].

2.2. Metabolite Extraction From the Extracellular Medium

All cell lines were calculated as containing 1 x 10^6 cells and seeded into four Petri dishes. Approximately 300 µl conditioned medium was obtained from the Petri dishes, and the cell number was determined using Thoma lame after 24, 48, 72, and 96 hours. The conditioned media were stored at -80 °C until the experiment. The mediums were centrifuged at 17.000 g and +4 °C for 15 minutes, and the supernatants were used for further analysis (HPLC method, glucose, and pyruvate determinations [27].

The released and consumed metabolite concentration levels were calculated for 1 x 10^6 cells following previously published protocols [28]. The calibration curve's equation and R^2 value, as well as the concentrations of the standard glucose solutions, are given in Table 1.

2.3. 3,5-Dinitro Salicylic Acid (DNS) Method

The DNS method [29] is used to determine glucose content in the growth mediums. The sample (50 µl) and

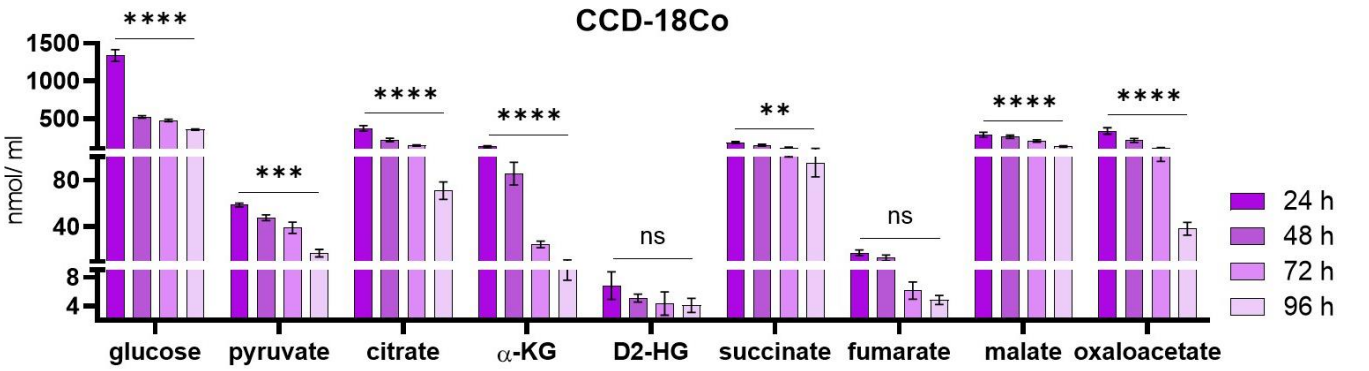

Figure 2. Time-dependent extracellular metabolite levels in CCD-18Co at 24-96 h. Metabolites were extracted from extracellular matrix of CCD-18Co cells in a time-dependent manner. Glucose and pyruvate levels were calculated by DNS method and pyruvate assay. The level of citrate, α-KG, D2-HG, succinate, fumarate, malate and oxaloacetate was determined by HPLC method. Abbreviations: ns, not significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

DNS reagent (1:1) were stirred at 140 rpm at 100 °C. After 10 minutes, it was incubated on ice for 1 minute. The standards and samples were measured at 540 nm [24] in a microplate reader (Multiskan™ GO, Thermo Scientific). The calibration curve's equality and R2 and the concentrations of the standard glucose solutions are given in Table 1.

2.4. Pyruvate Assay

The pyruvate assay determines the pyruvate content in the growth mediums [30] (Fig. 1). First, five mg of DNPH was dissolved in five ml of 2N HCl (37%), and then 10 µl was added to each well. Then, 30 µl of the samples were transferred to these wells and incubated for 5 minutes at room temperature. Finally, 60 µl of 2N NaOH was added and incubated at room temperature for 10 minutes. The results were obtained at 520 nm absorbance in a microplate reader (Multiskan™ GO, Thermo Scientific). The calibration curve's equality and R2 and the concentrations of the standard pyruvate solutions are given in Table 1.

2.5. UPLC System

A UPLC (Agilent) device with an Alltech OA-1000 column which runs at 42 °C was used. All HPLC-grade standards (Sigma Aldrich) are detected in a UV detector (210 nm). The flow rate was 0.4 mL/min, and the mobile phase was 9.0 mM H_2SO_4 [31]. Table 1 gives the calibration curve's equality, R2 value, and the concentrations of the standards for citrate, α-KG, oxaloacetate, malate, D2-HG, succinate, and fumarate.

Table 1. The calibration curve's equality and R2 and the concentrations of the standard solutions for glucose, pyruvate, citrate, α-KG, oxaloacetate, malate, D2-HG, succinate and fumarate.

	Calibration curve's equation	R^2	Concentrations of the standard (ppm)
glucose	y=0,0005x+0,0078	0,9998	500–1000–2000
	y=0,0005x+0,0074	0,9992	300–500–1000
	y=0,0004x+0,0388	0,9999	1250–2500–5000
pyruvate	y=0,0235x+0,073	0,9981	3.125–6.25–12.5–25
citrate	y=3,9292x+1,55	0,9975	12–24–48
α-KG	y=17,787x+6,9123	0,9998	2.5–5–10–25
oxaloacetate	y=2,1623x-9,1	1	25–50–100
malate	y=2,7077x-17,45	0,9995	25–50–100
D2-HG	y=3,5866x-24,45	0,9979	25–50–100
succinate	y=1,9914x-1,9	0,9998	5–10–20
fumarate	y=124,57x-16,3	0,9996	0.5–1–2

2.6. Statistical Analysis

The experiments were carried out in three independent repeats. GraphPad Prism 8.0 (GraphPad Software, CA, USA) was used for statistical analysis. The statistical analysis (Two-way ANOVA and then Tukey's multiple comparisons test) was performed. To denote statistical significance between the control and the sample groups, the asterisk(s) is shown on the graph.

3. Results

3.1. Glucose is Consumed More in The Colon Cancer Cell Lines During 96 Hours

In the present study, changes in the extracellular metabolite levels were investigated in the primary and metastatic colon cancer cell lines in a time-dependent manner. The previous study determined only the extracellular metabolite levels at the 24th hour [24].

Reducing glucose to pyruvate is the first part of energy generation [32]. The consumed glucose levels were 1337.7–353.1 nmol/ml in the healthy cell line, while they were 1426.5–616.2 and 1609.8–638.8 nmol/ml in the Caco-2 and SW620, respectively. At the 96 h, glucose was 1.7 and 1.8-fold ($p<0.05$) more consumed in the Caco-2 and SW620 than CCD-18Co, respectively (Fig. 2–Fig. 4).

3.2. The Pyruvate and TCA Cycle Metabolites Less Released from Colon Cancer Cell Lines During 96 Hours

Pyruvate, the end product of the glycolysis pathway, is converted to Acetyl CoA [32]. The released pyruvate levels were 58.6–17.2 nmol/ml in the healthy cell line,

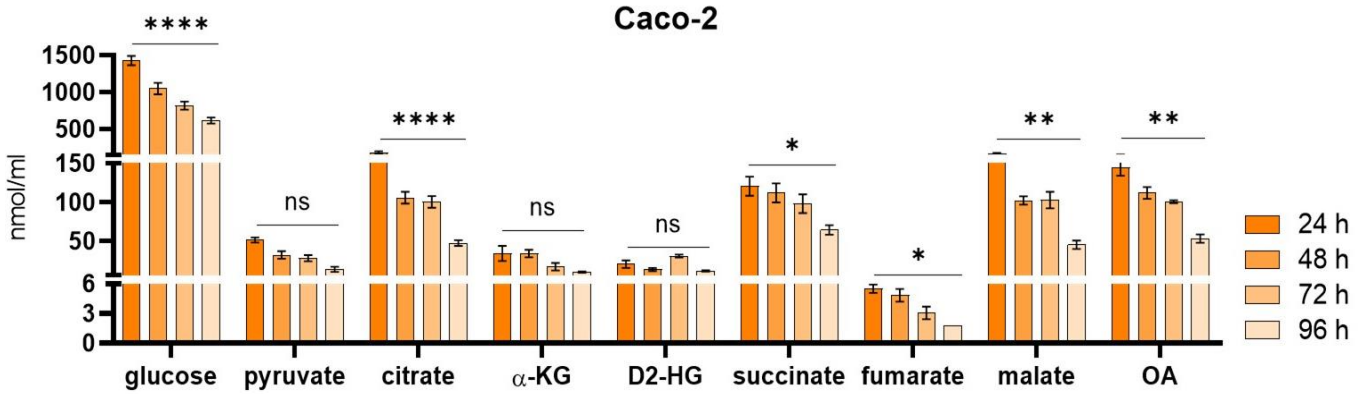

Figure 3. Time-dependent extracellular metabolite levels in Caco-2 at 24-96 h. Metabolites were extracted from extracellular matrix of Caco-2 cells in a time-dependent manner. Glucose and pyruvate levels were calculated by DNS method and pyruvate assay. The level of citrate, α-KG, D2-HG, succinate, fumarate, malate and oxaloacetate was determined by HPLC method. Abbreviations: ns, not significant. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

while they were 51.7–13.8 and 43.7–9.2 nmol/ml in the Caco-2 and SW620, respectively. At the 96 h, pyruvate was 1.9-fold (p<0.05) less released into the environment of SW620 than CCD-18Co (Fig. 2–Fig. 4).

The released citrate levels were determined as 370.2–70.9 nmol/ml in the CCD-18Co (Fig. 2), while they were 180.1–47.6 and 121.6–52.9 nmol/ml in the Caco-2 and SW620, respectively (Fig. 3–Fig. 4). In the next step, isocitrate is oxidized to α-KG and thus the first oxidative decarboxylation reaction occurs [32].

The released extracellular α-KG levels were 126.0–9.6 nmol/ml in the CCD-18Co (Fig. 2), while they were 34.3–10.3 and 12.7–4.1 nmol/ml in the Caco-2 and SW620, respectively (Fig. 3–Fig. 4). The extracellular α-KG levels were highly decreased in the SW620 cell line according to CCD-18Co because intracellular D2-HG was highly elevated in these cells [24]. At the 96 h, the α-KG was 2.3-fold (p<0.05) less released into the environment of metastatic colon cancer cells than normal cells (Fig. 2 and Fig. 4). The level of released extracellular succinate was 180.9–94.8 nmol/ml in the CCD-18Co (Fig. 2), while they were 120.6–64.6 and 94.8–6.8 nmol/ml in the Caco-2 and SW620, respectively (Fig. 3–Fig. 4). At the 96 h, the extracellular succinate level was highly decreased in the SW620 because we found that most intracellular α-KG is reduced to D2-HG [24]. The released extracellular fumarate levels were determined as 17.6–4.9 nmol/ml in the CCD-18Co (Fig. 2), while they were 5.5–1.8 and 12.7–3.3 nmol/ml in the Caco-2 and SW620, respectively (Fig. 3–Fig. 4). In the next step of the TCA cycle, malate was synthesized from fumarate [32]. The released extracellular malate levels were 287.7–128.7 nmol/ml in the CCD-18Co (Fig. 2), while they were 166.6–45.4 and 134.7–41.7 nmol/ml in the Caco-2 and SW620, respectively (Fig. 3–Fig. 4). At the 96 h, malate was 2.8 and 3.1-fold (p<0.05) less released into the Caco-2 and SW620 environment than CCD-18Co, respectively. In the last step of the TCA cycle, the oxaloacetate is synthesized from malate by the third oxidative decarboxylation reaction [32]. The released extracellular oxaloacetate levels were 335.7–38.2 nmol/ml in the CCD-18Co (Fig. 2), while they were 145–53.4 and 106.9–15.3 nmol/ml in the Caco-2 and SW620 cell lines, respectively (Fig. 3–Fig. 4). At the 96th h, oxaloacetate was 2.5–fold

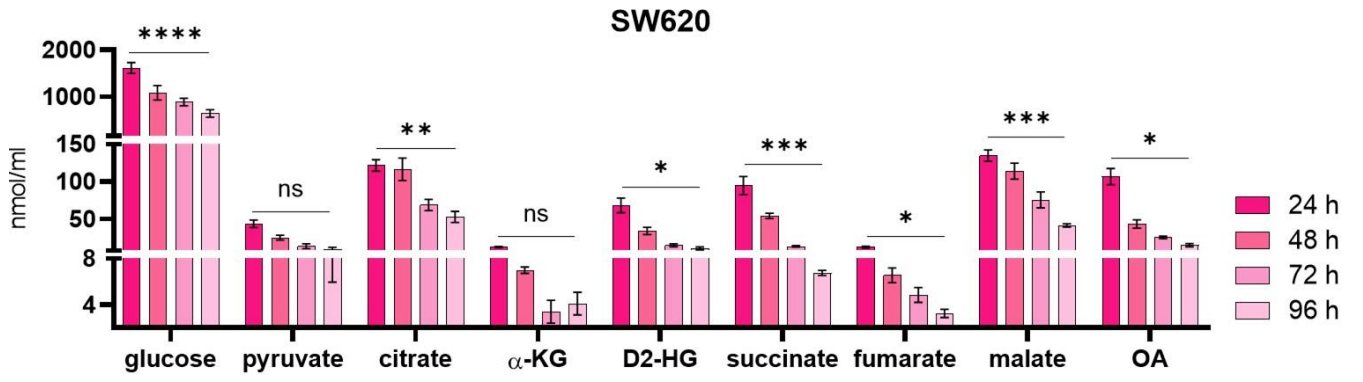

Figure 4. Time-dependent extracellular metabolite levels in SW620 at 24-96 h. Metabolites were extracted from extracellular matrix of SW620 cells in a time-dependent manner. Glucose and pyruvate levels were calculated by DNS method and pyruvate assay. The level of citrate, α-KG, D2-HG, succinate, fumarate, malate and oxaloacetate was determined by HPLC method. Abbreviations: ns, not significant. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

($p<0.05$) less released into the SW620 environment than CCD-18Co.

3.3. D2-HG Oncometabolite Released More From Colon Cancer Cell Lines

D2-HG is converted from α-KG by the wild-type or mutant isocitrate dehydrogenase (IDH) 1/2 enzymes and involves in the cancer progression [20]. In our study, significant results were obtained in the time-dependent extracellular D2-HG oncometabolite. The released levels of D2-HG by cancer cell lines were increased for 96 hours. The released extracellular D2-HG levels were 6.9–4.1 nmol/ml in the CCD-18Co (Fig. 2), while they were 20.6–11.7 and 68.5–12.3 nmol/ml in the Caco-2 and SW620 cell lines, respectively (Fig. 3–Fig. 4). At the 96 h, D2-HG was 2.8 and 3.0-fold ($p<0.01$) more released into the environment of Caco-2 and SW620 than the healthy cell.

4. Discussion

Cancer cells tend to Glycolysis, known as the Warburg effect, because they need more energy and intermediate. However, it has been found that cancer progression is not universal, and cancer cells exhibit metabolic diversity [33–35]. This allows cancer cells to adapt to changing metabolic pathways to survive and proliferate. Thus, metabolites produced in different forms due to mutations or changing their concentrations are significant therapeutic advantages for scientists [36]. Our study showed that the extracellular metabolite levels were significantly different between normal and cancer cells (Fig. 2–Fig. 4).

In the previous study, we investigated the level of intracellular metabolites produced in the TCA cycle and glycolysis in the cell lines (CCD-18Co, Caco-2, and SW620) and xenograft models generated with these cell lines. Unlike other metabolites, the elevated D2-HG level was found in the SW620 (152.6 μmol/10^6 cells), whereas it was not detected in the colon epithelial cells (CCD-18Co). In the xenograft models, the D2-HG level was 7.4 and 19.9-fold higher in Caco-2 and SW620 tumor tissues than in healthy tissue, respectively [24]. However, the time-dependent extracellular metabolite levels have not been detected until now. The present study, D2-HG-related metabolite levels were detected in the growth medium of CCD-18Co, Caco-2, and SW620 for 96 h. It was found that the D2-HG was 2.8 and 3.0-fold ($p<0.01$) more released into the environment of Caco-2 and SW620 than the healthy cell (Fig. 2–Fig. 4). In relation to that, the α-KG was 2.3-fold ($p<0.05$) less released into the environment of metastatic colon cancer cells than normal cells (Fig. 2–Fig. 4).

The TCA cycle is at the center of the mitochondrial metabolic pathway because coordinates energy generation, carbon metabolism, and biosynthetic pathways [37]. It has been observed that the abnormal level of TCA cycle metabolites is related to different diseases such as obesity, atrial fibrillation, heart failure, and cancer [9,10,18,38]. In the field of cancer, especially in colon cancer, the study of the amount of TCA cycle metabolites has recently increased [6]. As a result of our experiments, the levels of extracellular metabolites in cancer cells with a high metabolic rate differed significantly from healthy cells. In our study, Caco-2 and SW620 consumed 1.7 and 1.8-fold ($p<0.05$) more glucose than CCD-18Co respectively. Pyruvate was 1.9-fold ($p<0.05$) less released into the environment of SW620 than CCD-18Co (Fig. 2–Fig. 4). In addition, cancer cells released less TCA cycle intermediate than the healthy cells for 96 hours (Fig. 2–Fig. 4), consistent with the previous studies [39,40]. We showed that colon cancer cells exhibit the Warburg effect because of highly consumed glucose. On the other side, the TCA cycle intermediates is less released. All the results showed that metabolism does not proceed through Glycolysis, and the TCA cycle is active.

Based on the metabolic overflow concept, specific metabolites produced and accumulated inside the cell are secreted to the extracellular medium by specific metabolic overflow according to the needs of cell. Thus, information about the intracellular levels of these metabolites can be obtained by investigating the level of extracellular metabolites in the conditioned medium. When being studied experimentally, intracellular and extracellular metabolite levels must be determined simultaneously [14]. In the present study, unlike TCA cycle-related metabolites, the D2-HG oncometabolite, which is highly produced inside the cancer cells, was released more in the environment of Caco-2 and SW620 compared to normal cells for 24–96 hours (Fig. 2–Fig. 4). These results are consistent with the intracellular metabolite levels we obtained in the previous study [24].

During cancer development and progression, reversible reactions of metabolic pathways are optimized according to the conditions of the cell. Thus, the ATP and precursor molecules required for biosynthesis are highly synthesized in cancer cells [41]. As a result of the reorganization of energy metabolism, abnormal intracellular and extracellular levels of TCA cycle-related metabolites have been produced when cancer cells gain metastatic properties. In the previous studies, it has been observed that glucose consumption was decreased, and TCA cycle-related metabolites were more produced in the cancer cells according to a healthy cell. We know that D2-HG was highly produced in colon, breast, head and neck squamous metastatic cancer

cells [24,42,43]. In our study, the level of released D2-HG (in the conditioned medium) was increased when colon cancer cells gained metastatic potential, consistent with the earlier studies.

Our findings provide insight into the changes in time-dependent extracellular metabolite levels produced in glycolysis and the TCA cycle in both colon epithelial and colon cancer cell lines. We demonstrated that glucose is used to synthesize the TCA cycle intermediates which are then gradually released into the conditioned medium. Unlike TCA cycle-related metabolites, the D2-HG oncometabolite was released more in colon cancer than in normal cells. It is predicted that in the future, determining the levels of cancer-type-specific oncometabolite can be used for cancer diagnosis.

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References

[1] D. Hanahan, R.A. Weinberg, The hallmarks of cancer. Cell, 100, 2000, 57–70.

[2] R. Twombly, Cancer surpasses heart disease as leading cause of death for all but the very elderly. J Natl Cancer Inst, 2005, 97, 330–331.

[3] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA-Cancer J Clin, 71, 2021, 209–249.

[4] C. Joachim, J. Macni, M. Drame, A. Pomier, P. Escarmant, J. Veronique-Baudin, V. Vinh-Hung, Overall survival of colorectal cancer by stage at diagnosis: Data from the Martinique Cancer Registry, Medicine, 98, 2019.

[5] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2019, CA-Cancer J Clin, 69, 2019, 7–34.

[6] C. Neitzel, P. Demuth, S. Wittmann, J. Fahrer, Targeting altered energy metabolism in colorectal cancer: oncogenic reprogramming, the central role of the TCA cycle and therapeutic opportunities, Cancers, 12, 2020, 1731.

[7] A. Judge, M.S. Dodd, Metabolism, Essays Biochem, 64, 2020, 607–647.

[8] E. Holmes, I.D. Wilson, J.K. Nicholson, Metabolic phenotyping in health and disease, Cell, 134, 2008, 714–717.

[9] Y.J. Park, S.M. Han, J.Y. Huh, J.B. Kim, Emerging roles of epigenetic regulation in obesity and metabolic disease, J Biol Chem, 297, 2021.

[10] M. Bulló, C. Papandreou, J. García-Gavilán, M. Ruiz-Canela, J. Li, M. Guasch-Ferré, E. Toledo, C. Clish, D. Corella, R. Estruch, E. Ros, M. Fitó, C.H. Lee, K. Pierce, C. Razquin, F. Arós, L. Serra-Majem, L. Liang, M.A. Martínez-González, F.B. Hu, J. Salas-Salvadó, Tricarboxylic acid cycle related-metabolites and risk of atrial fibrillation and heart failure, Metabolisim, 125, 2021.

[11] D.R. Schmidt, R. Patel, D.G. Kirsch, C. Lewis, M.V. Vander Heiden, J. Locasale, Metabolomics in cancer research and emerging applications in clinical oncology, CA: Cancer J Clin, 71, 2021, 333–358.

[12] A. Galal, M. Talal, A. Moustafa, Applications of machine learning in metabolomics: Disease modeling and classification, Front Genet, 13, 2022.

[13] N. Paczia, A. Nilgen, T. Lehmann, J. Gätgens, W. Wiechert, S. Noack, Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms, Microb Cell Factories, 11, 2012, 1–14.

[14] N. Granucci, F.R. Pinu, T.L. Han, S.G. Villas-Boas, Can we predict the intracellular metabolic state of a cell based on extracellular metabolite data?, Mol Biosyst, 11, 2015, 3297–3304.

[15] D. Hunerdosse, D.K. Nomura, Activity-based proteomic and metabolomic approaches for understanding metabolism, Curr Opin Biotechnol, 28, 2014, 116–126.

[16] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell, 144, 2011, 646–674.

[17] I. Martínez-Reyes, N.S. Chandel, Mitochondrial TCA cycle metabolites control physiology and disease, Nat Commun, 11, 2020, 1–11.

[18] X.W. Hou, Y. Wang, C.W. Pan, Metabolomics in diabetic retinopathy: A systematic review, Investig Ophthalmol Vis Sci, 62, 2021, 4–4.

[19] M. Sciacovelli, E. Gonçalves, T.I. Johnson, V.R. Zecchini, A.S. Henriques da Costa, E. Gaude, A.V. Drubbel, S.J. Theobald, S.R. Abbo, M.G.B. Tran, V. Rajeeve, S. Cardaci, S. Foster, H. Yun, P. Cutillas, A. Warren, V. Gnanapragasam, E. Gottlieb, K. Franze, B. Huntly, E.R Maher, P.H. Maxwell, J. Saez-Rodriguez, C. Frezza, Fumarate is an epigenetic modifier that elicits epithelial-to-mesenchymal transition, Nature, 537, 2016, 544–547.

[20] L. Dang, D.W. White, S. Gross, B.D. Bennett, M.A. Bittinger, E.M. Driggers, V.R. Fantin, H.G. Jang, S. Jin, M.C. Keenan, K.M. Marks, R.M. Prins, P.S. Ward, K.E. Yen, L.M. Liau, J.D. Rabinowitz, L.C. Cantley, C.B. Thompson, M.G. Vander Heiden, S.M. Su, Cancer-associated IDH1 mutations produce 2-hydroxyglutarate, Nature, 462, 2009, 739–744.

[21] I.P. Tomlinson, N.A. Alam, A.J. Rowan, E. Barclay, E.E.M. Jaeger, D. Kelsell, I. Leigh, P. Gorman, H. Lamlum, S. Rahman, R.R. Roylance, S. Olpin, Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer, Nat Genet, 30, 2002, 406.

[22] H.X. Hao, O. Khalimonchuk, M. Schraders, N. Dephoure, J.P. Bayley, H. Kunst, P. Devilee, C.W.R.J. Cremers, J.D. Schiffman, B.G. Bentz, S.P. Gygi, D.R. Winge, H. Kremer, J. Rutter, SDH5, a gene required forflavination of succinate dehydrogenase, is mutated in paraganglioma, Science, 325, 2009, 1139–1142.

[23] H. Colvin, N. Nishida, M. Konno, N. Haraguchi, H. Takahashi, J. Nishimura, T. Hata, K. Kawamoto, A. Asai, K. Tsunekuni, J. Koseki, T. Mizushima, T. Satoh, Y. Doki, M. Mori, H. Ishii, Oncometabolite D-2-hydroxyglurate directly induces epithelial-mesenchymal transition and is associated with distant metastasis in colorectal cancer, Sci Rep, 6, 2016, 36289.

[24] E.B. Atalay, H.A. Kayali, The elevated D-2-hydroxyglutarate level found as a characteristic metabolic change of colon cancer in both in vitro and in vivo models, Biochem Biophys Res Commun, 627, 2022, 191–199.

[25] S.K. Manna, N. Tanaka, K.W. Krausz, M. Haznadar, X. Xue, T. Matsubara, E.D. Bowman, E.R. Fearon, C.C. Harris, Y.M. Shah, F.J. Gonzalez, Biomarkers of coordinate metabolic reprogramming in colorectal tumors in mice and humans, Gastroenterology, 146, 2014, 1313–1324.

[26] E. Subasi, E. Bulut Atalay, D. Erdogan, B. Sen, B. Pakyapan, H.A. Kayali, Synthesis and characterization of thiosemicarbazone-functionalized organoruthenium (II)-arene complexes: Investigation of antitumor characteristics in colorectal cancer cell lines, Mater Sci Eng C Mater Biol Appl, 106, 2020, 110152.

[27] S. Dietmair N.E. Timmins P.P. Gray, L.L. Nielsen, J.O. Krömer, Towards quantitative metabolomics of mammalian cells: development of a metabolite extraction protocol, Anal Biochem, 404, 2010, 155–164.

[28] E.B. Atalay, S. Senturk, H.A. Kayali, Wild-type IDH1 Knockout Leads to G0/G1 Arrest, Impairs Cancer Cell Proliferation, Altering Glycolysis, and the TCA Cycle in Colon Cancer, Biochem Genet, 2023, 1–17.
[29] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal Chem, 31, 1959, 426–428.
[30] K.S. Yoo, E.J. Lee, B.S. Patil, Underestimation of pyruvic acid concentrations by fructose and cysteine in 2, 4-dinitrophenylhydrazine-mediated onion pungency test, J Food Sci, 76, 2011, C1136–C1142.
[31] H. Ayar-Kayali, L. Tarhan, Vancomycin antibiotic generation and TCA-glyoxalate pathways depending on the glucose concentration in Amycolatopsis orientalis, Enzyme Microb Technol, 38, 2006, 727–734.
[32] G.M. Bodner, Metabolism Part II: The tricarboxylic acid (TCA), citric acid, or Krebs cycle, J Chem Educ, 63, 1986, 673.
[33] P.H. Chen, L. Cai, K. Huffmanet, C. Yang, J. Kim, B. Faubert, L. Boroughs, B. Ko, J. Sudderth, E.A. McMillan, L. Girard, D. Chen, M. Peyton, M.D. Shields, B. Yao, D.S. Shames, H.S. Kim, B. Timmons, I. Sekine, R. Britt, S. Weber, L.A. Byers, J.V. Heymach, J. Chen, M.A. White, J.D. Minna, G. Xiao, R.J. DeBerardinis, Metabolic diversity in human non-small cell lung cancer cells, Mol Cell, 76, 2019, 838–851.
[34] A.N. Lau, Z. Li, L.V. Danai, A.M. Westermark, A.M. Darnell, R. Ferreira, V. Gocheva, S. Sivanand, E.C. Lien, K.M. Sapp, J.R. Mayers, G. Biffi, C.R. Chin, S.M. Davidson, D.A. Tuveson, T. Jacks, N.J. Matheson, O. Yilmaz, M.G. Vander Heiden, Dissecting cell-type-specific metabolism in pancreatic ductal adenocarcinoma, Elife, 9, 2020, e56782.
[35] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, Nat Rev Cancer, 11, 2011, 85–95.
[36] E.D. Montal, R. Dewi, K. Bhalla, L. Ou, B.J. Hwang, A.E. Ropell, C. Gordon, W.J. Liu, R.J. DeBerardinis, J. Sudderth, W. Twaddel, L.G. Boros, K.R. Shroyer, S. Duraisamy, R. Drapkin, R.S. Powers, J.M. Rohde, M.B. Boxer, K.K. Wong, G.D. Girnun, PEPCK coordinates the regulation of central carbon metabolism to promote cancer cell growth, Mol Cell, 60, 2015, 571–583.
[37] H. Krebs, W.A. Johnson, The role of citric acid in intermediate metabolism in animal tissues, FEBS letters, 117, 1980, K2-K10.
[38] J. Eniafe, S. Jiang, The functional roles of TCA cycle metabolites in cancer, Oncogene, 40, 2021, 3351–3363.
[39] K. Dettmer, F.C. Vogl, A.P. Ritter, W. Zhu, N. Nürnberger, M. Kreutz, P.J. Oefner, W. Gronwald, E. Gottfried, Distinct metabolic differences between various human cancer and primary cells, Electrophor, 34, 2013, 2836–2847.
[40] D.A. Scott, A.D. Richardson, F.V. Filipp, C.A. Knutzen, G.G. Chiang, Z.A. Ronai, A.L. Osterman, J.W. Smith, Comparative metabolic flux profiling of melanoma cell lines beyond the warburg effect, J Biol Chem, 286, 2011, 42626–42634.
[41] G. Czibik, V. Steeples, A. Yavari, H. Ashrafian, Citric acid cycle intermediates in cardioprotection, Circ Cardiovasc Genet, 7, 2014, 711–719.
[42] K. Smolková, A. Dvořák, J. Zelenka, L. Vítek, P. Ježek, Reductive carboxylation and 2-hydroxyglutarate formation by wild-type IDH2 in breast carcinoma cells, Int J Biochem Cell Biol, 65, 2015, 125–133.
[43] P.K. Mukherjee, P. Funchain, M. Retuerto, R.J. Jurevic, N. Fowler, B. Burkey, C. Eng, M.A. Ghannouma, Metabolomic analysis identifies differentially produced oral metabolites, including the oncometabolite 2-hydroxyglutarate, in patients with head and neck squamous cell carcinoma, BBA Clin, 7, 2017, 8–15.