Significance of the glycolytic pathway and glycolysis related-genes in tumorigenesis of human colorectal cancers

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Received June 13, 2007; Accepted August 17, 2007

Abstract. We investigated gene expressions involved in the glycolytic pathways in colorectal cancer. The study was designed to use gene ontology and its relevant bioinformatics tools to analyze the microarray data obtained from CRC tissues and their corresponding normal tissues, in order to explore the correlation between the glycolytic metabolic pathway and possible pathogenesis of this disease. The overexpression of glycolysis-related genes was observed in over 76% of CRC tissues. In addition, we stimulated the SW480 and SW620 CRC cell lines with 15 mM D-(+)-glucose and 10 mM 2-deoxy-D-glucose respectively. The results indicate that the proliferation response of both the SW480 and SW620 cell lines increased remarkably with a time-dependent effect by D-(+)-glucose administration. In contrast, the proliferation response of both the SW480 and SW620 cell lines was significantly inhibited by 2-DG administration. Likewise, further analyses of the expression of related genes triggered by the D-(+)-glucose *in vivo* show that the activation process of these eight genes - GLUT1, HK1, GPI, GAPD, PGK1, PGK2, ENO2, PKM2 - prominently increased with a time-dependent effect. In conclusion, this study demonstrates that the glycolytic pathway and glycolysis-related genes may play an important role in the tumorigenesis of CRC, but their molecular mechanisms need further investigation to verify this.

Introduction

Colorectal cancer (CRC) is a primary cause of morbidity and mortality in Europe and the USA. Each year, about 300,000 new cases and 200,000 deaths due to CRC are reported in these areas (1-3). CRC is also one of the most frequent malignancies and has the third highest mortality rate of all the cancers in Taiwan, affecting and causing over 7,000 new cases and 3,000 deaths per year (4-6). As investigative studies have reported, approximately 30-40% of CRC patients who have undergone curative resection afterwards develop metastatic disease and die within 5 years (7,8).

Little is known about the details of the molecular mechanism of CRC development; therefore, difficulties exist in its prevention and treatment. Consequently, knowing how to rapidly organize new techniques for exploring the molecular mechanisms of CRC is key to providing researchers with the information regarding detailed functional analyses of turmorigenesis. In order to explore the molecular mechanisms underlying the tumorigenesis of CRC, high-throughput and efficient tools are used, such as comparative genomic hybridization (CGH), microarray and proteomics, which can analyze the activation of differential molecular factors between the normal mucosal tissues and the cancerous tissues. Afterwards bioinformatics tools are employed to predict the relevant mechanisms of CRC turmorigenesis.

Previously, we have observed that constructed membrane-array techniques can be effective and efficient for the confirmation of overexpression of related genes in human cancerous tissues (9-11). Recently, we have demonstrated that fatty acid metabolism plays an important role in turmorigenesis of human CRCs by microarray-bioinformatics analysis (12). We have also obtained preliminary results from the same experiments that indicate the glycolysis pathway might be imperative for tumorigenesis of CRC. Although several studies have proved that malignant tumor cells increase the glycolytic activity in many malignant diseases *in vivo* and *in vitro* (13-16), whether the glycolytic pathway and glycolysis-related genes participate in tumorigenesis of human CRC has yet to be elucidated.

In this study therefore, we first attempted to verify the activation of these genes relevant to the glycolytic pathway in human CRC tissues. Moreover, we stimulated the CRC cell lines by D-(+)-glucose and 2-deoxy-D-glucose (2-DG, a non-metabolizable glucose analogue) and observed their effect on cell proliferation; in addition we inhibited the CRC cell line by 2-DG administration. Finally, we sought to clarify

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Key words: glycolytic pathways, glycolysis related-genes, membrane array, 2-deoxy-D-glucose, turmorigenesis, colorectal cancer

Table I. Clinicopathological characteristics of CRC patients.

No.	Sex	Age (years)	Size (cm)	Differentiation[a]	Location	UICC
3	F	54	4.2x3.0	MD	Sigmoid colon	II
9	F	48	8.8x5.6	PD	Descending colon	III
17	M	61	4.5x3.9	PD	Descending colon	III
20	M	70	2.0x1.5	WD	Ascending colon	I
25	F	58	6.7x2.9	MD	Descending colon	II
31	M	76	8.15x6.5	PD	Rectum colon	IV
45	M	27	5.0x3.8	MD	Descending colon	II
49	F	62	5.7x4.6	PD	Descending colon	III
64	F	55	3.0x4.5	WD	Ascending colon	I
75	M	71	10.5x5.7	MD	Rectum	II
78	M	70	5.85x4.1	PD	Sigmoid colon	IV
82	M	51	5.0x4.5	MD	Descending colon	II
85	F	59	7.2x6.2	PD	Sigmoid colon	IV
89	M	67	12.0x9.8	PD	Rectum colon	III
91	F	73	7.5x6.7	WD	Rectum	I
93	M	38	3.5x4.5	MD	Rectum	II
99	M	55	7.85x5.3	PD	Sigmoid colon	IV
102	F	93	4.0x3.5	MD	Transverse colon	II
116	F	85	8.2x3.7	PD	Sigmoid colon	IV
127	M	68	6.1x2.9	WD	Ascending colon	I
134	F	79	11.5x6.5	PD	Sigmoid colon	IV
143	M	71	10.5x7.8	PD	Transverse colon	III
157	M	66	5.8x4.0	PD	Rectosigmoid colon	III
179	F	53	3.8x7.2	WD	Ascending colon	I
207	F	66	3.2x2.9	MD	Ascending colon	II

[a]WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

the possible molecular mechanisms underlying the turmorigenesis of CRC and its association to the glycolytic pathway and glycolysis-related genes.

Patients and methods

Sample collection. Twenty-five patients, ranging in age from 27 to 93 years (mean, 63.04), who had undergone resection of CRC, were selected randomly from a patient group at the Department of Surgery of Kaohsiung Medical University Hospital between August 2004 and January 2006. None of these patients had received any preoperative radiotherapy or chemotherapy. Soon after resection, the specimens were opened in the operating room and photographed for documentation. According to the prospective protocol, the tumors and all the lymph nodes were cut at different levels and embedded in paraffin, and sections were taken for routine H&E staining. Senior pathologists checked all the slides and documented the pathological characteristics of the tumor and lymph nodes. The tumor stage was defined according to the criteria of the American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC, 2002). The results showed 5 patients with UICC stage I, 8 with UICC stage II, 6 with UICC stage III and 6 with UICC stage IV CRC (Table I). The surgical tissue samples upon acquisition were frozen instantly in liquid nitrogen, and then stored at -80˚C until analysis. All sample acquisition and subsequent use was also approved by the Institutional Review Board of the Kaohsiung Medical University Hospital.

Total-RNA extraction and first strand cDNA synthesis. Total-RNA was isolated from patient tissue and cell specimen samples with Isogen™ (Nippon Gene Co., Ltd., Toyama, Japan) and QIAmp® RNA Blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions (17). The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm (Beckman, DU800, USA). First strand cDNA was synthesized from total-RNA by using a RT-PCR kit (Promega Corp., Madison, WI). The reverse transcription was carried out in a reaction mixture consisting of 1X transcription optimized 5X buffer, 25 μg/ml oligo(dT)15mer primer, 100 mmol/l PCR nucleotide mix, 200 μmol/l M-MLV reverse transcriptase, and 25 μl of recombinant RNasin® ribonuclease inhibitor (Promega). The

reaction mixtures with RNA were incubated at 42˚C for longer than 2 h, heated to 95˚C for 5 min, and then stored at -80˚C until analysis.

Oligo membrane array preparation. The procedure of the membrane-array method for the detection of CRC-related genes was performed in accordance with our previous work (9-11). Visual OMP3 (oligonucleotide modeling platform, DNA Software, Ann Arbor, MN) was applied to design oligonucleotide probe sequences for target genes and ß-actin, and the latter served as an internal control (Table II). The newly synthesized oligonucleotide fragments were dissolved in diswater to a concentration of 200 mM and then loaded to a BioJet Plus 3000 nanoliter dispense system (BioDot Inc., Irvine, CA), which blotted the 33 target oligonucleotides and TB, ß-actin control sequentially (0.05 μl per spot and 1.5 mm between spots) on SuPerCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate. DMSO was also dispensed onto the membrane as a blank control (Fig. 1A). After rapid drying and cross-linking procedures, the preparation of CRC diagnostic membrane array was accomplished (10).

Preparation of digoxigenin-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were produced using SuperScript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD) in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany). After the pre-hybridization procedure, the gene chips were subjected to hybridization. The lifts were covered with Express Hyb Hybridization Solution (BD Biosciences, Palo Alto, CA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics GmbH) and blocking. For hybridization, the arrays were incubated at 42˚C for 12 h in a humidity chamber. After washing, the arrays were exposed to light. For signal detection, the gene chips were incubated in chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro3-indoyl-phosphate (NBT/BCIP) for 15 min. The hybridize arrays were then scanned with an Epson Perfection 1670 flat bed scanner (SEIKO EPSON Corp., Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out using AlphaEase® FC software (Alpha Innotech Corp., San Leandro, CA). Spots consistently carrying a factor of two or more were considered as differentially expressed. These array analysis tools facilitated the measurement of relative gray levels of objects in a uniformly spaced array, such as dot blots. A deformable template extracted the gene spots and quantified their expression levels by integrated intensity of spot after background subtraction. The fold ratio for each gene was calculated as follows: spot intensity ratio = mean intensity of target gene/mean intensity of ß-actin. The membrane array was used to detect the expression of glycolysis-associated genes in CRC tissues and in CRC cell lines after treatment with D-(+)-glucose and 2-DG.

Cell culture and cell treatment. A human colon adenocarcinoma cell line SW480 (Dukes' B) and SW620 (Dukes' C) was obtained from American Type Culture Collection (CCL-228 and CCL-227; ATCC, Rockville, MD). The cells were maintained in Leibovitz's L-15 medium (Gibco Life Sciences, BRL, Grand Island, NY) supplemented with 10% (v/v) of fetal bovine serum (FBS) at 37˚C in humidified atmospheric air without CO_2 addition. All media was supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin and 0.025 mg/ml fungizone. Cell density was maintained at ~10,000 cells per milliliter of medium in T-25 flasks (Corning, NY). When grown to confluent monolayer, the cells were harvested by washing the dishes once with phosphate-buffered saline (PBS), pH 7.3, and then incubated in phosphate-buffered saline containing 0.53 mmol/l EDTA and 0.05% trypsin (Gibco-BRL) for 10-15 min at 37˚C. The trypsinized cells were counted and cell viability was assessed by trypan blue dye exclusion. Furthermore, for treatment with 2-DG (Sigma), cells were prepared as above, with the addition of 15 mM D-(+)-glucose (18,19) and 10 mM 2-DG (final) (20) to the culture media immediately preceding 37˚C in a CO_2-free humidified atmosphere.

Cell proliferation assay. The MTT cell proliferation assay determined the ability of viable cells to reduce the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) to blue-colored formazan crystals by mitochondrial enzymes. The concentration of formazan crystals can be spectrophotometrically determined when dissolved in an organic solvent (21). To counteract the glycolytic effect of D-(+)-glucose, 2-DG (Sigma), a non-metabolizable glucose analogue that could block glycolysis and inhibit protein glycosylation, was used. Cell suspensions were plated at 10,000 cells per well in (100 μl/well) 96-well microplate (Corning, NY); the cell proliferation effect of D-(+)-glucose and 2-DG against SW480 and SW620 cells were analyzed by the MTT assay kit (Roche, NJ). The growth inhibitory rate of treated cells was calculated using test data by (ODtest-ODcontrol)/ODcontrol x 100%. The experiments were carried out using a previously reported procedure (22). Absorbance was measured at 570 nm and readings expressed as viability percentages using DMSO-treated as negative controls (100% of viability). Three independent experiments were performed in triplicate and the results shown in the figures represent the means of one of these reproducible experiments.

Lactate concentration measurements. After cells were rinsed twice with phosphate-buffered saline, fresh medium was added to the cultures with and without each of the glucose analogs tested, at the indicated concentrations, and the cells were incubated at 37˚C in humidified atmospheric air without CO_2 addition for 6, 12, 16 and 20 h. Then, SW480 and SW620 cell media were collected for lactate release measurements. The supernatant was centrifuged three more times, to yield a final clear supernatant. Aliquots of the culture medium were removed for content analysis. Then, the absorbance was measured at 540 nm using a Multiskan® EX ELISA Reader (Thermo Electron Corp., Finland) with a linear range of stander lactate concentrations according to the procedures recommended by the manufacturer (Trinity Biotech, Jamestown, NY). The intra-assay coefficient of variation was 1.1%. Samples were analyzed in triplicate.

Table II. Oligonucleotides for membrane arrays.

Gene name[a]	Oligonucleotides (5'-3')
HK1	TGGTACAAAGTTTACTAGGTCATACGACACGGCTCACAAAGCGGTGGGAA
GCK	AGCCACTCAGTGATGGTATGGGAGAGAAAAGGTCTCTGGGGGGTATTCAA
GPI	AATGTTGGAGACATACCAGACGCGGGGACCTCCTGAAGAGTATGGCTTAA
PFKL	ACGAGAGGACTCTCGACCTCTACCCTGGTTACACATGCTTCTCATCCGCT
PFKM	AACCACTCTTAGATACCGGGGGTCTGACATGAGCTAGAAGCTTGTACACC
PFKP	TCAAACACGCCTTTGCCCTCTTCTGAATACAGCTGGTAAATGAAGTCGGT
ALDOA	AGGTGCCTTCCAGGTAGATGTGGTGGTCACTCAGAGCCTTGTAGACAGCA
ALDOB	ATTGGACCCTAGTAGGTATAGCAGGCTGTGAAGAGCGACTGGGTGGAAGC
ALDOC	AGGGCTGTGGTATGGAGTGGATACTCAGTAGGCATGGTTGGCAATGTAGA
TPI1	CAGGACGACCTTGCTCCAGTCCTTCACGTTATCTGCGATGACCTTTGTCT
GAPD	AGTGGGTGTCGCTGTTGAAGTCAGAGGAGACCACCTGGTGCTCAGTGTAG
GAPDS	GAAAGAAAGGACCTTCCCGTTTCACTTGTCTCGGCTGAACATGTAGCGGA
PGAM1	TCTGCATACCTGCGATCCTTACTGATGTTGCTGTAGAAAGGATGGTCGGG
PGAM2	ACCGACGCTCCTTGCTAATGGAGTTGTAGTAGGGGTGCTTCTCGTCCATC
BPGM	AGCTTTCCGACCGTGGCAGTTGATCCAAGGGCACATCGCATACTTTATAC
PGK1	CATTCAAATACCCCCACAGGACCATTCCACACAATCTGCTTAGCCCGAGT
PGK2	TTATCTTCAGTGTTCCATTTGGCACAGCAAGTAGCAGTGTCTCCACCCCC
ACYP1	ACCCAGCCTACCAATCCCAGCTTTTTACCCTCAGCCTGAGTATGCTTACG
ACYP2	CTGTCACGGTGCCTTTGCTGGTATTCTTCACCCAGCCAACCACTCCTATT
ENO1	TGACCACCTTATCAGTGTAGCCAGCTTTCCCAATAGCAGTCTTCAGCAGC
ENO2	ACGTGGGACAAGAGCAAAGCACAAGTGACACATGGTCCCTCTCTAACACC
ENO3	AGATCGTACTTCCCATTGCGATAGAACTCAGATGCTGCCACATCCATGCC
PKM2	AGGAACTGGACAGAGTACACACAGGAAAGGAAGCTGTCACCCTCTTGCCA
PKLR	GGTCAGGAATAGAGAAGAGAGGACTTAAAGGTGGGGCTTTGGAGGGGTGT
GLUT1	CAACCCCACTTACTTCTGTCTCACTCCCATCCAAACCTCCTACCCTCAAT
GLUT3	AGAAATGGGACCCTGCCTTACTGCCAACCTACTGTTTGAGGAGCCAGAAA
GALM	TGCTGTATAGAGACAGGTCCATCTCCTAGCATGCAGGGTGAGGCTAATGT
PGM1	AATTGAGATTGATCTAAGCCCGCAGGTCCTCTTTCCCTCACAGCTCCCAA
PGM3	TAGAGAGCATCCCACTGTTGTACAGTCAAGCCCTTCAGAGCCAAGATTGC
LDHA	ACAGCATTGGCAGTGGTGCGTCAGAGGTGGCAGAACTATTTCACACTAAC
LDHB	TGGATGTCCCACAGGGTATCTGCACTTTTCTTGAGCTGAGCAACCTCATC
LDHC	TCTGAGACACCATTCCGCCCCAAGACACAAGGGATACTGAGAAAGAGTTC
G6PC	AAGGAGTCAAAGACGTGCAGGAGGACGAGGGAGGCTACAATAGAGCTGAG
TB	GAGTTCACGGATATTGCGTTCGATACTGCTGGCGATGAGTTCGAGGACAT
ß-actin	TGCATTGTTACAGGAAGTCCCTTGCCATCCTAAAAGCCACCCCACTTCTCTCTAAGGAGA

[a]HK1, nuclear gene encoding mitochondrial protein, transcript variant 1; GCK, hexokinase 4 (maturity onset diabetes of the young 2), transcript variant 1; GPI, glucose phosphate isomerase; PFKL, phosphofructokinase, liver, transcript variant 1; PFKM, phosphofructokinase, muscle; PFKP, phosphofructokinase, platelet; ALDOA, aldolase A, fructose-bisphosphate, transcript variant 1; ALDOB, aldolase B, fructose-bisphosphate; ALDOC, aldolase C, fructose-bisphosphate; TPI1, triosephosphate isomerase 1; GAPD, glyceraldehyde-3-phosphate dehydrogenase; GAPDS, glyceraldehyde-3-phosphate dehydrogenase, spermatogenic; PGAM1, phosphoglycerate mutase 1 (brain); PGAM2, phosphoglycerate mutase 2 (muscle); BPGM, 2,3-bisphosphoglycerate mutase, transcript variant 1; PGK1, phosphoglycerate kinase 1; PGK2, phosphoglycerate kinase 2; ACYP1, acylphosphatase 1, erythrocyte (common) type; ACYP2, acylphosphatase 2, muscle type; ENO1, enolase 1 (α); ENO2, enolase 2 (γ, neuronal); ENO3, enolase 3 (ß, muscle), transcript variant 1; PKM2, pyruvate kinase, muscle, transcript variant 1; PKLR, pyruvate kinase, liver and RBC, nuclear gene encoding mitochondrial protein, transcript variant 1; GLUT1, solute carrier family 2 (facilitated glucose transporter), member 1; GLUT3, solute carrier family 2 (facilitated glucose transporter), member 3; GALM, galactose mutarotase (aldose 1-epimerase); PGM1, phosphoglucomutase 1; PGM3, phosphoglucomutase 3; LDHA, lactate dehydrogenase A; LDHB, lactate dehydrogenase B; LDHC, lactate dehydrogenase C, transcript variant 1; G6PC, glucose-6-phosphatase, catalytic (glycogen storage disease type I, von Gierke disease); TB, Mycobacterium tuberculosis; ß-actin, actin, ß (ACTB).

Statistical analysis. The data were analyzed using the Statistical Package for the Social Sciences Ver. 12.0 software (SPSS Inc., Chicago, IL). Student's t-test was used to compare SW480 and SW620 cell proliferation ratios at various timings in the presence of 15 mM D-(+)-glucose and 10 mM 2-DG. The data are reported as mean ± SE of at least three independent experiments. Statistical analysis was performed using unpaired Student's t-tests to determine significance between two means. A probability of <0.05 was accepted as significant.

A

ß-actin	Blank	TB	HK1	GCK	GPI	PFKL	PFKM	PFKP	ALDOA	ALDOB	ALDOC
ß-actin	Blank	TB	HK1	GCK	GPI	PFKL	PFKM	PFKP	ALDOA	ALDOB	ALDOC
ß-actin	Blank	TB	HK1	GCK	GPI	PFKL	PFKM	PFKP	ALDOA	ALDOB	ALDOC
TPI1	GAPD	GAPDS	PGAM1	PGAM2	BPGM	PGK1	PGK2	ACYP1	ACYP2	ENO1	ENO2
TPI1	GAPD	GAPDS	PGAM1	PGAM2	BPGM	PGK1	PGK2	ACYP1	ACYP2	ENO1	ENO2
TPI1	GAPD	GAPDS	PGAM1	PGAM2	BPGM	PGK1	PGK2	ACYP1	ACYP2	ENO1	ENO2
ENO3	PKM2	PKLR	GLUT1	GLUT3	GALM	PGM1	PGM3	LDHA	LDHB	LDHC	G6PC
ENO3	PKM2	PKLR	GLUT1	GLUT3	GALM	PGM1	PGM3	LDHA	LDHB	LDHC	G6PC
ENO3	PKM2	PKLR	GLUT1	GLUT3	GALM	PGM1	PGM3	LDHA	LDHB	LDHC	G6PC

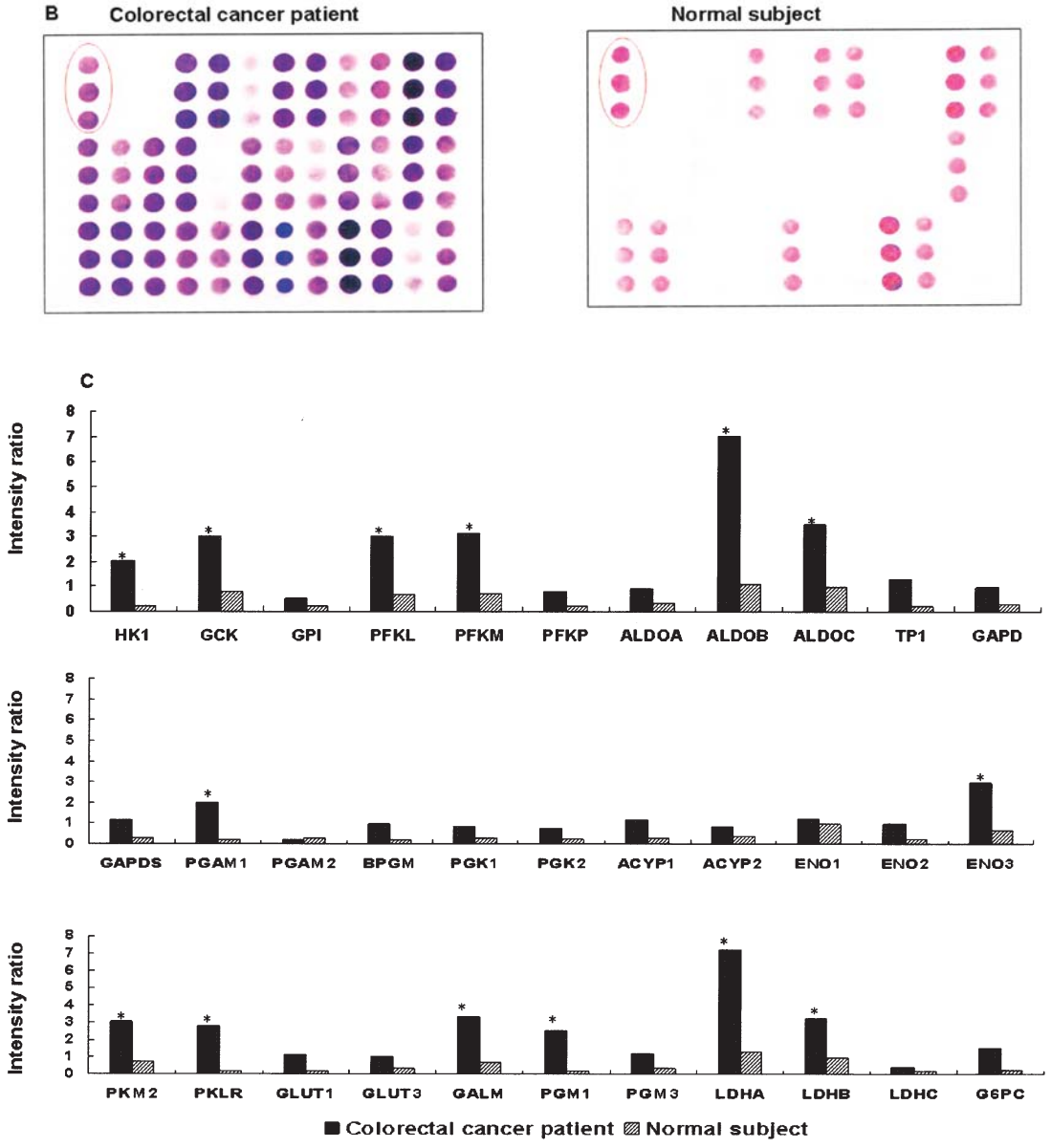

Figure 1. Gene expression profile of human CRC (case 91) was detected with the membrane array. (A) Thirty-three target genes, the position of correlation of the blank and positive control as well as the negative control in the nylon membrane is pointed out by spots. (B) The concentration of each spot is 200 μM. The membrane array includes purified positive control of human ß-actin, negative control of TB and target genes, (HK1, GCK, GPI, PFKL, PFKM, PFKP, ALDOA, ALDOB, ALDOC, TPI1, GAPDH, GAPDHS, PGAM1, PGAM2, BPGM, PGK1, PGK2, ACYP1, ACYP2, ENO1, ENO2, ENO3, PKM2, PKLR, GLUT1, GLUT3, GALM, PGM1, PGM3, LDHA, LDHB, LDHC and G6PC) which have been spotted in triplicate, and detected by a DIG detection kit (as described in Patients and methods). The fold ratio for each gene was calculated as follows: spot intensity ratio = mean intensity of target gene/mean intensity of ß-actin. On the left side, the chart shows the gene expression of CRC patients. On the right side, the chart shows the gene expression of normal subjects. The genes within red circle at the right bottom corner of each image represent ß-actin (positive control). (C) Quantification of each gene as shown in (B) (n=3; mean ± SEM). *P<0.05 vs. control.

Table III. Over- and under-expressed genes involved in glycolysis pathway in colorectal cancer.

Gene	HK1	GCK	GPI	PFKL	PFKM	PFKP	ALDOA	ALDOB	ALDOC	TPI1	GAPD	GAPDS	PGAM1	PGAM2	BPGM	PGK1	PGK2
Ratio[a] >1.0	19	25	20	20	20	20	25	25	20	23	20	19	25	23	23	23	25
Ratio <1.0	6	0	5	5	5	5	0	0	5	2	5	6	0	2	2	2	0
Overexpression (%)	76	100	80	80	80	80	100	100	80	92	80	76	100	92	92	92	100

Gene	ACYP1	ACYP2	ENO1	ENO2	ENO3	PKM2	PKLR	GLUT1	GLUT3	GALM	PGM1	PGM3	LDHA	LDHB	LDHC	G6PC
Ratio >1.0	23	25	25	25	25	22	19	25	23	23	25	25	25	23	25	25
Ratio <1.0	2	0	0	0	0	3	6	0	2	2	0	0	0	2	0	0
Overexpression (%)	92	100	100	100	100	88	76	100	92	92	100	100	100	92	100	100

[a]Gene expression ratio of adenocarcinoma/normal tissue.

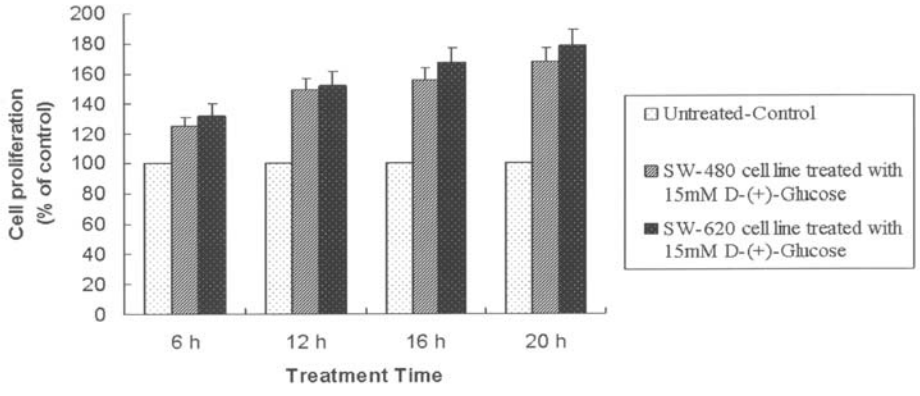

Figure 2. Cell proliferation ratio of SW480 and SW620 human colon cancer cells supplemented with 15 mM D-(+)-glucose. The cells were seeded at 10,000 cells/ well in 96-well plates and cultured for 6, 12, 16 and 20 h respectively, and tested by MTT assay as described in Patients and methods. The values of the D-(+)-glucose-treated samples were compared with the DMSO-treated control. Values are the pooled means with SEM of three experiments.

Results

Differentially expressed genes in the glycolytic pathway. Our recent work has shown that the possible genes involved in metabolic pathways of CRC development were further sub-categorized to be bile acid (25.1%), glycolysis (23.1%), fatty acid (22.6%), pyruvate (10.2%) and others (19.0%) (12). In the context of gene ontology, these genes might play single or multiple roles contributing to the biological phenomena in cells. In the current experiment, we exclusively focused on the abnormality of gene expression relevant to the metabolic pathway of glycolysis. Therefore, we investigated in more depth not yet fully analyzed genes in the whole pathway to determine if the expression of these genes produced alteration in a consistent manner with CRC. In 25 clinical samples, the ratio of the gene's activation was as follows: GCK (100%), GPI (80%), PFKL (80%), PFKM (80%), PFKP (80%), ALDOA (100%), ALDOB (100%), ALDOC (80%), TPI1 (92%), GAPD (80%), PGAM1 (100%), PGAM2 (92%), BPGM (92%), PGK1 (92%), PGK2 (100%), ACYP1 (92%), ACYP2 (100%), ENO1 (100%), ENO2 (100%), ENO3 (100%), PKM2 (88%), GLUT1 (100%), GLUT3 (92%), GALM (92%), PGM1 (100%), PGM3 (100%), LDHA (100%), LDHB (92%), LDHC (100%), and G6PC (100%) genes (Fig. 1B and C). Overall, 80% of these genes were activated in CRC (Table III).

Cell proliferation assay by D-(+)-glucose and 2-deoxy-D-glucose. Results of cell proliferation analysis of the two cell lines are shown in the ideogram in Fig. 2. When SW480 and SW620 cells were exposed to various concentrations of D-(+)-glucose and 2-DG and treated for 6, 12, 16 and 20 h, cell proliferation significantly increased and decreased in a time-dependent manner, having stimulated the SW480 cell by the D-(+)-glucose, the viability values were 25, 49, 56 and 68%. However, having stimulated the SW620 cell by D-(+)-glucose, viability values were 32, 52, 67 and 78% in the MTT assay. Thus, both D-(+)-glucose (Fig. 2) and 2-DG (data not shown) may stimulate or affect the proliferation of the cells. In addition, the prominent overexpression of 33 glycolytic-associated genes was detected by using a membrane array after treatment with D-(+)-glucose. Membrane array showed

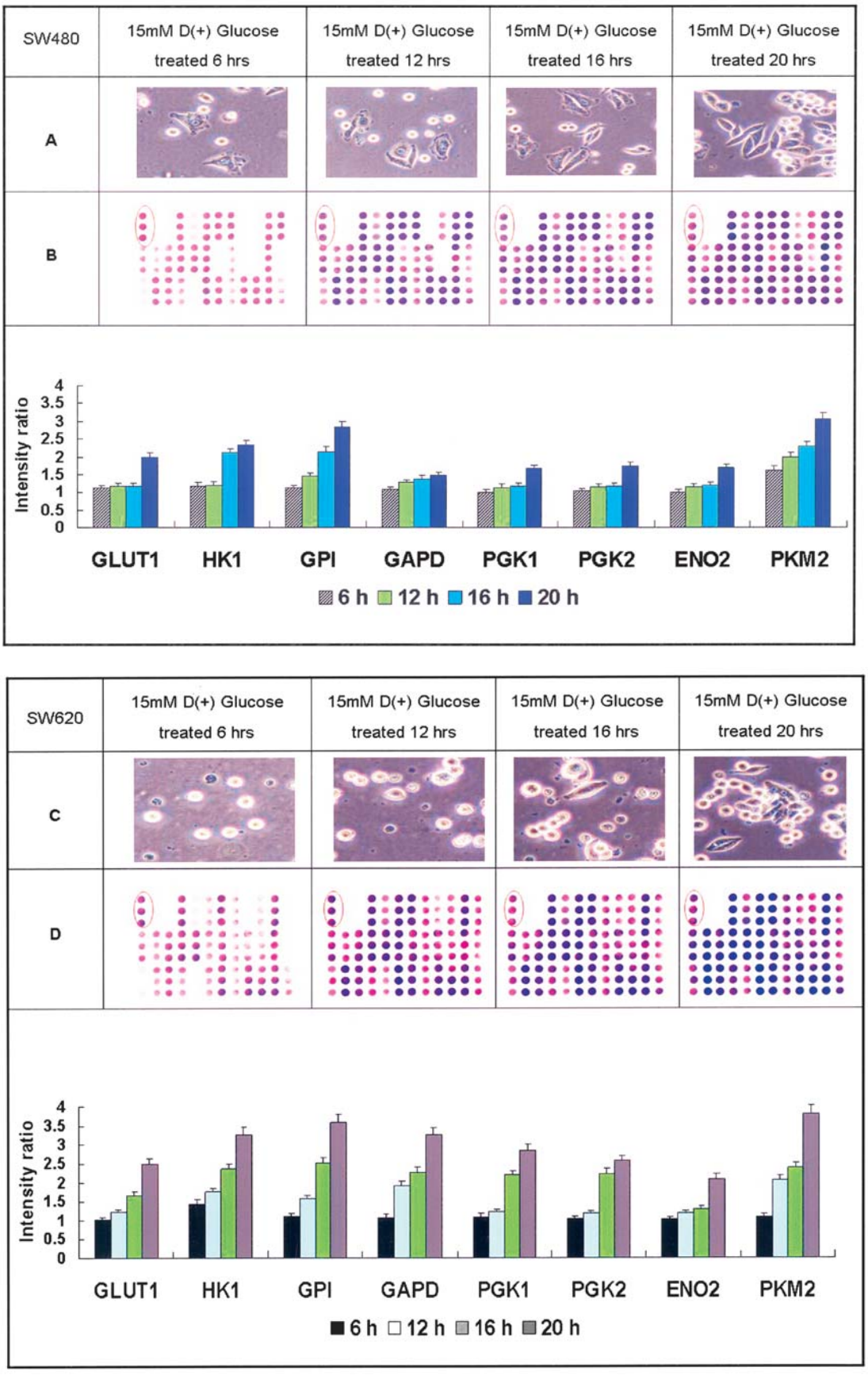

Figure 3. Effect of D-(+)-glucose on cell proliferation of gene expression of SW480 and SW620 cells were detected by using membrane array. In the MTT assays process, its experimental condition has been quoted as evidence at the same time. The cells were seeded at 10,000 cells/well in 6-well plates and cultured for 6, 12, 16 and 20 h respectively, and tested by membrane array as described in Patients and methods. Rows (A) and (C) show the situation of the growth of the cell line in the SW480 and SW620 human colon cancer cells stimulated by the D-(+)-glucose (15 mM) after 6, 12, 16 and 20 h, respectively. Rows (B) and (D) demonstrate the reaction of the glycolysis-associated genes in SW480 and SW620 human colon cancer cells stimulated by the D-(+)-glucose (15 mM) after 6, 12, 16 and 20 h, respectively. The genes within red circle at the right bottom corner of each image represent ß-actin (positive control).

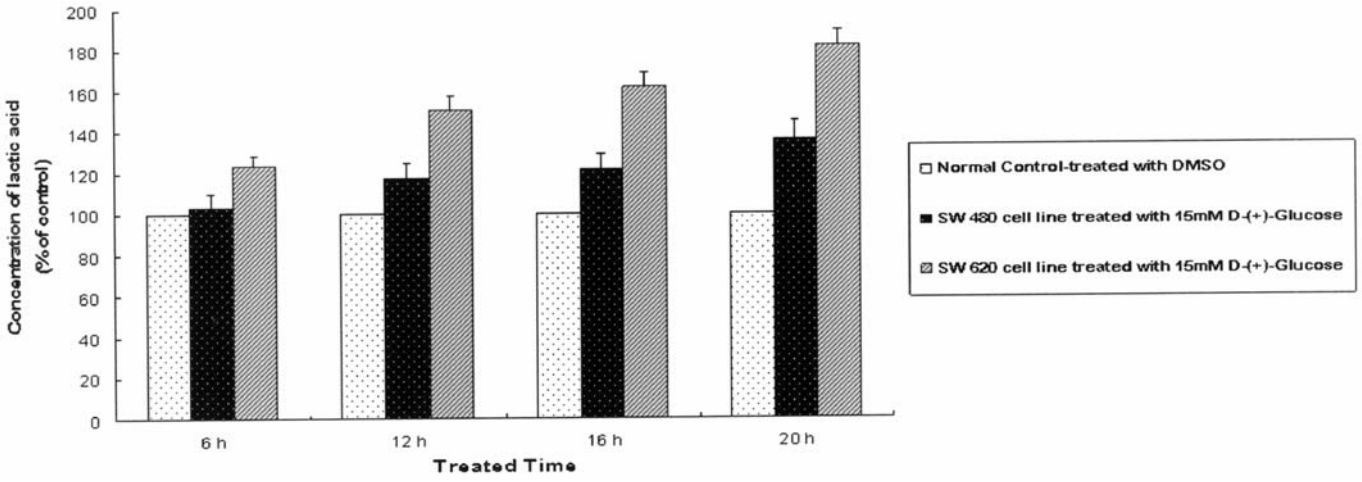

Figure 4. The results show that the concentration of lactate in the SW480 and SW620 cell lines is stimulated differently by the D-(+)-glucose (15 mM) after 6, 12, 16 and 20 h, respectively. The increase of the concentration of lactate is shown by the D-(+)-glucose administration. Afterwards, the stimulation of the D-(+)-glucose was conducted as described in Patients and methods. Values are the means ± SD of 10 independent experiments with triplicate dishes.

Table IV. Effect of glycolysis activity for lactate concentration by D-(+)-glucose of colorectal cancer cell lines.

	SW480 cell line		SW620 cell line	
Time (h)	Concentration (mg/dl)	Increase (%)	Concentration (mg/dl)	Increase (%)
6	20.39±0.06	3.25	26.86±0.11	23.39
12	27.56±0.23	17.11	39.36±0.20	50.95
16	31.64±0.20	21.14	46.40±0.22	62.04
20	38.13±0.15	35.84	57.98±0.30	81.63

gene expressions involving a total of 33 target gene in the two cell lines. Overall, gene expression of glycolytic related-genes were detected by using membrane array, and all the genes were determined to be overexpressed (Fig. 3).

Expression of glycolytic pathway related-genes in CRC cell lines. Lactate is assumed as the intermediate product of glycolysis pathway. Having stimulated the SW480 and SW620 cell lines by either the 15 mM D-(+)-glucose or the control DMSO at 6, 12, 16 and 20 h, the concentrations of lactate increased 3.25, 17.11, 21.14 and 35.84%, respectively by D-(+)-glucose administration in the SW480 cell line (Fig. 4

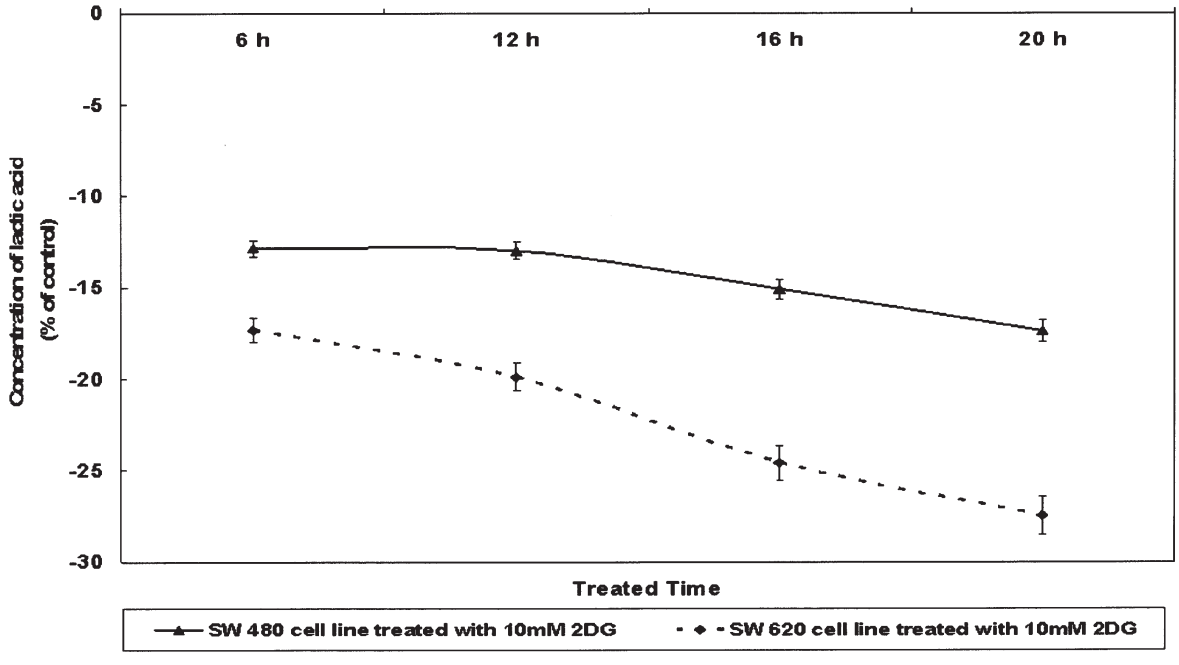

Figure 5. The results demonstrate that the decrease of the concentration of lactate in the SW480 and SW620 cell lines was individually stimulated by the 2-DG (10 mM) after 6, 12, 16 and 20 h, respectively. Afterwards, the stimulation of the 2-DG was conducted as described in Patients and methods. Values are the means ± SD of 10 independent experiments with triplicate dishes.

Table V. The stimulation of the D-(+)-glucose in the reaction of the CRC cell lines and their relevant genes.

		Gene expression of CRC cell lines	
		SW480 with 15 mM D-(+)-glucose	SW620 with 15 mM D-(+)-glucose
Pathway	Gene name	6/12/16/20 h	6/12/16/20 h
Glycolysis	GLUT1	1.12/1.17/1.19/1.99	1.01/1.23/1.67/2.49
	HK1	1.18/1.21/2.10/2.32	1.44/1.75/2.35/3.26
	GPI	1.13/1.46/2.14/2.84	1.10/1.58/2.52/3.58
	GAPD	1.08/1.27/1.38/1.48	1.07/1.93/2.27/3.25
	PGK1	1.01/1.14/1.17/1.68	1.09/1.22/2.19/2.84
	PGK2	1.02/1.15/1.18/1.75	1.04/1.19/2.22/2.56
	ENO2	1.01/1.16/1.21/1.69	1.02/1.18/1.29/2.09
	PKM2	1.62/1.99/2.29/3.07	1.08/2.05/2.37/3.99

After the stimulation, the results show the individual analysis of the SW480 and SW620 cell lines and their reaction. Eight genes, GLUT1, HK1, GPI, GAPD, PGK1, PGK2, ENO2 and PKM2, are coherently and successively increasing.

Table VI. The stimulation of the 2-DG in the reaction of the CRC cell lines and their relevant genes.

		Gene expression of CRC cell lines	
		SW480 with 10 mM 2-DG	SW620 with 10 mM 2-DG
Pathway	Gene name	6/12/16/20 h	6/12/16/20 h
Glycolysis	GLUT1	1.22/1.27/1.99/2.22	1.01/1.25/1.77/2.99
	HK1	1.20/1.48/2.26/2.49	1.41/1.89/2.03/2.97
	GPI	1.15/1.08/0.57/0.36	1.58/1.11/0.85/0.56
	GAPD	1.23/1.12/0.89/0.77	1.22/1.10/1.02/0.78
	PGK1	1.25/1.15/0.95/0.70	1.12/0.99/0.88/0.79
	PGK2	1.28/1.08/0.86/0.65	1.35/1.09/0.67/0.55
	ENO2	1.99/1.81/1.26/0.98	1.28/1.14/1.02/0.59
	PKM2	1.52/1.40/1.22/0.71	1.24/1.05/0.91/0.38

After the stimulation, the results show the individual analysis of the SW480 and SW620 cell lines and their reaction. Six genes, GPI, GAPD, PGK1, PGK1, ENO2 and PKM2, are coherently and successively decreasing.

and Table IV). In addition, the corresponding figures in the SW620 cell line by D-(+)-glucose administration increased 23.39, 50.59, 62.04 and 81.63% respectively. Moreover, when using either the 10 mM 2-DG or the control DMSO to inhibit the CRC cell lines at 6, 12, 16 and 20 h, the concentrations of the lactate decreased 12.86, 12.98, 15.09 and 17.41%, respectively in the SW480 cell line by 2-DG administration; similarly, they decreased 17.32, 19.39, 24.59 and 27.49%, respectively in the SW620 cell line by 2-DG administration (Fig. 5). Under the same conditions of stimulation, it was found that the activation of the CRC cells successively increased the activation of eight genes by D-(+)-glucose administration, including GLUT1, HK1, GPI, GAPD, PGK1, PGK2, ENO2, PKM2 (Table V). In contrast, by 2-DG administration, expression of six genes including GPI, GAPD, PGK1, PGK2, ENO2, and PKM2 was gradually decreased; whereas GLUT1 and HK1 were serially increased (Table VI).

Discussion

The turmorigenesis of CRC includes the possible participation of multiple molecular mechanisms, including the glycolysis pathway. With accumulated evidence, more investigators have demonstrated that high blood sugar levels may be correlated to the development of CRC (23-26). The molecular mechanisms leading to constitutive up-regulation of aerobic glycolysis are not properly clarified. From our previous microarray-bioinformatics analysis (12), this present research has subsequently demonstrated a remarkable and meaningful activation in the glycolytic pathway and glycolysis-related genes in CRC *in vivo* and *in vitro* through the verification of the glycolytic pathway and glycolysis-related genes. Moreover, cell proliferation of CRC cell lines significantly increases by D-(+)-glucose administration, whereas cell proliferation considerably decreases by 2-DG administration. The above observation has demonstrated that glucose may potentially be involved in the tumorigenesis of CRC. Meanwhile, a panel of glycolysis-related genes was found to be overexpressed in CRC tissues and CRC cell lines following the D-(+)-glucose and 2-DG treatments. Consequently, glycolysis-related genes may participate in the tumorigenesis of CRC.

Furthermore, this study attempts to establish a possible association between the glycolytic pathway and turmorigenesis of CRC. In the present study, the overexpression of GLUT1, HK1, GPI genes was identified in 100, 76 and 80% of CRC tissues. Concurrently, we found significant changes to glycolysis-related genes in SW480 and SW620 cell lines, including the up-regulation of GLUT1, HK1, GPI, GAPD, PGK1, PGK2, ENO2 and PKM2 following treatment with D-(+)-glucose; but found down-regulation of GPI, GAPD, PGK1, PGK2, ENO2 and PKM2 following treatment with 2-DG. The above glycolysis-related genes have been shown to be remarkably correlated to human cancers. For example, some investigators have indicated that GLUT1 mRNA is overexpressed in CRC tissues (27,28), and that both GLUT1 and hexokinase are also overexpressed in esophageal cancer *in vivo* (29,30). Consequently, high carbohydrates, through the up-regulation of a series of glycolysis-related genes, may subsequently activate the glycolytic pathway and is closely related to the tumorigenesis of CRC. GAPD is overexpressed in both ovarian and prostate cancers, and is proved to be crucial in metastatic properties (31-33). In addition, GAPD is associated with the proliferation and aggressiveness of breast cancer cells (34). The overexpression and elevated serum levels of phosphoglycerate kinase 1 are shown in pancreatic ductal adenocarcinoma (35). Moreover, serum ENO2 is considered to be a prognostic factor in small cell lung, breast, and prostrate cancer (36-39). Finally, PKM2 seems to be a more sensitive marker than conventional tumor markers such as CEA and CA72-4 for the post-operative surveillance of gastrointestinal cancer (40-44).

Alternatively, 2-DG is a glucose analogue, which acts as a competitive inhibitor of glucose metabolism (45), where 2-DG competes with D-glucose to be transferred to the cells as a target for the hexokinase in the first step of the

glycolytic pathway (46). Upon transport into the cells, glucose and 2-DG are phosphorylated to glucose-PO4 and 2-DG-PO4 respectively by the hexokinase. However, unlike glucose-PO4, the 2-DG-PO4 cannot further be metabolized by the GPI. In addition, 2-DG could inhibit GPI (47), and then down-regulate its downstream glycolysis-related genes. Consistent with results from the glycolytic pathway regulated by 2-DG, our data revealed that the upstream genes of GLUT1 and HK1 are overexpressed subsequent to 2-DG administration, and the downstream genes of GPI, GAPD, PGK1, PGK2, ENO2 and PKM2 are underexpressed in CRC cell lines. Therefore, this further discloses that glycolysis-related genes are critical in the tumorigenesis of CRC.

In conclusion, these results are consistent with the viewpoint that glycolysis has a promoting role in CRC development, and provides evidence of the modulation of cell proliferation by D-(+)-glucose. Future studies should focus on the marking of these relevant molecules that can potentially be helpful for CRC diagnosis or prevention.

Acknowledgements

The authors wish to express their thanks to the generous research support provided by Drs T.F. Chen and O.L. Hsu (the founders of Sunrider International Co.), through Give2Asia Foundation, to Kaohsiung Medical University Education and Development Fund. Thanks are also expressed to Dr K.-H. Chang, National Health Research Institute for his assistance in performing the statistical analysis.

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