Type 2 diabetes is more closely associated with risk of colorectal cancer based on elevated DNA methylation levels of ADCY5

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Received February 16, 2022; Accepted April 19, 2022

DOI: 10.3892/ol.2022.13327

Abstract. Type 2 diabetes mellitus (T2DM) has an increased risk of cancer. In the present study, the relationship between T2DM and 13 types of cancer was analyzed and key methylation genes were searched. First, DNA methylation and mRNA expression were obtained data for T2DM and 13 types of cancer from The Cancer Genome Atlas and Gene Expression Omnibus. The t-test was used to screen the differentially methylated expression overlapping genes (DE-MGs) in T2DM and cancer on both methylation and expression levels. DE-MGs are weighted based on the methylation and projected into the human protein interaction network. The correlation between T2DM and each type of cancer was analyzed, and key genes were identified. The results showed that 293 DE-MGs were related to T2DM and 3307 were related to cancer. The network found that T2DM is more related to colorectal cancer (CRC) compare with the other 12 types of cancer. A total of 5 from 8 candidate genes were associated with CRC. A total of 28 clinical patients were used to validate these 5 genes. A CRC tissue sample was collected from each patient, as well as a paracancerous sample that served as a control. A total of 56 tissue samples were divided into 4 groups: control group, T2DM group, CRC group and T2DM with CRC group (combination group). Compared with the control group, the methylation level of adenylate cyclase 5 (ADCY5), neuregulin 1 and ELAV-like RNA-binding protein 4 in the combination group was significantly upregulated, and the mRNA level was significantly downregulated. Furthermore, based on the methylation level of ADCY5, the correlation coefficient between the combination group and the T2DM group was greater than that of the CRC group. In conclusion, T2DM is most likely to be associated with CRC among 13 common types of cancer based on methylation characteristics. An upregulated methylation of ADCY5 in T2DM may have a higher risk of CRC.

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Key words: type 2 diabetes, colorectal cancer, DNA methylation, mRNA, biomarkers, adenylate cyclase 5

Introduction

Type 2 diabetes mellitus (T2DM) and cancer are the most common causes of death in the world, and the incidence of certain cancers is increasing in patients with T2DM, such as a total incidence of 0.95% for endometrial cancer in 2007 and 0.75% for colorectal cancer in 2011 (1-3). Meanwhile, a mortality rate of 7.5% in patients with T2DM with cancer was also observed in 2010 (1). Among them, the incidence of T2DM combined with digestive system tumors ranks first, for instance, the incidence of colorectal cancer (CRC) is 1.3-1.5% (4). As the risk of T2DM complicated with cancer has varied in recent years, it is necessary to systematically examine their relationship to discover potential biomarkers to detect cancer early and help understand pathogenesis.

Both T2DM and cancer are multifactorial diseases caused by the interaction of genes and the environment. On the one hand, the risk of these diseases is strongly influenced by genetic factors (5-7). Over the past 10 years, genome-wide association studies have found important applications in the genetics of complex diseases (8). Both share similar underlying pathophysiological pathways, including hyperinsulinemia, hyperglycemia, or chronic inflammation (9). Therefore, genes and their molecular mechanisms deserve attention. On the other hand, there is large heterogeneity in the clinical phenotypes of these diseases, mainly caused by environmental factors such as lifestyle. Epigenetic modifications, particularly DNA methylation modifications (10), are necessary mechanisms to connect the internal genetic genome with the external environment and influence pathogenesis (11).

The regulation of gene expression by DNA methylation has also been extensively studied in T2DM and cancer. DNA methylation may be involved in the development of obesity and insulin resistance in T2DM by regulating the expression of glycolysis and adipogenesis genes (12). Concurrently, the hypermethylation of CpG islands in the promoter region of tumor suppressor genes and the hypomethylation of oncogenes are involved in the occurrence and development of cancer by affecting different stages of cell growth and development (13). DNA methylation, which is associated

with both T2DM and cancer, was chosen as the entry point for the present study.

Finding the cancer types most likely to be associated with T2DM and their reliable DNA methylation genes as biomarkers can help predict disease risk in advance. In the present study, high-throughput omics data of DNA methylation and gene expression in T2DM and 13 cancers were first systematically integrated and analyzed, and then genes with differences in DNA methylation levels and mRNA expression levels were screened. Second, a weighted association network was constructed and analyzed based on methylation levels to identify the types of cancer and their key genes most associated with T2DM. Subsequently, tissue samples from clinical cancer patients were collected to verify the results.

Materials and methods

Data collection. The RNA-SeqV2 gene expression and DNA methylation data of human types of cancer were downloaded from The Cancer Genome Atlas database (TCGA; https://cancergenome.nih.gov/). Cancer with fewer than 10 cases in the case or control group were removed. Prostate cancer was also removed due to its inverse association with diabetes, unlike others reported positively associated. After sorting, a total of 13 types of cancer were included in the present study including breast cancer (BRCA), CRC, liver cancer (LIHC), pancreatic cancer (PAAD), bladder cancer (BLCA), esophageal cancer (ESCA), head and neck cancer (HNSC), renal clear cell carcinoma (KIRC), renal papillary cell carcinoma (KIRP), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), endometrioid carcinoma (UCEC) and thyroid cancer (THCA). Human T2DM mRNA expression datasets (GSE9006, GSE15653, GSE15932, GSE23343, GSE25462, GSE25724 and GSE55650) and DNA methylation datasets (GSE65057, GSE34008, GSE21232 and GSE38291) were downloaded from the Gene Expression Omnibus database (GEO; https: //www.ncbi.nlm.nih.gov/geo/). A summary of the data obtained in the TCGA database is revealed in Table I, and a summary of the data obtained in the GEO database is presented in Table II. Illumina HiSeq 2000 RNA was used to measure gene expression profiles, and Illumina Human Methylation 450 Beadchip (450 K array) (both from Illumina, Inc.) was used to measure DNA methylation data.

Screening of differentially methylated genes and differentially expressed genes. The levels of mRNA expression and DNA methylation were compared between the cancer and the normal control group to screen out cancer-related differentially expressed genes (cDEGs) and differentially methylated genes (cDMGs). The same approach was used to screen each dataset for cDEGs and cDMGs associated with T2DM. When using t-tests, the thresholds for defining DEGs were false discovery rate (FDR) <0.05 and |log2 fold difference [fold change (FC)]|>1.5. DMGs are those genes that satisfy the conditions of FDR<0.05 and |absolute difference|>0.2. Due to the large number of dDMGs, those in the top 10% of FDR values and located in the promoter region were selected. For each disease, results from different datasets were first united, and then DEGs and DMGs were intersected to obtain differentially methylated expression overlapping genes (DE-MGs).

Pathway enrichment and functional annotation of DE-MGs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Gene Ontology (GO) gene function annotations were performed on DE-MGs using David web tools (https://david.ncifcrf.gov/tools.jsp). Thresholds were FDR <0.05 and gene count >2.

Construction of gene interaction network. Spearman's correlation analysis was performed on the methylation levels of DE-MGs for each type of cancer. Each methylation data was perturbed 1,000 times and correlation coefficients were calculated. P<0.01 was considered to indicate a statistically significant difference and random coefficients with connectivity less than 0.2 were removed. Thus, significant gene pairs and their correlation coefficients were received. Then, the human protein-protein interaction (PPI) network was downloaded from the Human Protein Reference Database (http://www.hprd.org/). Significant gene pairs were mapped to a PPI network and correlation coefficients were used as weights for their edges. The DE-MG and its one-step neighbors were extracted as a sub-network, and different diseases were marked with different colors. Topological properties of the sub-networks were analyzed to identify candidate genes for T2DM-associated types of cancer.

Clinical sample collection. Samples were collected from 28 patients with CRC including 14 with T2DM and 14 without T2DM. There were no significant differences in mean age and sex distribution between the two groups. Each group included 10 males and 4 females. The mean age of the two groups was 66±4.45 and 62.5±7.07 years, respectively. There were no statistically significant differences in age and sex between the two groups. The clinicopathological characteristics of the patients are presented in Table III. All patients underwent CRC resection at the Affiliated Hospital of Harbin Medical University (Harbin, China) from March 2016 to December 2018 and the diagnosis was based on the pathology report. The present study was approved (approval no. KY2016-017) by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Written informed consent was obtained from all patients.

A CRC cancer tissue and a paracancerous tissue were collected from each patient. A total of 56 tissue samples were divided into 4 groups: i) adjacent tissues without T2DM (control group), ii) CRC tissues without T2DM (CRC group), iii) adjacent tissues with T2DM (T2DM group) and iv) CRC tissues with T2DM (combination group).

RNA extraction and related gene expression detection. Primer3 (http://primer3.ut.ee/) was used to design primers. The NCBI online primer design tool (primer-blast, http://www.ncbi.nlm.nih.gov/tools/primer-blast) and NCBI-Basic Local Alignment Search Tool were used for sequence alignment. Primers were synthesized by Shanghai Bioengineering Company (http://www.sangon.com). Primer sequences for mRNA and DNA methylation are revealed in Tables SI and SII, respectively.

All cancer tissues were frozen in liquid nitrogen immediately after resection and stored at -80˚C until analysis. Total RNA was extracted from tissues with TRIzol® reagent

Table I. Number of cases for each cancer type in The Cancer Genome Atlas database.

	Gene expression dataset		DNA methylation dataset	
Type of malignancy	Cancer	Normal	Cancer	Normal
Breast cancer	1104	114	793	96
Colorectal cancer	383	51	398	45
Liver cancer	373	50	373	48
Pancreatic cancer	179	4	185	10
Bladder cancer	407	19	419	21
Esophageal cancer	185	11	184	16
Head and neck cancer	522	44	527	50
Renal clear cell carcinoma	534	72	324	159
Renal papillary cell carcinoma	291	32	276	45
Lung adenocarcinoma	517	59	469	32
Lung squamous cell carcinoma	502	51	370	42
Endometrioid carcinoma	177	24	425	45
Thyroid cancer	513	59	501	56

Table II. Data summary of mRNA and DNA methylation of T2DM in GEO database.

Accession/ID	Platform	Tissue	T2DM	Normal	Gene/DNA methylation
GSE25724	GPL96	Pancreatic islet	6	7	Gene
GSE9006	GPL96, GPL97	Blood	24	48	Gene
GSE15932	GPL570	Blood	8	8	Gene
GSE55650	GPL570	Skeletal muscle	12	11	Gene
GSE25462	GPL570	Skeletal muscle	10	40	Gene
GSE23343	GPL570	Liver	10	7	Gene
GSE15653	GPL96	Liver	9	9	Gene
GSE65057	GPL13534	Liver	9	15	DNA methylation
GSE21232	GPL8490	Pancreatic islet	5	11	DNA methylation
GSE34008	GPL8490	Blood	12	12	DNA methylation
GSE38291	GPL8490	Skeletal muscle	11	11	

DNA methylation T2DM, type 2 diabetes mellitus.

(Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed to generate cDNA using the PrimeScript® 1st Strand cDNA Synthesis kit according to the manufacturer's protocol (Takara Bio, Inc.). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the mRNA expression levels. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal reference to normalize sample-to-sample variations. qPCR was performed in triplicate using SYBR®Premix Ex Taq™ Master Mix (Takara Bio, Inc.) and a 7300 real-time PCR system (Thermo Fisher Scientific, Inc.). The qPCR analysis was performed as follows: A 10-μl aliquot of the PCR reaction mixture was prepared for each reaction, which included 5 μl of SYBR Premix Ex Taq II (2x), 0.2 μl of ROX Reference Dye (50x), 2 μl of each primer (2 μM), 1 μl of the template and 1.8 μl of DNase-free water. The reaction mixture was incubated in the wells of a 96-well plate at 95˚C for 1 min, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. Dissociation curve analysis of the PCR products was performed at the final stage of 60˚C to 95˚C. All reactions were performed in triplicate. The relative expression levels of the candidate genes were normalized to that of GAPDH and fold changes among the samples were calculated using the $2^{-\Delta\Delta Cq}$ method (14).

Detection of methylation levels. Primer3 (http://primer3.ut.ee/) was used to design primers. Further processing was conducted using the software (copyright number SW110703) of Shanghai Tianhao Biology (http://www.geneskybiotech.com). Sample processing was performed using the EZ DNA Methylation Gold kit (Zymo Research Corp.) to convert unmethylated cytosine to uracil in genomic DNA. The optimized multiplex PCR primer panel to was used to perform multiplex PCR amplification using the transformed sample genome as a template. After quality control, the amplification products of all multiplex

Table III. Clinicopathological characteristics of the patients.

Clinicopathological characteristics	CRC without T2DM	CRC with T2DM
Age, years	66±4.45	62.5±7.07
Sex		
Male	10	10
Female	4	4
Tumor stage		
T3	12	9
T4	2	5
Lymph node metastasis		
N0	10	9
N1	2	4
N2	2	1
Total	14	14

T2DM, type 2 diabetes mellitus; CRC, colorectal cancer.

PCR primer sets were pooled using the same sample genomic DNA as template, and it was ensured that the amount of primer amplification product at each site was equal. Specific index sequences compatible with the Illumina platform were introduced into the library ends by PCR amplification using primers with index sequences. The reaction uses an 11-cycle PCR program to minimize PCR tendency. Exponential PCR amplification products from all samples were equally mixed and tapped to obtain the final methyl target sequencing library. The fragment length distribution of this library was verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). After accurate quantification of library molarity, the library was finally sequenced in 2x150 bp paired-end sequencing mode on the Illumina Hiseq (Illumina, Inc.) platform to obtain FastQ data.

Statistical analysis. The unpaired t-test was used for comparison between two groups. One-way ANOVA followed by Tukey's post-hoc test was used for comparison between multiple groups. Correlation analysis was performed using Spearman's correlation analysis. All statistical analyses were performed using SPSS v. 21.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Acquisition and functional annotation of DE-MGs. Based on the data in the GEO database, there were a total of 293 DE-MGs associated with T2DM. There are 3307 cancer-related DE-MGs in the TCGA database, including 794 in BLCA, 599 in BRCA, 975 in CRC, 479 in ESCA, 604 in HNSC, 302 in KIRC, 303 in KIRP, and 586 in LIHC. There were 364 in LUAD, 902 in LUSC, 63 in PAAD, 74 in THCA, and 1023 in UCEC.

DE-MGs for each disease annotated GO gene function and enriched the KEGG pathway separately. For example, DE-MGs of T2DM annotated 20 important GO gene functions (Fig. 1), including c-AMP metabolism and regulation of adenylate cyclase activity, but no important KEGG pathway. DE-MGs of CRC were annotated to 412 important GO gene functions and 11 important KEGG pathways. The top 20% of GO functions and all KEGG pathways are revealed in Fig. 2. The complete results are revealed in Figs. S1-4.

Establishment and analysis of gene interaction network. As background, the PPI network consists of 13,368 proteins and 80,977 pairs of interactions. Spearman's correlation analysis was performed pairwise according to the methylation level of DE-MGs in each disease, and significant gene pairs ($P<0.01$) were mapped to PPI. Then, the DE-MGs and their one-step neighbors were extracted to obtain a sub-network (Fig. 3), including 373 nodes and 416 edges. The topological properties of the sub-network were analyzed, and the size of the nodes represents the connection value. Different colors are used to mark different diseases, such as T2DM in red.

In the sub-network, a node with multiple colors means that it participates in the process of multiple diseases. In Fig. 3, there are 8 comorbid genes of T2DM with types of cancer. Of these, 5 were associated with CRC, which was the highest frequency among all 8 comorbid genes. Therefore, at the DNA methylation level, T2DM was more closely related to CRC than the other 12 types of cancer. Adenylate cyclase 5 (ADCY5), neuregulin 1 (NRG1), mediator complex subunit 12L (MED12L), drive protein family member 5A (kinesin family member 5A, KIF5A) and ELAV-like RNA-binding protein 4 (ELAVL4) genes were further examined.

Clinical validation results. Compared with the control group, the methylation levels of candidate genes in the disease groups (CRC group, T2DM group, combined group) were increased. Compared with non-cancer group (control group, T2DM group), the methylation level of candidate genes in the cancer group (CRC group, combination group) was higher. At the same time, the mRNA expression levels were completely opposite (Fig. 4).

First, compared with the control group, ADCY5, NRG1 and ELAVL4 were significantly upregulated in the combination group, and their mRNA levels were significantly downregulated. However, MED12L only differed at the methylation level, and KIF5A only differed at the mRNA level. Furthermore, ADCY5 exhibited no significant difference between the combination group and the T2DM group and between the combination group and the CRC group regarding both methylation or mRNA level. However, the methylation and mRNA levels of NRG1 were different between the combination group and the T2DM group, and the methylation levels of ELAVL4 were different between the two aforementioned groups (Fig. 4). In addition, analysis of the correlation between ADCY5 and methylation levels in the combination, the T2DM group and the CRC group was also conducted. The correlation coefficient between the combination and the T2DM group was $r=0.732$. The correlation coefficient between the combination and the CRC group was $r=0.688$. The former is greater than the latter. The scatter plot for these correlation analyses is revealed in Fig. S5.

The data generated in the present study are also presented in Table SIII.

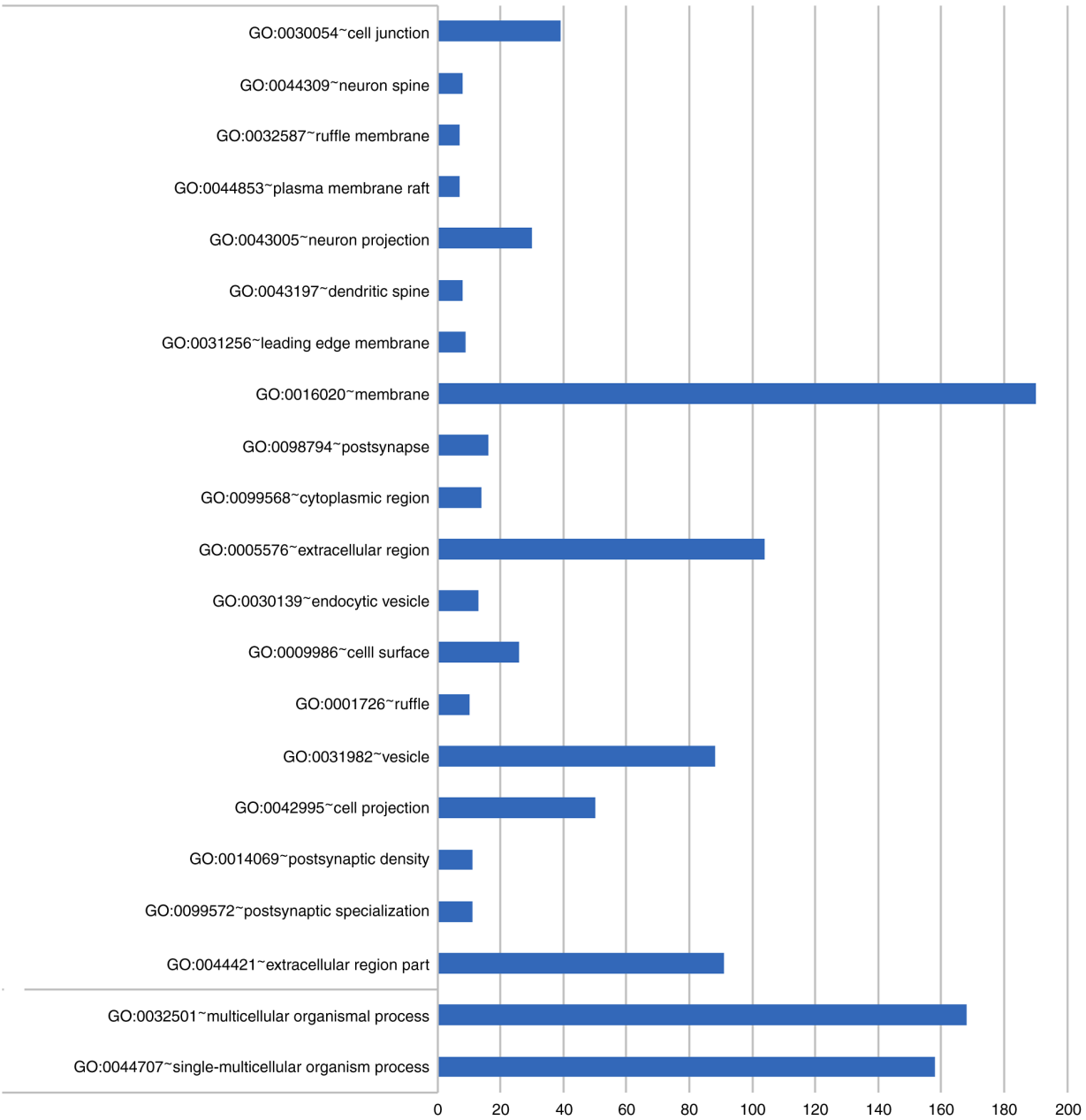

Figure 1. GO enrichment terms of dDE-MGs. The corrected P-value of false discovery rate is displayed in-log10 ratio. GO, Gene Ontology; DE-MGs, differentially methylated expression overlapping genes.

Discussion

DNA methylation is specific, particularly in gene promoter regions, and can be used as cancer marker (15). In the present study, DNA methylation and mRNA expression data were retrieved from databases for T2DM and 13 types of cancer and bioinformatics analysis was used to identify key genes associated with differential gene expression and differential methylation. Gene interaction weighted networks were constructed and analyzed using the correlations of methylation levels of these genes, to understand the interactions between genes in an improved way. Ultimately, 8 genes were identified as co-morbid genes for T2DM and cancer.

Since the information of different diseases is integrated in our network, the types of cancer involved in comorbid genes and their frequencies, as well as the connectivity values of gene nodes, all reflect the close relationship between T2DM and cancer. The results showed that among these 8 genes, the frequency of CRC was the highest (5/8). Therefore, it is considered that T2DM is more likely to be comorbid with CRC than the other 12 types of cancer, at least in terms of DNA methylation signatures.

Then, these 5 genes were examined. There were significant differences in the methylation and mRNA levels of ADCY5, NRG1 and ELAVL4 between the combination and the control group, suggesting that they may be pathogenic methylated genes in T2DM with CRC. Furthermore, only ADCY5 showed no difference between the combination and the T2DM group or the CRC group, indicating that its methylation status and mRNA expression pattern in the combination group were similar to both T2DM and CRC. Therefore, ADCY5 may be a

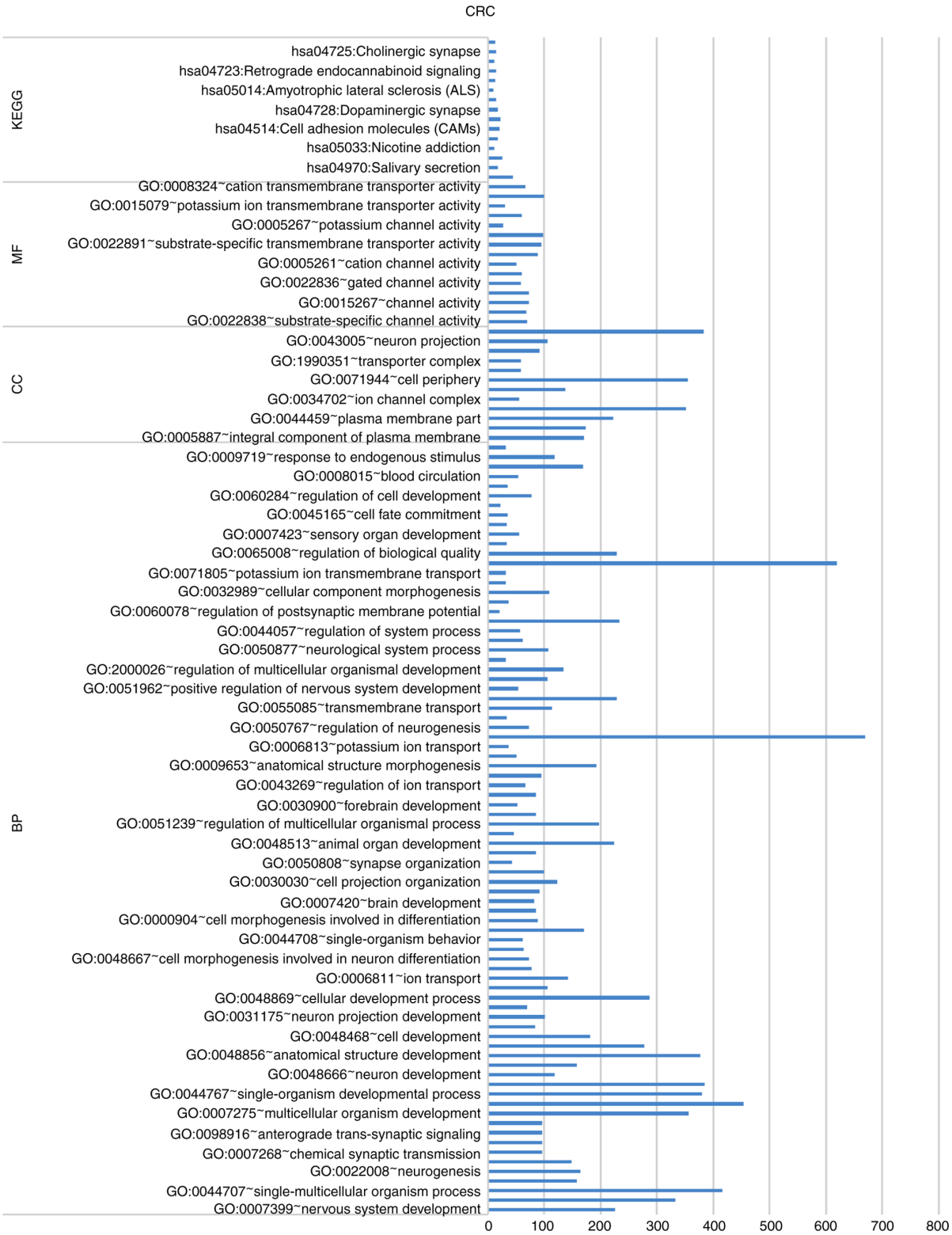

Figure 2. GO enrichment terms and KEGG pathways of CRC-related cDE-MGs. The top 20% of GO functions and all KEGG pathways are demonstrated. BP means the GO function of BP. CC means the GO function of CC. MF means the GO function of MF. KEGG means the KEGG pathways. The corrected P-value of false discovery rate is displayed in-log10 ratio. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CRC, colorectal cancer; cDE-MGs, cancer-related differentially methylated expression overlapping genes; BP, biological process; CC, cellular component; MF, molecular function.

comorbid gene in T2DM with CRC. Finally, the ADCY5 of the combined group was found to have a greater correlation coefficient with the T2DM group than the CRC group, indicating that changes of DNA methylation level of ADCY5 in T2DM with CRC were more likely related to T2DM.

ADCY5 is a member of the membrane-bound adenylate cyclase family. ADCY5 can affect glucose metabolism through glucose-coupled insulin secretion in human islets (16). Studies have shown that the levels of ADCY5 mRNA (17), methylation (12) and single nucleotide polymorphism (16)

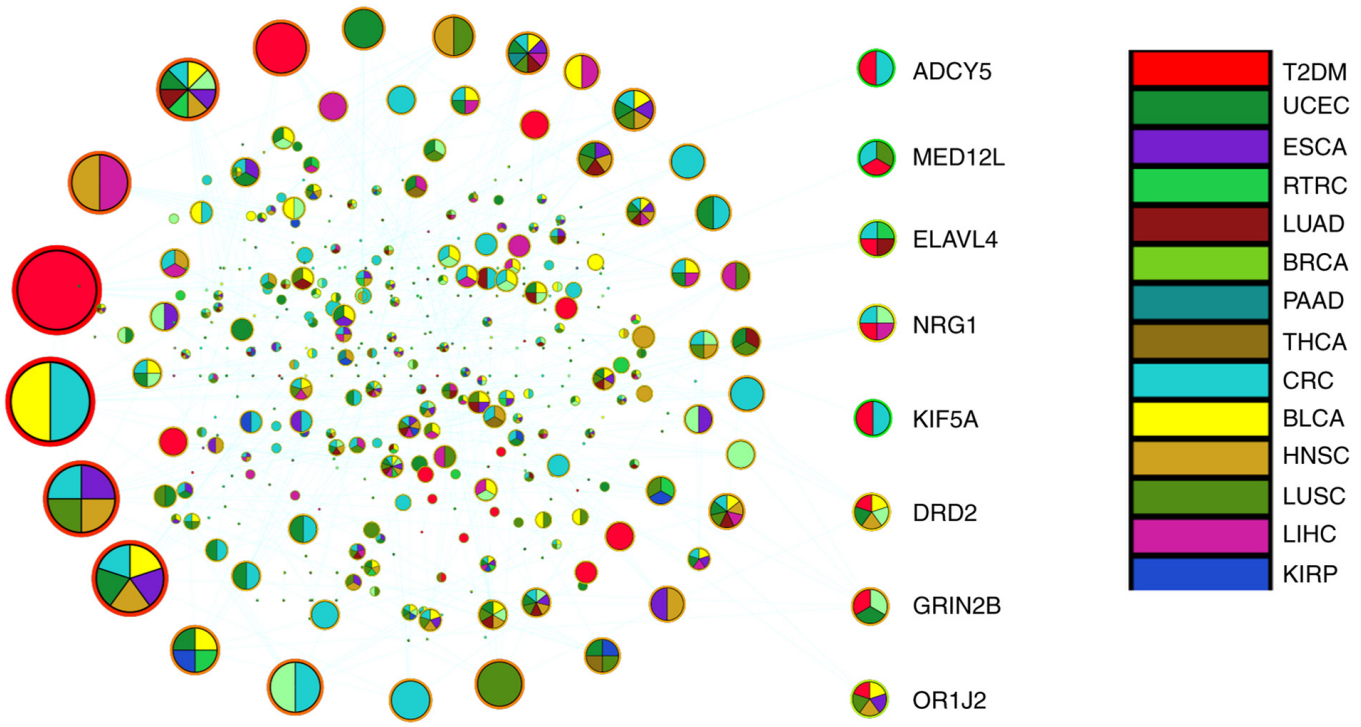

Figure 3. Visualization of the sub-network for T2DM with CRC. The node size is proportional to the degree value. Different diseases are represented in different colors. A node containing different colors means that it is involved in different kinds of diseases. Those nodes that contain both red and at least one other color are comorbid genes of T2DM and certain types of cancer. A total of 8 genes were further amplified to be clearly displayed since they are comorbid genes of T2DM with cancer. T2DM, type 2 diabetes mellitus; CRC, colorectal cancer; ADCY5, adenylate cyclase 5.

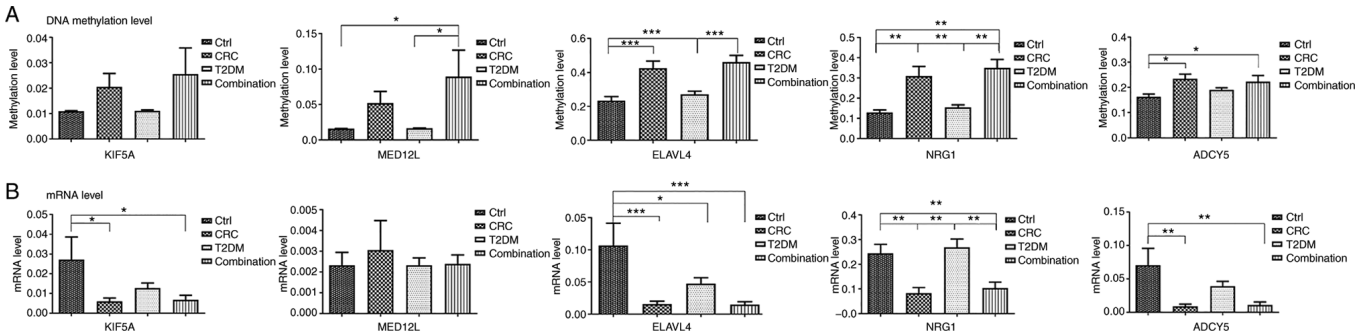

Figure 4. Expression differences of candidate genes between groups. (A) DNA methylation level and (B) mRNA level. *P<0.05, **P<0.01 and ***P<0.001. ADCY5, adenylate cyclase 5; KIF5A, kinesin family member 5A; ELAVL4, ELAV-like RNA-binding protein 4; MED12L, mediator complex subunit 12L.

are closely related to T2DM, and the differences between different ethnic groups are consistent (16). Furthermore, ADCY3 of the same family is at present a promising target for anti-obesity therapeutics. Although it was reported that intervention with ADCY5 in rodents does not improve insulin sensitivity (18), evidence is still lacking in primates, particularly in humans.

In addition, ADCY5 has been reported in numerous types of tumors, such as glioma (19), prostate cancer (20) and lung cancer (21), and is involved in neovascularization and inflammatory infiltration (22). However, there are few reports on CRC, and the present findings suggested a new direction.

Most previous studies have investigated the relationship between T2DM and one type of cancer, lacking a systematic and holistic view. In addition, studies involving numerous types of cancer are often limited to a certain molecular level, thus the molecular mechanism cannot be well reflected. The present study integrated data regarding T2DM and 13 types of cancer in public databases; it was found that T2DM was more closely related to CRC than other types of cancer from the perspective of methylation signatures and their regulation of mRNA expression. This finding provided new ideas for exploring the possible mechanisms of T2DM and CRC.

The present study has certain limitations. The bioinformatics analysis included reported data from different populations, and the validated samples were all from China, which may have biased the results. In addition, since normal human tissue samples could not be obtained, the present study only used paracancerous tissue as a control. In future studies, the sources and types of samples will be enriched in order to obtain more credible conclusions.

In conclusion, from the perspective of methylation characteristics, T2DM is most likely to be associated with CRC among 13 common types of cancer. When high DNA methylation level of ADCY5 was detected, patients with T2DM were more likely to develop CRC, and the possible mechanism was that methylation was involved in regulating mRNA expression.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 82073491 and 81872560).

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA822645).

Authors' contributions

JW and YW completed the downloading and sorting of data together, constructed the network and analyzed statistics. XZ, JS and JianL collected clinical samples and performed statistical analyses based on the information of the clinical characteristics of the contributors of these samples. JinL and XY detected sample expression level. HQ participated in the determination of the research direction and the design of the project, and put forward constructive opinions in the analysis of the results. JW and YW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. KY2016-017) by the Ethics Committee of the Second hospital of Harbin Medical University (Harbin, China). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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